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Pearl millet growth and biochemical alterations determined by mycorrhizal inoculation, water availability and atmospheric CO_2 concentration

Eliseu G. Fabbrin[1], Yolanda Gogorcena[2], Átila F. Mogor[1], Idoia Garmendia[3] and Nieves Goicoechea[4*]

[1] Departamento de Fitotecnia e Fitossanitarismo, Setor de Ciências Agrárias, Universidade Federal do Paraná. Rua dos Funcionários, 1540. Juvevê, Curitiba, Brasil.

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

[3] Departamento Ciencias de la Tierra y del Medio Ambiente, Facultad de Ciencias, University of Alicante, Ctra. San Vicente del Raspeig, s/n. Apdo. Correos 99, E-03080 Alicante, Spain

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

*** Corresponding author:**

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

Running head: Biotic & abiotic factors affecting millet growth

Abstract

Pennisetum glaucum is an important fodder and may be a potential feedstock for fuel ethanol production in dry areas. Our objectives were to assess the effect of elevated CO_2 and/or reduced irrigation on biomass production and levels of sugars and proteins in leaves of *P. glaucum* and to test if mycorrhizal inoculation could modulate the effects exerted by these abiotic factors on growth and metabolism. Results showed that mycorrhizal inoculation and water regime were the factors that most influenced biomass of shoots and roots; however, their individual effects were dependent on the atmospheric CO_2 concentration. At ambient CO_2, mycorrhizal inoculation helped alleviating effects of water deficit on *P. glaucum* without significant decreases in biomass production, which contrasted with the low biomass of mycorrhizal plants under restricted irrigation and elevated CO_2. Mycorrhizal inoculation enhanced water content in shoots while reduced irrigation decreased water content in roots. The triple interaction between CO_2, arbuscular mycorrhizal fungi (AMF) and water regime significantly affected the total amount of soluble sugars and determined the predominant soluble sugars in leaves. Under optimal irrigation, elevated CO_2 increased the proportion of hexoses in pearl millet non-inoculated with AMF, thus improving the quality of this plant material for bioethanol production. In contrast, elevated CO_2 decreased the levels of proteins in leaves thus limiting the quality of pearl millet as fodder and prime matter for cattle feed.

Additional keywords: arbuscular mycorrhizal fungi, biomass, climatic change, carbohydrates, *Pennisetum glaucum*, proteins.

Introduction

Pearl millet (*Pennisetum glaucum*) (L.) R. Brown belongs to the Poaceae family and has its origins as a cereal crop adapted to the harshest growing conditions in sub-Saharan African. Nowadays is a major staple food crop in the drier parts of Africa and Asia (Purseglove 1972) because it is highly tolerant to drought and salt (Maiti and Wesche-Ebeling 1997). According to FAOSTAT (FAO Statistics Division) 2012, millet was grown over 31 million ha area worldwide and the total production of *P. glaucum* accounts for approximately 50% of the total world production of millets (Borde *et al.* 2011). Pearl millet was introduced in Brazil in the 1960s and its cultivation has become more widespread in no tillage crop farming systems in central regions of the country (de Carvalho *et al.* 2006). Moreover, it is an important fodder and prime matter for cattle feed, in the rainy or dry season, in Brazil (Netto 1998). According to Andrews *et al.* (1996) feeding tests in cattle, swine, laying hens, ducks, and catfish showed that pearl millet is either superior to, or as good as, feed corn. In addition, in a study performed to test the potential of different genotypes of pearl millet as raw material for fuel ethanol production, Wu *et al.* (2006) concluded that pearl millets could be a potential feedstock for fuel ethanol production in areas too dry to grow corn or grain sorghum.

Arbuscular mycorrhizal fungi (AMF) are soil inhabitants belonging to the phylum Glomeromycota, with a presumed origin at least 460 million years ago (Schüβler *et al.* 2001). These fungi colonize the roots of over 80% of plant species (including millet) mostly to the mutual benefit of both the plant host and the fungus. The association between AMF and plant roots develops in two functional phases (Smith and Read 2008): the extraradical phase extending from the root into the soil and the intraradical

73 phase with intercellular hyphae and specialized intracellular structures called
74 'arbuscules'. Arbuscules are the structures where exchanges of carbon to the fungus
75 and nutrients to the host plant take place. In a recent work, Borde *et al.* (2011)
76 concluded that mycorrhizal association can help *P. glaucum* to perform better under
77 moderate salinity levels by enhancing the antioxidant activity and proline
78 accumulation as compared to non-mycorrhizal plants.

79 Levels of atmospheric CO_2 have been constantly increasing since the industrial
80 revolution due to anthropogenic activities, including burning of fossil fuels,
81 deforestation and intensive animal husbandry. The enhanced CO_2 concentration
82 increases the potential net photosynthesis in C3 plants (Drake *et al.* 1997) and
83 therefore can improve yield (Oliveira *et al.* 2010) over short-term exposures. In
84 contrast, net CO_2 assimilation rates in C4 species should not be directly stimulated by
85 elevated CO_2 under optimal conditions of temperature, water availability and nutrient
86 supply (Ghannoum *et al.* 2000). However, C4 plants in natural and agricultural
87 ecosystems frequently grow in conditions of limiting water availability and/or limiting
88 nitrogen (N) supply. In this context of rising atmospheric CO_2, AMF are predicted to be
89 important in defining plant responses to elevated CO_2 concentrations. In fact, lower
90 concentrations of phosphorus (P) in tissues of plants when grown under elevated CO_2
91 can be alleviated by the formation of AMF and any improvements in plant N nutrition
92 resulting from the formation of AMF may be also important in determining plant
93 responses to atmospheric CO_2 enrichment (Cavagnaro *et al.* 2011). In alfalfa cultivated
94 under elevated CO_2, Baslam *et al.* (2014) found that AMF increased levels of glucose
95 and fructose in stems of inoculated plants compared with non-mycorrhizal plants,
96 which may result in enhanced potential for bioethanol conversion in mycorrhizal

97 alfalfa cultivated under elevated CO_2. In arid and semiarid areas (i.e, Mediterranean
98 regions), rising atmospheric CO_2 concentrations may increase the severity of drought
99 conditions under future climate change scenarios (Gregory *et al.* 2003). Kholer *et al.*
100 (2009) found that the contribution of AMF (together with the plant-growth-promoting
101 rhizobacterium *Pseudomonas mendocina*) to soil aggregate stability under elevated
102 atmospheric CO_2 was largely enhanced by soil drying. However, the role that AMF play
103 in ecosystems responding to global climatic change is not still well understood (Mohan
104 *et al.* 2014).

