1   **Running page heading:** Mycorrhized wheat, high CO_2 and drought

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

4   environmental scenarios

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Abstract

A greater understanding of how climate change will affect crop photosynthetic performance has been described as a target goal to improve yield potential. Other concomitant stressors can reduce the positive effect of elevated atmospheric CO_2 on wheat yield. Arbuscular mycorrhizal fungi (AMF) are symbiotic fungi predicted to be important in defining plant responses to rising atmospheric CO_2, but their role in response to global climatic change is still poorly understood. This study aimed to assess if increased atmospheric CO_2 interacting with drought can modify the effects of mycorrhizal symbiosis on flag leaf physiology in winter wheat. The study was performed in climate-controlled greenhouses with ambient (400 ppm, ACO_2) or elevated (700 ppm, ECO_2) CO_2 concentrations in the air. Within each greenhouse half of the plants were inoculated with *Rhizophagus intraradices*. When ear emergence began, half of the plants from each mycorrhizal and CO_2 treatment were subjected to terminal drought. At ACO_2 AMF improved the photochemistry efficiency of PSII compared with non-mycorrhizal plants, irrespective of irrigation regime. Mycorrhizal wheat accumulated more fructan than non-mycorrhizal plants under optimal irrigation. The level of proline in the flag leaf increased only in mycorrhizal wheat after applying drought. Mycorrhizal association avoided photosynthetic acclimation under ECO_2. However, nitrogen availability to flag leaves in mycorrhizal plants was lower under ECO_2 than at ACO_2. Results suggest that the mechanisms underlying the interactions between mycorrhizal association and atmospheric CO_2 concentration can be crucial for the benefits that this symbiosis can provide to wheat plants undergoing water deficit.

1. Introduction

Wheat is one of the major crops in temperate regions. Its production in the world reached nearly 730 million tonnes (Tm) in 2014/2015 and the estimated production for 2015/2016 is 733 million Tm. The European Union (EU), together with Argentina, Australia, Canada, Kazakhstan, the Russian Federation and Ukraine, is one of the major wheat exporters (FAO 2016). Despite an expected increase in cereal production from the projected increase in CO_2 (Manderscheid and Weigel 1995; Amthor 2001), the interaction of CO_2 with other limiting environmental factors (mainly temperature and low water and/or nitrogen availability) might decrease or eliminate the positive effect of elevated CO_2 (ECO_2) on plant production (Aranjuelo and others 2011, 2013). The phenophases in which wheat plants are exposed to drought strongly influence the effect of elevated CO_2 concentrations in the atmosphere on the yield (Varga and others 2016). Indeed, as observed by Oury and

3

72   others (2012), the beneficial effects expected from the increase in $[CO_2]$ 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 (Schüβler

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 CO_2, AMF are predicted to be important in defining plant responses to

87   ECO_2 concentrations. According to Cavagnaro and others (2011), lower contents of

88   phosphorus (P) in tissues of plants when grown under ECO_2 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 CO_2 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 $[CO_2]$ conditions

94   higher than those currently existing in air. Although AMF improved carbon (C)

95   accumulation and N uptake in wheat under ECO_2 (Zhu and others 2016), the

4

96 accumulation of some mineral nutrients (P, Cu, Fe) and antioxidant compounds

97 induced by AMF in leaves of lettuces cultivated at current [CO_2] levels decreased or

98 even disappeared under ECO_2 (Baslam and others 2012*a*). Moreover,

99 photosynthetic acclimation was increased in alfalfa associated with AMF under

100 ECO_2 (Goicoechea and others 2014). In addition, the effects of AMF on host plants

101 may depend on other limiting environmental factors interacting with ECO_2. While

102 AMF improved vegetative growth of *Triticum aestivum* subjected to salinity stress

103 under ECO_2 (Zhu and others 2016), the confluence of water deficit and ECO_2 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 ECO_2 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 ECO_2, drought and mycorrhizal symbiosis. We have only

116 found one study focusing on the contribution of AMF for wheat plants grown under

117 ECO_2 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

5

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 CO_2 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 CO_2 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 (Conviron 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 P_2O_5 and 140-220 mg L^{-1} K_2O and it was previously sterilized at

6

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 μmolm^{-2} 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 & Schüβler 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 [CO_2] controlled

167   greenhouses located on the Universidad de Navarra campus (42.80 N, 1.66 W;

168   Pamplona, Spain) (two ambient CO_2 (ACO_2) and two elevated CO_2 (ECO_2)

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 ACO_2 greenhouses (eight –M and eight +M pots in each ACO_2

172   greenhouse). Sixteen –M pots (32 plants) and sixteen +M pots (32 plants) were

173   placed in ECO_2 greenhouses (eight –M and eight +M pots in each ECO_2 greenhouse).