105 The objectives of our study were (1) to assess the effect of climate change
106 scenarios (elevated CO_2 and/or restricted irrigation) on biomass production, sugars
107 accumulation and proteins levels in leaves of *P. glaucum* and (2) to test if mycorrhizal
108 inoculation could modulate the effects exerted by elevated CO_2 and/or restricted
109 irrigation on growth and metabolism of *P. glaucum*.

110

111 **Materials and methods**

112

113 *Plant material and growth conditions*

114

115 Seeds from pearl millet (*Pennisetum glaucum*) (L.) R. Brown were germinated on a
116 mixture of light peat (Floragard, Vilassar de Mar, Barcelona, Spain) and siliceous sand
117 (on 26th March 2013). Peat had a pH of 5.2-6.0, 70-150 mg L^{-1} of nitrogen, 80-180 mg L^{-1}
118 P_2O_5 and 140-220 mg L^{-1} K_2O and it was previously sterilized at 100ºC for 1 h on three
119 consecutive days. After sowing (on 8th April 2013), seedlings were transferred to 48
120 pots of 13 L (three plants per pot) filled with a mixture of vermiculite- siliceous sand-

121 light peat (2.5:2.5:1, v:v:v) and divided into eight groups (six pots per group, three
122 plants per pot) for an experimental design 2 × 2 × 2 as explained below. Main factors
123 were 'mycorrhizal inoculation, AMF' (inoculated, +M or non-inoculated, -M, plants);
124 'water regime, W' (well watered, WW, or water regime equivalent to ½ of well
125 watered conditions, ½ WW); and 'CO_2 concentration in the atmosphere, CO_2' (ambient,
126 ACO_2, or elevated, ECO_2, carbon dioxide concentration in the air).
127
128 a) Mycorrhizal inoculation, AMF
129 At transplanting, half of the plants (72 plants in 24 pots) were inoculated with the
130 mycorrhizal inoculum 'Glomygel Intensivo' (Mycovitro S.L., Pinos Puente, Granada,
131 Spain) (+M plants). The concentrated commercial inoculum derived from an *in vitro*
132 culture of the AMF *Rhizophagus intraradices* (Schenck and Smith) Walker & Schüβler
133 comb. nov. (Krüger et al., 2012) and contained around 2,000 mycorrhizal propagules
134 (inert pieces of roots colonized by AMF, spores and vegetative mycelium) per mL of
135 inoculum. In order to facilitate its application, the concentrated commercial inoculum
136 was diluted with distillate water until obtaining a resultant mycorrhizal inoculum with
137 around 250 propagules per mL. Each +M plant received 8 mL of the diluted mycorrhizal
138 inoculum close to the roots thus making a total of 2,000 propagules. A filtrate was
139 added to plants that did not receive the mycorrhizal inoculum (-M plants, 72 plants in
140 24 pots) in an attempt to restore other soil free-living microorganisms accompanying
141 AMF. The filtrate was obtained by passing diluted mycorrhizal inoculum through a
142 layer of 15-20 µm filter papers (Whatman, GE Healthcare, UK) and each –M plant
143 received 8 mL of filtrate close to the roots.
144

145 b) Water regime, W

146 Two different irrigation regimes were imposed at transplanting. Twelve pots with
147 plants inoculated with AMF (36 +M plants in total) and 12 pots with plants non-
148 inoculated with AMF (36 –M plants in total) were always maintained under optimal
149 irrigation and kept as well-watered (WW) controls. Well-watered plants received 2 L of
150 Hoagland nutrient solution (Arnon and Hoagland 1939) and 4 L of distilled water per
151 pot and week. Distilled water was added in order to avoid excessive salt accumulation.
152 Other 12 pots with plants inoculated with AMF (36 +M plants in total) and 12 pots with
153 plants non-inoculated with AMF (36 –M plants in total) were grown under an irrigation
154 regime equivalent to 1/2 of optimal irrigation (1/2 WW) and received 2 L of Hoagland
155 nutrient solution and 1 L of distilled water per pot and week.

156

157 c) CO_2 concentration in the atmosphere, CO_2

158 At transplanting all pots were transferred to four [CO_2] controlled greenhouses
159 located at the University of Navarra campus (42.80 N, 1.66 W; Pamplona, Spain). The
160 design of the greenhouses was similar to that described by Sanz-Sáez *et al.* (2012) and
161 based on Aranjuelo *et al.* (2005). Inside the greenhouses, the pots were placed in holes
162 made in the soil in order to provide for natural temperature fluctuations, thus
163 simulating the temperature differences observed between shoots and roots under
164 field conditions (Rawson *et al.* 1995). In the two ambient CO_2 (ACO_2) greenhouses no
165 CO_2 was added and [CO_2] in the atmosphere was maintained at ambient conditions
166 (~360 µmol mol^{-1}). In the two greenhouses with elevated CO_2 (ECO_2), [CO_2] was
167 increased to ~700 µmol mol^{-1} by injecting pure CO_2 (purity up to 99.99%) from cylinder-
168 gases (34 L of CO_2 per cylinder) at the two inlet fans during the light hours. Injection of

169 CO_2 to greenhouses began when light intensity was equal or superior to 5 watts m^{-2} as
170 measured by a Silicon Pyranometer PYR-S (APOGEE Instruments, Inc., Logan, UT, USA)
171 making a total of 13-15 h of high CO_2 a day from April to June. The CO_2 was provided
172 by Air Liquide (Bilbao, Spain). The [CO_2] was continuously monitored using a Guardian
173 Plus gas monitor (Edinburgh Instruments Ltd, Livingston, UK). The monitor's signal was
174 fed into a proportional integrative differential controller that regulated the opening
175 time (within a 10 s cycle) of a solenoid valve that injected CO_2 into both inlet fans. Six
176 –M WW pots (18 plants), six –M ½ WW pots (18 plants), six +M WW pots (18 plants)
177 and six +M ½ WW pots (18 plants) were placed either at ACO_2 or ECO_2 greenhouses
178 thus making a total of eight different treatments: –M WW ACO_2; –M ½ WW ACO_2; +M
179 WW ACO_2; +M ½ WW ACO_2; –M WW ECO_2; –M ½ WW ECO_2; +M WW ECO_2; +M ½ WW
180 ECO_2. In order to prevent the CO_2 effect being confounded with greenhouse effects
181 (De Luis *et al.* 1999), we used two ACO_2 greenhouses and two ECO_2 greenhouses and
182 the six pots belonging to the same treatment were divided into the two greenhouses
183 with equal atmospheric CO_2 concentration (three pots, nine plants in every
184 greenhouse). Data obtained for the same treatment from the two equivalent
185 greenhouses were then mixed for statistical analyses.
186
187 *Growth and water status parameters and mycorrhizal analyses*
188
189 Plants were harvested at tillering on 12th June 2013, 65 days after transplanting to
190 pots, when they had main shoot and three tillers (growth stage 23 according to Zadoks
191 scale, 1974). Number of leaves and tillers per plant were recorded. Then shoots and
192 roots from all plants (18 plants per treatment) were immediately separated in order to

193 estimate their fresh weight (FW). Afterwards, shoots of ten plants randomly chosen
194 per treatment were frozen (-20ºC) pending analyses of proline, proteins and starch;
195 roots of these ten plants were kept fresh and then cleared and stained according to
196 Phillips and Hayman (1970) for visualizing mycorrhizal structures. The other eight
197 plants of each treatment were used for estimating biomass and water content in
198 shoots and roots and sugars in leaves. Dry matter (DM) of shoots and roots was
199 determined after drying plant material in the oven at 80ºC until weight was constant.
200 Water content (WC) was calculated as shoot or root FW – shoot or root DM/shoot or
201 root DM and results were expressed as grams of water per gram of shoot or root DM.
202
203 *Biochemical analyses*
204 Four samples (each one equivalent to 0.2 g DM of leaves from a pool of eight plants)
205 for soluble carbohydrate analyses were freeze crushed and polar compounds were
206 extracted into 1 mL aqueous 80% ethanol at 80ºC, in three steps, each lasting 20 min
207 (Jiménez *et al.* 2011). The mixture of each step was centrifuged for 5 min at 14,000 x *g*
208 and slurries were pooled. Ethanol was evaporated under vacuum in a speed vac
209 system (Thermo Fisher Scientific Inc., Waltham, MA, USA) and dry extracts were
210 solubilized in 500 µL double-distilled water. The soluble carbohydrates of the samples
211 were purified using about 3.5 g g^{-1} plant material ion exchange resins (Bio-Rad AG 50
212 W-X8 Resin 200-400 mesh hydrogen form, Bio-Rad AG 1-X4 Resin 200-400 chloride
213 form). The samples were concentrated to 200 µL, filtered through a 0.22 µm filter and
214 20 µL were injected and analyzed by high-performance liquid chromatography (HPLC),
215 using Ca-column (Aminex HPX-87C 300 mm x 7.8 mm column Bio-Rad) flushed with 0.6
216 mL min^{-1} double distilled water at 85ºC with a refractive index detector (Waters 2410,

217 Milford, MA, USA). Concentrations of the main carbohydrates, raffinose, sucrose,
218 galactinol, glucose, xylose, fructose and sorbitol were calculated for each sample using
219 mannitol as an internal standard since it was not present in pearl millet samples.
220 Carbohydrate quantification was performed with the Empower Login software, Waters
221 (Millford, Mass, USA) using standards of analytical grade from Panreac Química S.A.
222 (Barcelona, Spain) and Sigma-Aldrich (Schnelldorf, Germany). Concentrations of
223 carbohydrates were expressed as mg g^{-1} DM.

224 Starch, proline and total soluble proteins were quantified in potassium phosphate
225 buffer (KPB) (50 mM, pH = 7.5) extracts of leaves (1 g FW, ten samples per treatment).
226 These extracts were filtered through four cheese cloth layers and centrifuged at 38,720
227 x *g* for 10 min at 4ºC. The pellet was used for starch determination (Jarvis and Walker
228 1993). The supernatant was collected and stored at 4ºC for protein and proline
229 determinations. Total soluble proteins were measured by the protein dye-binding
230 method of Bradford (1976) using bovine serum albumin (BSA) as standard. Free proline
231 was estimated by spectrophotometric analysis at 515 nm of the ninhydrine reaction
232 (Irigoyen *et al.* 1992). Results were expressed as mg of starch or total soluble proteins
233 per gram of DM and µmol of proline per gram of DM.

234

235 *Statistical analysis*

236

237 Data were subjected to a three-factor ANOVA (factorial 2 x 2 x 2) (SPSS v. 15.0). The
238 variance was related to the main treatments (atmospheric CO_2 concentration, CO_2,
239 water regime, W, and AMF inoculation, AMF) and to the interaction between them
240 (CO_2 × W, CO_2 × AMF, W × AMF, CO_2 × W × AMF). Means ± standard errors (SE) were

241 calculated and, when the F ratio was significant ($P \leq 0.05$), a Duncan Multiple Range
242 Test was applied. Tests were considered significant at $P \leq 0.05$.
243
244 **Results**
245
246 *Growth and water status parameters and mycorrhizal analyses*
247
248 When cultivated at ACO_2 and optimal irrigation (WW), the inoculation of AMF (+M)
249 decreased dry matter production in both shoots and roots in comparison with the non-
250 inoculated controls (-M) (Table 1). Limited irrigation (½ WW) strongly decreased shoot
251 FW of -M millet plants compared with the well-watered controls, being the reduction
252 in shoot FW a consequence of decreased shoot biomass; the accumulation of water in
253 aerial tissues was similar under optimal and restricted irrigation (Table 1). However,
254 limited irrigation did not have a significant negative effect on plant biomass when
255 millet was inoculated with AMF (+M plants). Inoculated plants subjected to limited
256 water supply (+M, ½ WW) achieved similar development of shoots and roots than the
257 well-watered non-inoculated (-M, WW) plants (Table 1).
258 The exposition of -M plants to ECO_2 under optimal irrigation (WW) decreased the
259 number of leaves per plant and reduced the root FW in comparison with the non-
260 inoculated well-watered plants (-M, WW) grown at ACO_2 (Table 1). When exposed to
261 ECO_2, mycorrhizal inoculation (+M) caused decreases in both shoot and root biomass
262 under either well-watered or restricted water supply conditions compared to the non-
263 inoculated (-M) controls (Table 1).