174   In order to prevent the CO_2 effect being confounded with greenhouse effects (De

175   Luis and others 1999), we used two ACO_2 greenhouses and two ECO_2 greenhouses

176   and the sixteen pots belonging to the same treatment were divided between the

177   two greenhouses with equal atmospheric CO_2 concentrations. Data obtained for the

178   same treatment from the two equivalent greenhouses were then pooled for

179   statistical analyses. In the two ACO_2 greenhouses, no CO_2 was added and the $[CO_2]$

180   in the atmosphere was approximately 392 $\mu mol \; mol^{-1}$. In the other two

181   greenhouses (ECO_2), $[CO_2]$ was fixed at ~700 $\mu mol \; mol^{-1}$ by injecting pure CO_2

182   (purity up to 99.99%) from cylinder-gases (34 L of CO_2 per cylinder) through the two

183   inlet fans during the light hours. Injection of CO_2 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 CO_2 a day from March to June. The CO_2 was provided by Air Liquide

187   (Bilbao, Spain). The $[CO_2]$ 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 CO_2 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 cm^3 cm^{-3}. The applied drought level corresponded to

203   50% θ_v of well-watered plants (around 0.22 cm^3 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 ACO_2; –M D ACO_2; +M WW ACO_2; +M D ACO_2; –

208   M WW ECO_2; –M D ECO_2; +M WW ECO_2; +M D ECO_2, 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 (CO_2 exchange rate, leaf conductance, intercellular CO_2

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 (δ^{15}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   (δ^{15}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 ($\delta^{13}C$, $\delta^{15}N$) 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. $\delta^{13}C$, $\delta^{15}N$ 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 $^{15}N/^{14}N$ ratio (R) in plant material was expressed in δ notation ($\delta^{15}N$) with

255 respect to atmospheric N_2, and measured with an analytical precision of 0.2‰:

256 $$\delta^{15}N = (R_{sample} - R_{standard})/R_{standard} \times 1000$$

257 $\delta^{15}N$ accuracy was monitored using international secondary standards of known

258 $^{15}N/^{14}N$ ratios (IAEA-N_1 and IAEA-N_2 ammonium sulphate and IAEA-NO_3 potassium

259 nitrate, IAEA, Austria). The nitrogen isotope composition was then converted to a

260 percentage: $\%^{15}N = R_{sample}/(1+R_{sample})$ where R_{sample} was obtained with

261 $R_{standard} \times (\delta^{15}N+1)$, with $R_{standard} = 0.003667$.

262   The $^{13}C/^{12}C$ ratio (R) in plant material was expressed in δ notation ($δ^{13}C$) with

263   respect to Vienna Pee Dee Belemnite calcium carbonate (V-PDB), and measured

264   with an analytical precision of 0.1‰:

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

266     $δ^{13}C$ accuracy was monitored using international secondary standards of known

267     $^{13}C/^{12}C$ 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 µmol mol^{-1} CO_2 for plants grown at ACO_2 or 700 µmol mol^{-1} CO_2 for

277   plants grown at ECO_2. The gas exchange system was maintained at 25°C and 1200

278   µmol m^{-2} 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 µmol mol^{-}

283   ^{1} 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 µL 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 µL, filtered through a 0.22 µm filter and 20 µL 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 [CO$_2$] treatment.

326 Two-factor ANOVA analyses (IBM SPSS v. 21) (between the same [CO$_2$]

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 [CO$_2$]

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

333   inoculation, AMF; atmospheric CO_2 concentration, CO_2 and water regime, W) and to

334   the interaction between these parameters (AMF × CO_2, AMF × W, CO_2 × W, AMF ×

335   CO_2 × W). Means ± standard errors (SE) were calculated and, when the F ratio was

336   significant ($P \leq 0.05$), a Duncan Multiple Range Test was applied. The acclimation of

337   conductance and photosynthesis was tested by using single-group t-tests,

338   evaluating whether the mean ratios of each treatment differed significantly from a

339   value of one ($P \leq 0.05$). Data on mycorrhizal colonization were analysed by Chi-

340   square ($\chi2$) test and were subjected to arc-sin transformation before applying $\chi2$-

341   test. Tests results were always considered significant at $P \leq 0.05$.

342

343   **3. Results**

344

345   *3.1. Plant growth, gas exchange and chlorophyll fluorescence parameters*

346   Neither the concentration of CO_2 in the atmosphere nor the water regime applied

347   to plants altered the percentage of wheat roots showing mycorrhizal structures at

348   growth stage 50 (Zadoks 1974) (Fig. 1). Fungal structures were never found in roots

349   of plants not inoculated with AMF.