264 Microscopic observations of cleared and stained roots revealed that there were
265 very few fungal structures (vesicles) colonizing root tissues (Fig. 1).
266
267 *Non-structural sugars in leaves*
268
269 Table 2 shows the concentrations of individual soluble sugars, total soluble sugars
270 (TSS) and starch determined in leaves of *P. glaucum*. Raffinose, sucrose, glucose,
271 xylose, fructose and sorbitol were present in leaves of pearl millet plants, regardless
272 they were (+M) or not (-M) inoculated with AMF, the water regime and the
273 concentration of CO_2 in the atmosphere.
274 At ACO_2 and optimal irrigation (WW), the levels of non-structural sugars (soluble
275 sugars and starch) were significantly lower in plants inoculated with AMF (+M) than in
276 -M plants. Restricted water supply (½ WW) caused a significant decrease in the levels
277 of soluble sugars in –M plants, being reductions especially strong in sucrose and
278 glucose. In contrast, +M plants accumulated higher quantities of TSS when subjected
279 to water deficit and increases mainly affected to the levels of sucrose.
280 ECO_2 modified the proportion of most individual sugars. Under full irrigation (WW),
281 -M plants accumulated similar amounts of TSS at ambient (25.68 mg g^{-1} DM) and
282 elevated (25.32 mg g^{-1} DM) CO_2; however, ECO_2 strongly decreased sucrose
283 concentrations and sharply enhanced fructose levels. In well-watered inoculated
284 plants (WW, +M), ECO_2 favoured the accumulation of TSS (20.48 mg g^{-1} DM under
285 ECO_2 compared with 11.40 mg g^{-1} DM at ACO_2). The application of limited irrigation (½
286 WW) to -M millet plants under ECO_2 increased the concentration of TSS in leaves
287 (33.28 mg g^{-1} DM) in comparison with well-watered –M plants (25.32 mg g^{-1} DM),

288 being such enhancement mainly due to the significant increase in sucrose (12.63 mg g^{-1}
289 DM under ECO_2 and 3.79 mg g^{-1} DM at ACO_2). In inoculated plants (+M) cultivated
290 under ECO_2, water restriction (½ WW) induced the accumulation of starch and the
291 reduction of TSS (10.84 mg starch g^{-1} DM, 11.58 mg TSS g^{-1} DM) in comparison with
292 well-watered inoculated (WW, +M) plants (3.67 mg starch g^{-1} DM, 20.48 mg TSS g^{-1}
293 DM).
294
295 *Proline and total soluble proteins in leaves*
296
297 Reduction of water amount induced the accumulation of proline in shoots of both non-
298 inoculated (-M) and inoculated (+M) pearl millet plants when grown at ACO_2 (Fig. 2*a*)
299 (Table 3); under these conditions (ACO_2 and ½ WW) +M plants had higher levels of
300 proline in leaves than –M plants. Under ECO_2, +M plants always accumulated higher
301 amount of proline than –M plants (Fig. 2*a*), although the levels were lower than those
302 measured in +M plants subjected to restricted irrigation at ACO_2. Non-inoculated –M
303 plants grown at ECO_2 showed lower proline leaf concentration than –M plants
304 cultivated at ACO_2.
305 Total soluble proteins in leaves of –M plants decreased as a consequence of the
306 interaction between reduced water supply and ECO_2 (Fig. 2*b*)(Table 3). In +M plants,
307 ECO_2 was the factor that caused reductions in the levels of proteins under either
308 optimal or restricted irrigation (Fig. 2*b*) (Table 3).
309
310
311

312 **Discussion**

313

314 Water deficit is one of the major factors limiting crops production in the world and it
315 also affects forage yield and biomass production of millet (Winkel *et al.* 1997, 2001),
316 which is in accordance with the strong decreases in both shoot and root DM and
317 number of leaves observed in –M millet plants grown at ACO_2 and subjected to
318 restricted irrigation in comparison with plants cultivated under optimal water regime.
319 Our results also demonstrated the beneficial influence of mycorrhizal inoculation on
320 plant growth when millet was subjected to water deficit at ACO_2 conditions. This
321 positive effect was not only a consequence of improved water content in aerial tissues
322 of +M plants but was also due to enhanced biomass in +M plants. It has been
323 described that the beneficial effect of AMF on the development of host plants is more
324 evident under adverse than under optimal growth conditions (Goicoechea *et al.* 2004).
325 Enhanced water uptake by fungal hyphae and/or improved whole plant, soil-to-root or
326 root–to-leaf hydraulic conductance have been found to favour water status in plants
327 associated with AMF and subjected to drought (Augé 2001). In addition, mycorrhizal
328 symbiosis can help plants to maintain levels of mineral nutrients in tissues under water
329 deficit (Goicoechea *et al.* 1997). The increased biomass in +M than in –M plants
330 subjected to limited water supply at ACO_2 could also be a consequence of improved
331 photosynthesis as suggested by the higher concentration of TSS in pearl millet plants
332 inoculated with AMF (Sánchez-Díaz *et al.* 1990). This accumulation of TSS together
333 with increased proline concentrations in shoots of +M millet could help these plants to
334 store greater amount of water in tissues than –M plants grown at ACO_2 and exposed to
335 reduced irrigation (Seki *et al.* 2007). Increased proline accumulation in *P. glaucum*

associated with AMF has also been reported by Borde *et al.* (2011) in plants grown under salinity stress condition. In addition, +M plants showed higher concentrations of proteins in shoots than –M plants when cultivated under restricted irrigation and ACO_2, which indicates greater quality of +M pearl millet to be used as forage for cattle (Lara and Andreo 2011). Reduced protein contents have been found in plants undergoing water stress and cultivated either at ambient or under elevated CO_2 (Irigoyen *et al.* 1992; De Luis *et al.* 1999; Baslam and Goicoechea 2012) and such decreases can be alleviated by mycorrhizal symbiosis (Baslam and Goicoechea 2012).

The abovementioned positive effects of AMF on growth and physiology of plants undergoing water deficit at ACO_2 occurred without being the roots highly colonized by AMF at harvesting. Vesicles were the main fungal structures observed in pearl millet roots suggesting that mycorrhizal symbiosis in our plants was at final stages of its development. Some authors have reported that, in cereals, the percentage of mycorrhizal colonization can strongly vary according to the phenological stage of the host plant (Mohammad *et al.* 1998). In addition, Krishna *et al.* (1985) reported that percentage of root colonized by mycorrhizal fungi strongly differed between different genotypes of *Pennisetum americanum* and so did phosphorus uptake and growth responses of host plants to mycorrhizal symbiosis. Moreover, the mere presence of AMF in the rhizosphere may have affected rooting patterns of inoculated pearl millet as well as the supply of available nutrients to plants, thereby modifying the quality and quantity of root exudates, which may have affected fungal and microbial activity (Barea *et al.* 2005). However, the beneficial effect of mycorrhizal inoculation on growth, water status and contents of sugars and proteins in leaves of pearl millet plants subjected to water restriction disappeared when plants were exposed to ECO_2

360 in the atmosphere. Baslam *et al.* (2012), working with lettuces grown either at ambient
361 or under elevated CO_2, noted that AMF increased the levels of some secondary
362 metabolites in the edible part of lettuces only when plants were cultivated at ACO_2 and
363 suggested that carbon partitioning between primary and secondary metabolism in
364 mycorrhizal plants was conditioned by the level of CO_2 in the atmosphere. Plants
365 frequently allocate more resources to mycorrhizal fungi under increased CO_2, which
366 may lead to greater extraradical hyphal growth and increased mycorrhizal respiration
367 (Mohan *et al.* 2014), presumably in detriment of plant growth and accumulation of
368 carbohydrates and other primary metabolites in host plant tissues.

369 Hamerlinck *et al.* (1997) found that photosynthetic rates increased in the C4 grass
370 *Andropogon gerardii* exposed to ECO_2 but only when plants were also subjected to
371 drought. Cody Markelz *et al.* (2011), working with maize, did not observe any
372 stimulation of photosynthetic rates by ECO_2 when water availability was high;
373 however, ECO_2 delayed and relieved both stomatal and non-stomatal limitations to
374 photosynthesis during water deficit. Likewise, the review by Lara and Andreo (2011)
375 mentions several scientific works in which C4 plants grown under Free-Air Carbon
376 dioxide Enrichment (FACE) exhibited increased photosynthetic rates only during
377 drought or under conditions of atmospheric vapour pressure deficits. In our study, the
378 highest concentrations of TSS (together with high levels of starch) were found in –M
379 pearl millet plants simultaneously exposed to ECO_2 and limited water supply,
380 suggesting improved photosynthesis in these plants in comparison with –M plants
381 grown at ACO_2 or under both ECO_2 and optimal irrigation. However, when determined
382 the individual carbohydrate composition of –M plants grown under ECO_2 and different
383 water regimes, we found that amount of sucrose accounted 38% of TSS in plants

subjected to restricted irrigation whereas the contribution of glucose and fructose to TSS was 44%; in well-watered plants, only 15% of TSS corresponded to sucrose and more than 70% of TSS corresponded to glucose (21%) and fructose (52%). The larger concentration of monosaccharides (glucose and fructose) in leaves of -M pearl millet exposed to ECO_2 under high water availability might be advantageous for a more efficient bioethanol production because hexoses can be converted at higher yields to ethanol than most other carbohydrates (Dien *et al.* 2006, 2011). In contrast with findings of Baslam et al. (2014) in the forage legume alfalfa (a C3 species), mycorrhizal symbiosis did not enhance the potential of pearl millet for bioethanol conversion in plants cultivated under high atmospheric CO_2 concentration, irrespective of irrigation regime.

Conclusions

Our results demonstrate that biomass production and biochemical characteristics of *P. glaucum* foliage can be modulated by biotic and abiotic factors applied to plants thus affecting the quality of this crop for different applications. When plants are cultivated at ACO_2, inoculation of AMF in the substrate helped alleviating effects of water deficit on *P. glaucum* without any significant decrease in biomass production and leaf protein content, being this effect significant even without achieving high mycorrhizal colonization of roots. However, this beneficial effect of AMF inoculation disappeared under ECO_2. Under optimal irrigation, ECO_2 in the atmosphere can enhance the proportion of monosaccharides in leaves of pearl millet non-inoculated with AMF (-M plants), thus improving the quality of this plant material for bioethanol production.

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550

Table 1 Growth and water status in *Pennisetum glaucum* non-inoculated (-M) or inoculated (+M) with arbuscular mycorrhizal fungi (AMF), cultivated either under well-watered conditions (WW) or limited irrigation (½ WW), and grown either at ambient (ACO_2) or under elevated (ECO_2) CO_2. Values are means (n = 18 for FW, leaves per plant and tillers per plant, n = 8 for DM and WC) ± SE separated by Duncan Multiple Range Test ($P \leq 0.05$); different letters indicate significant differences within treatments as affected by the main factors 'atmospheric CO_2, CO_2, 'water regime, W' and 'mycorrhizal inoculation, AMF' and their interactions. ns = not significant; * and ** = significant at $P \leq 0.05$ and $P \leq 0.01$, respectively. FW = fresh weight; DM = dry matter; WC = water content.