350   At ECO_2, shoot biomass of wheat plants was enhanced, especially when plants

351   were well-watered (CO_2, $P \leq 0.001$; CO_2 × W, $P \leq 0.01$) (Table 1). However, the effect

352   of growth [CO_2] was not significant in root and spike DM (CO_2, $P > 0.05$), although

353   ECO_2 increased the number of spikes in well-watered -M plants at growth stage 69

354   (Zadoks, 1974) (CO_2, $P \leq 0.01$; CO_2 × W, $P \leq 0.05$). In relation to mycorrhizal

355   symbiosis, there was no response in growth parameters, with the exception of a

356   decreased root DM in +M plants grown with limited irrigation together with ACO_2

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    $P \leq 0.05$; $CO_2 \times W$, $P \leq 0.01$).

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 ($CO_2 \times AMF$, $P \leq 0.01$ 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 Fv/Fm),

367   with a significant interaction between the three factors that were analyzed in this

368   study related to the photochemistry efficiency of PSII ($CO_2 \times AMF \times W$, $P \leq 0.01$ for

369   Fv/Fm).

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 ($CO_2 \times AMF \times W$, $P \leq 0.05$)

379   (Table 4). Thus, while −M well-watered plants increased $\delta^{15}N_{non\text{-}lab}$ under ECO_2

380   conditions, +M plants with limited irrigation levels were decreased. In addition,

381 wheat plants grown at elevated [CO$_2$] reduced foliar $\delta^{15}N_{lab}$, $\delta^{13}C$ and N

382 concentration (CO$_2$, $P \leq 0.001$ for $\delta^{15}N_{lab}$, $\delta^{13}C$ and N). On the other hand, plants

383 associated with mycorrhizal fungi and grown at ACO$_2$ showed higher $\delta^{15}N_{lab}$ than

384 their respective non-mycorrhizal controls (AMF, $P \leq 0.001$). In contrast, when plants

385 were subjected to limited irrigation, $\delta^{15}N_{lab}$ 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; CO$_2$ × 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, $P \leq 0.001$; AMF × 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

405   6). The interaction between the three factors in protein concentration resulted in

406   the highest values in droughted -M plants and well-watered +M plants grown at

407   ACO_2 in comparison with their respective controls ($CO_2 \times AMF \times W$, $P \leq 0.01$). Levels

408   of proline increased in plants subjected to limited irrigation (W, $P \leq 0.001$),

409   regardless of whether they were inoculated or not with AMF. However, WW plants

410   exposed to ECO_2 showed the lowest concentrations of proline in flag leaves (CO_2, P

411   ≤ 0.01; $CO_2 \times W$, $P \leq 0.001$).

412

413   **4. Discussion**

414

415   *4.1. Effects of mycorrhizal association at ACO_2*

416   Under optimal irrigation (WW) the development of vegetative organs (shoot and

417   root) was similar in –M and +M wheat but the number of spikes was slightly higher

418   in +M than in –M plants (Table 1), indicating that phenology may be accelerated in

419   wheat inoculated with AMF. In agreement with this hypothesis, Baslam and others

420   (2012b) also observed that the association of alfalfa with AMF can shorten the

421   vegetative period of this forage legume. However, while the application of water

422   deficit (D) accelerated the production of spikes in –M plants, it did not affect the

423   number of spikes in +M plants, suggesting that water restriction only accelerated

424   life cycle in –M wheat plants. Mycorrhizal symbiosis can delay (Goicoechea and

425   others 1995) or accelerate (Goicoechea and others 2004) the senescence of

426   vegetative organs in host plants subjected to drought depending on the type of

427   plant and its own ecological adaptations to cope with water deficit. Under water

428   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 ACO_2 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 ACO_2 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 ACO_2 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 ACO_2 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 ACO_2 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 ACO_2 (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 ACO_2, 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 ACO_2 was the

474   increased uptake of N (demonstrated by the higher $\delta^{15}N$) (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 ACO_2 (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 ECO_2*

496   In well watered (WW) –M wheat, the greater photosynthetic activity under ECO_2

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 ACO_2. However, the increased level

21

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 ECO_2 in −

503 M plants (Long and others 2004). In contrast, enhanced photosynthetic rates in +M

504 plants cultivated under optimal irrigation (WW) under ECO_2 (+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 ECO_2, 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 ECO_2 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 ACO_2) (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 ECO_2

526 (Table 6). However, in contrast with results at ACO_2, 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 ECO_2 and water deficit.