Treatments			Shoot FW	Shoot DM	Root FW	Root DM	Leaves per plant	Tillers per plant	Shoot WC	Root WC
			g plant^{-1}						g H_2O g^{-1} DM	
ACO_2	WW	-M	68.37 ± 2.40 ab	6.03 ± 0.22 ab	20.02 ± 0.88 a	1.56 ± 0.07 a	10.77 ± 0.12 a	3.50 ± 0.06 a	11.20 ± 0.38 ab	11.90 ± 0.51 bc
		+M	56.41 ± 1.12 bc	4.41 ± 0.07 cd	16.73 ± 0.63 ab	1.03 ± 0.05 cd	8.62 ± 0.36 b	2.91 ± 0.10 ab	12.04 ± 0.35 a	14.91 ± 0.69 ab
	½ WW	-M	45.55 ± 1.54 c	3.61 ± 0.11 e	10.89 ± 0.32 c	0.71 ± 0.02 ef	8.75 ± 0.11 b	2.75 ± 0.10 ab	11.53 ± 0.23 ab	14.07 ± 0.64 b
		+M	63.14 ± 1.19 ab	5.20 ± 0.18 ab	17.97 ± 1.01 ab	1.18 ± 0.05 bc	10.85 ± 0.12 a	2.91 ± 0.05 ab	11.11 ± 0.39 ab	14.04 ± 0.53 b
ECO_2	WW	-M	76.26 ± 1.30 ab	6.35 ± 0.13 a	12.79 ± 0.33 bc	1.28 ± 0.03 ab	8.89 ± 0.27 b	3.16 ± 0.10 a	9.85 ± 0.34 b	9.01 ± 0.41 c
		+M	56.36 ± 1.46 bc	5.04 ± 0.17 bc	12.97 ± 0.31 bc	0.85 ± 0.02 de	7.72 ± 0.13 b	2.25 ± 0.07 b	10.69 ± 0.28 ab	14.87 ± 0.61 ab
	½ WW	-M	70.17 ± 1.43 ab	6.45 ± 0.09 a	19.75 ± 0.65 a	1.47 ± 0.03 ab	9.60 ± 0.15 ab	3.08 ± 0.02 a	10.13 ± 0.31 b	12.33 ± 0.55 bc
		+M	49.13 ± 1.00 c	3.82 ± 0.07 de	8.50 ± 0.22 c	0.44 ± 0.22 f	8.56 ± 0.26 b	2.91 ± 0.09 ab	12.40 ± 0.37 a	18.03 ± 0.76 a
Main effects										
CO_2										
	ACO_2		59.12 ± 1.93	4.87 ± 0.17	16.77 ± 0.60	1.16 ± 0.07	9.65 ± 0.18	2.92 ± 0.08	11.47 ± 0.27	18.56 ± 2.97
	ECO_2		62.42 ± 1.40	5.50 ± 0.15	14.03 ± 0.46	1.06 ± 0.05	8.59 ± 0.20	2.75 ± 0.07	10.77 ± 0.33	13.56 ± 0.92
W										
	WW		64.55 ± 1.98	5.58 ± 0.19	15.74 ± 0.33	1.21 ± 0.07	8.90 ± 0.22	2.85 ± 0.08	10.94 ± 0.28	17.51 ± 2.02
	½ WW		56.99 ± 1.23	4.79 ± 0.13	15.06 ± 0.74	1.00 ± 0.05	9.34 ± 0.16	2.81 ± 0.07	11.29 ± 0.34	14.62 ± 0.72
AMF										
	-M		64.36 ± 1.83	5.69 ± 0.19	16.22 ± 0.62	1.30 ± 0.06	9.40 ± 0.16	3.02 ± 0.07	10.68 ± 0.30	11.83 ± 0.48
	+M		57.18 ± 1.42	4.68 ± 0.29	14.59 ± 0.47	0.92 ± 0.04	8.84 ± 0.22	2.64 ± 0.08	11.56 ± 0.30	20.30 ± 2.95
	CO_2		ns	ns	*	ns	*	ns	ns	ns
	W		*	*	ns	*	ns	ns	ns	*
	AMF		**	**	ns	**	ns	*	*	**
	CO_2 × W		ns	ns	*	ns	ns	*	ns	ns
	CO_2 × AMF		**	**	**	**	ns	ns	ns	*
	W × AMF		*	ns	ns	**	*	*	ns	ns
	CO_2 × W × AMF		**	**	**	**	*	**	ns	ns

Table 2 Carbohydrates in leaves of *Pennisetum glaucum* non-inoculated (-M) or inoculated (+M) with arbuscular mycorrhizal fungi (AMF), cultivated either under well-watered conditions (WW) or limited irrigation (½ WW), and grown either at ambient (ACO_2) or under elevated (ECO_2) CO_2. Values are means (n = 4) ± SE separated by Duncan Multiple Range Test ($P \leq 0.05$); different letters indicate significant differences within treatments as affected by the main factors 'atmospheric CO_2, CO_2, 'water regime, W' and 'mycorrhizal inoculation, AMF' and their interactions. ns = not significant; * and ** = significant at $P \leq 0.05$ and $P \leq 0.01$, respectively. DM = dry matter; TSS = total soluble sugars.

Treatments			Raffinose	Sucrose	Glucose	Xylose	Fructose	Sorbitol	TSS	Starch
			mg g^{-1} DM							
ACO_2	WW	-M	2.08 ± 0.20 a	12.59 ± 1.46 a	4.06 ± 0.71 a	1.09 ± 0.21 b	5.81 ± 1.11 c	0.12 ± 0.02 a	25.68 ± 3.59 b	11.54 ± 1.39 ab
		+M	1.25 ± 0.30 c	5.72 ± 1.29 c	1.64 ± 0.16 d	0.51 ± 0.08 c	2.02 ± 0.21 d	0.13 ± 0.01 a	11.40 ± 1.98 e	8.39 ± 1.10 b
	½ WW	-M	1.61 ± 0.61 c	6.74 ± 3.23 c	2.58 ± 0.35 cd	0.67 ± 0.10 bc	3.71 ± 0.73 cd	0.13 ± 0.01 a	14.81 ± 4.90 d	9.35 ± 1.34 b
		+M	2.08 ± 0.33 a	10.79 ± 1.89 b	2.59 ± 0.38 cd	0.69 ± 0.10 bc	3.63 ± 0.60 cd	0.11 ± 0.01 ab	20.96 ± 2.70 c	8.46 ± 1.04 b
ECO_2	WW	-M	0.65 ± 0.05 d	3.79 ± 0.43 d	5.43 ± 1.11 a	2.05 ± 0.40 a	13.12 ± 2.89 a	0.13 ± 0.04 a	25.32 ± 4.79 b	10.54 ± 1.33 ab
		+M	1.59 ± 0.28 b	9.65 ± 2.22 b	3.44 ± 0.81 bc	1.04 ± 0.22 b	4.63 ± 1.34 cd	0.08 ± 0.02 b	20.48 ± 4.74 c	3.67 ± 0.47 c
	½ WW	-M	1.64 ± 0.65 b	12.63 ± 3.42 a	5.69 ± 1.66 a	1.79 ± 0.48 a	9.10 ± 2.67 b	0.14 ± 0.03 a	33.28 ± 6.47 a	13.89 ± 1.84 a
		+M	0.57 ± 0.29 d	2.94 ± 0.95 d	1.79 ± 0.24 d	0.72 ± 0.14 bc	3.10 ± 1.57 d	0.12 ± 0.01 a	11.58 ± 2.18 e	10.84 ± 0.31 ab
Main effects										
CO_2										
	ACO_2		1.76 ± 0.10	9.01 ± 0.77	2.72 ± 0.24	0.74 ± 0.07	3.80 ± 0.36	0.13 ± 0.01	18.21 ± 1.47	9.44 ± 0.49
	ECO_2		1.12 ± 0.14	7.26 ± 1.07	4.09 ± 0.44	1.41 ± 0.16	7.49 ± 1.13	0.12 ± 0.01	22.67 ± 2.09	9.74 ± 1.25
W										
	WW		1.40 ± 0.14	7.94 ± 0.89	3.65 ± 0.37	1.18 ± 0.15	6.40 ± 1.09	0.12 ± 0.01	20.72 ± 1.56	8.54 ± 0.83
	½ WW		1.48 ± 0.15	8.32 ± 1.02	3.17 ± 0.41	0.97 ± 0.15	4.89 ± 0.78	0.13 ± 0.01	20.16 ± 2.18	10.64 ± 0.99
AMF										
	-M		1.50 ± 0.14	8.94 ± 1.03	4.45 ± 0.35	1.41 ± 0.16	7.94 ± 1.00	0.13 ± 0.01	24.78 ± 1.77	11.33 ± 0.91
	+M		1.38 ± 0.14	7.32 ± 0.83	2.37 ± 0.22	0.75 ± 0.07	3.35 ± 0.40	0.12 ± 0.01	16.11 ± 1.25	7.85 ± 0.76
	CO_2		**	**	**	**	**	ns	**	ns
	W		ns	*	*	ns	*	ns	ns	*
	AMF		ns	**	**	**	**	*	**	**
	CO_2 × W		*	ns	ns	ns	*	*	ns	**
	CO_2 × AMF		*	ns	**	**	**	ns	**	ns
	W × AMF		*	**	ns	ns	*	ns	ns	ns
	CO_2 × W × AMF		**	**	**	ns	ns	ns	**	ns

Table 3 Significance of the main factors 'atmospheric CO_2, CO_2, 'water regime, W' and 'mycorrhizal inoculation, AMF' and their interactions (Duncan Multiple Range Test, $P \leq 0.05$) on proline and protein concentrations in leaves of *Pennisetum glaucum* non-inoculated (-M) or inoculated (+M) with arbuscular mycorrhizal fungi (AMF), cultivated either under well-watered conditions (WW) or limited irrigation (½ WW), and grown either at ambient (ACO_2) or under elevated (ECO_2) CO_2. ns, not significant; * and **, significant at $P \leq 0.05$ and $P \leq 0.01$, respectively.

Treatments	Proline	Proteins
Main effects		
CO_2		
ACO_2	3.03 ± 0.45	176.92 ± 8.38
ECO_2	2.09 ± 0.40	146.49 ± 10.84
W		
WW	2.01 ± 0.27	165.97 ± 9.68
½ WW	3.11 ± 0.53	157.44 ± 11.07
AMF		
-M	2.13 ± 0.35	158.87 ± 11.23
+M	2.99 ± 0.50	164.54 ± 9.55
CO_2	**	**
W	**	ns
AMF	**	ns
CO_2 × W	**	**
CO_2 × AMF	ns	*
W × AMF	*	ns
CO_2 × W × AMF	ns	ns

Figure captions

Figure 1 Microscopic images (× 100) of roots belonging to plants inoculated (+M) with arbuscular mycorrhizal fungi (AMF). Fungal structures: v = vesicle.

Figure 2 Concentrations of proline (µmol g^{-1} DM) (*a*) and total soluble proteins (mg g^{-1} DM) (*b*) in leaves of *Pennisetum glaucum* inoculated (+M, black bars) or not (-M, white bars) with arbuscular mycorrhizal fungi (AMF), cultivated under either well-watered (WW) or restricted irrigation (½ WW) conditions, and grown at either ambient (ACO_2) or under elevated (ECO_2) CO_2. Values are means (n = 10) ± SE. Within each graph, histograms with the same letter indicate that values did not differ significantly ($P \leq 0.05$).