530 It has been reported that plant responses to ECO_2 lead to stomatal closure

531 (Long and others 2004) and this also occurred in the flag leaves of wheat cultivated

532 under ECO_2 independently of mycorrhizal inoculation and water regime (Table 2).

533 However, compared to values measured at ACO_2, decreases of g_w under ECO_2 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 ECO_2 (as reported by the recovery of

537 N^{15}) (Table 4) were found in +M plants (decreases of around 54-56% depending on

538 irrigation regime), which contrasts with results obtained at ACO_2. The lack of

539 relationship between g_w and N^{15} 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 ACO_2 or ECO_2. 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 ECO_2 as compared to plants cultivated at ACO_2. Moreover, Jauregui and

545 others (2015) also show that expression of nitrate can be negatively affected by

546 ECO_2. 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 ECO_2 than at ACO_2 and, in agreement with our results, Chen and others

23

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

583

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734

Figure legends

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 (ACO$_2$) or under elevated (ECO$_2$) CO$_2$. Values are means (n = 8 plants per treatment) ±

742 SE. Data were subjected to arc-sin transformation before applying the χ2-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.
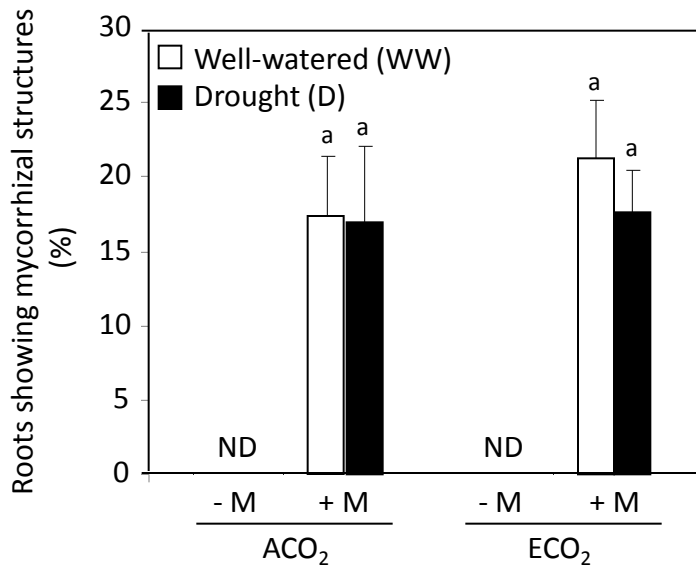
Figure 1

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 (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			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
$CO_2 \times$ AMF			ns	ns	ns	ns
$CO_2 \times$ W			**	ns	ns	*
AMF × W			ns	ns	*	ns
$CO_2 \times$ 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 CO_2 exchange rate (CER), total leaf conductance to water vapour (g_w), intercellular CO_2 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 (ACO_2) or elevated (ECO_2) CO_2. Photosynthetic rates were measured at 400 µmol mol^{-1} CO_2 for plants grown at ACO_2 and at 700 µmol mol^{-1} CO_2 for plants grown under ECO_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			CER (µmol CO_2 m^{-2} s^{-1})	g_w (mol H_2O m^{-2} s^{-1})	C_i (µmol mol^{-1} 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 CO_2 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 (ACO$_2$) or elevated (ECO$_2$) CO_2. The acclimatory responses of stomatal conductance and photosynthesis rate are expressed as the values for plants grown under ECO$_2$ relative to those of plants cultivated at ACO$_2$ 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 $P \leq 0.05$

Table 4 Nitrogen isotopic composition ($\delta^{15}N$) corresponding to $K^{15}NO_3$-non-labeled ($\delta^{15}N_{non-lab}$) or $K^{15}NO_3$-labeled ($\delta^{15}N_{lab}$) wheat plants, carbon isotopic composition ($\delta^{13}C$) 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			$\delta^{15}N$ (‰)		$\delta^{13}C$ (‰)	N (%)	C (%)
			($\delta^{15}N_{non-lab}$)	($\delta^{15}N_{lab}$)			
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 (ACO$_2$) or elevated (ECO$_2$) CO$_2$. Total sugars: 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			Glucose (G)	Xilose	Fructose (F)	Sorbitol	Sucrose(S)	Fructans (mg g^{-1} DM)	Fructans DP>3	Fructans DP=3	S+G+F	Fructans+S+F+G	Total sugars
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 (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			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	***
$CO_2 \times$ AMF			**	ns	ns
$CO_2 \times$ W			*	ns	***
AMF $\times$ W			*	ns	ns
$CO_2 \times$ AMF $\times$ 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.