Figure 1

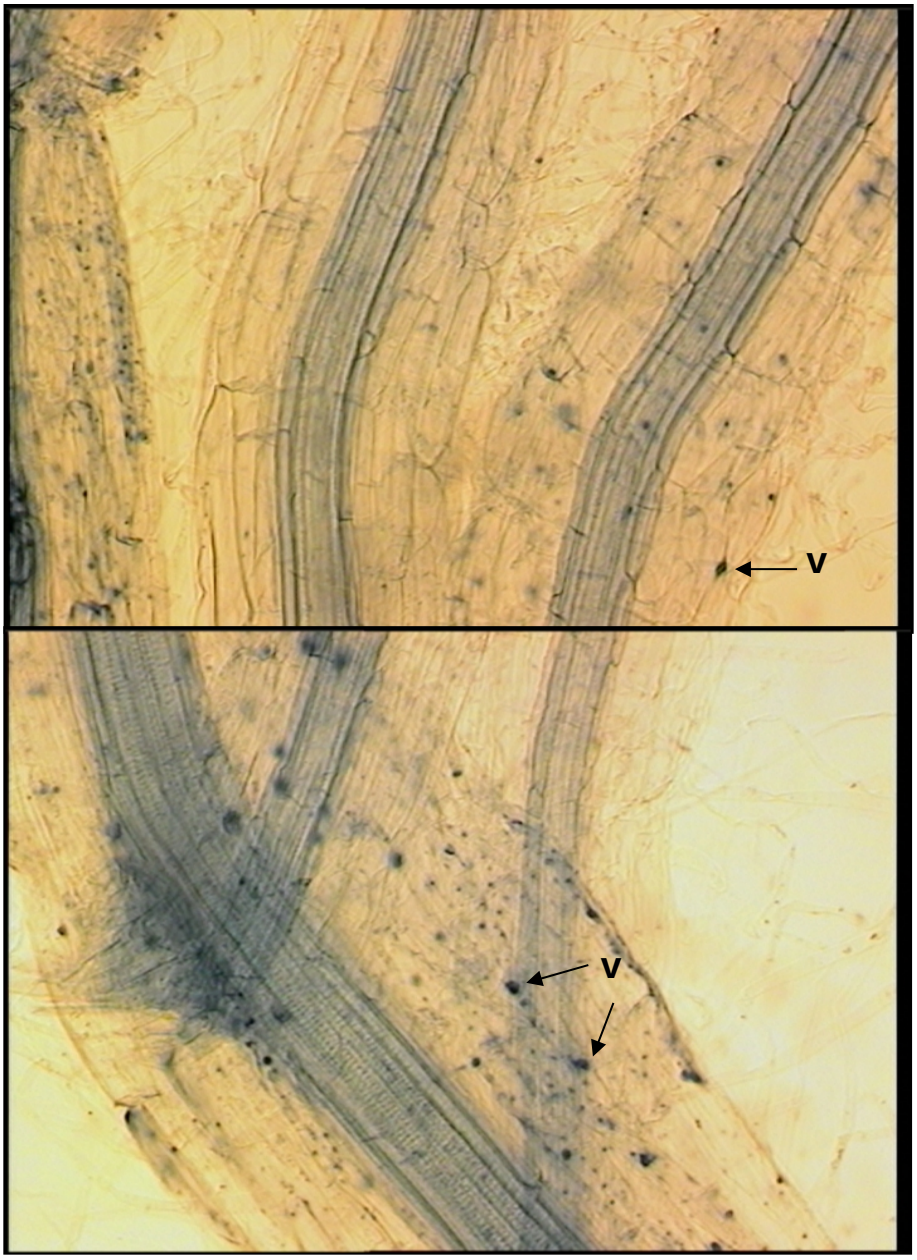
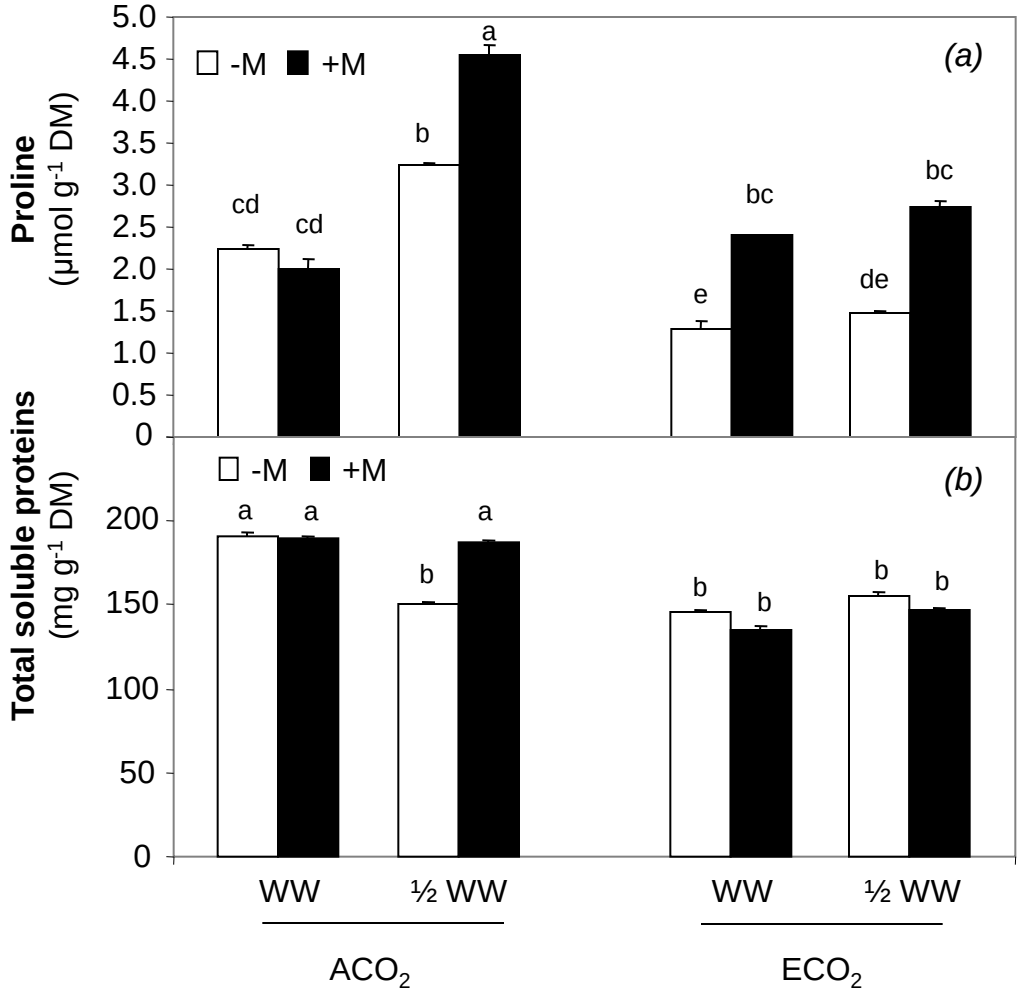


Figure 2


Proline
(µmol g-1 DM)
-M
+M
(a)
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
cd
cd
b
a
e
bc
de
bc
Total soluble proteins
(mg g-1 DM)
(b)
200
150
100
50
0
a
a
b
a
b
b
b
b
WW
½ WW
WW
½ WW
ACO2
ECO2