1 **Climate change in forest ecosystems: a field experiment addressing the effects of raising**

2 **temperature and reduced rainfall on early life cycle stages of oaks**

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1

19 **Abstract**

20 Higher temperatures and reduced rainfalls that are expected with the advance of climate change

21 can impair the emergence and establishment of tree seedlings in forest ecosystems. These

22 climatic changes can also decrease the availability of soil resources and reduce the performance

23 of seedlings. We evaluated these effects in a temperate forest from Mexico with two native oak

24 species (*Quercus crassifolia* and *Quercus eduardii*). As recently emerged oak seedlings are

25 highly sensitive to changing environmental conditions, our field experiment was conducted

26 across the season in which seedling emergence occurs (October-February). In the field, we used

27 open-top chambers to increase temperature and rainout shelters to reduce rainfall, while controls

28 were exposed to the current climate. Experimental plots of both treatments were established

29 beneath the forest canopy because most oaks recruit in understory habitats. In these plots, we

30 sowed acorns of both species in October 2015 and recorded seedling emergence and survival

31 until February 2016, also monitoring temperature, precipitation and contents of water and

32 nitrogen in the soil. On seedlings that survived until the end of the experiment we measured their

33 growth, photosynthetic efficiency and foliar contents of water, carbon and nitrogen. Both the

34 emergence and survival of *Q. crassifolia* seedlings were lower in climate change plots than in

35 controls, but no differences were found for *Q. eduardii*. However, seedlings of both species had

36 lower growth rates, photosynthetic efficiencies and contents of water, nitrogen and carbon in

37 climate change simulation plots. These results indicate that climate change can impair tree

38 seedling establishment in oak forest, also suggesting that their development will be constrained

39 by reduced water and nitrogen availability.

40

1. Introduction

The prevalence of forest ecosystems mainly depends on the emergence and establishment of tree seedlings, but climate change can alter these processes (Clark et al., 2016). In temperate forests from North America, most global circulation models predict that the average air temperature will rise 5° C in the course of this century, while annual precipitation levels in these ecosystems are projected to decrease up to 20% (IPCC, 2013). These ecosystems will then face warmer and drier conditions in the future, but field experiments assessing how tree seedlings will deal with these changes are still scarce.

Water availability is a key factor that regulates the emergence of tree seedlings in forests and climate change can strongly impair this process (Pérez-Ramos et al., 2013; Clark et al., 2016). This is particularly critical for tree species that produce recalcitrant seeds because higher temperatures and lower precipitation levels can enhance seed water loss and induce embryo mortality, hence reducing seedling emergence (Zavala-Chávez, 2008; Joët et al., 2013). Further, if seedlings emerge under these conditions, they can display a number of functional responses that would ultimately reduce their performance in climate change scenarios. Increased temperatures usually enhance foliar transpiration but, when plants are also subjected to water deficits, stomatal closure is induced to avoid excessive water loss and carbon fixation rates are reduced due to low gas exchange (Chaves et al., 2002; Haldimann and Feller, 2004; Rodriguez-Dominguez et al., 2016). Climate change can also affect light-dependent processes of photosynthesis in tree seedlings. This is because elevated thermal and/or water stress reduce the flow of electrons in the thylakoid membrane of chloroplasts and this decreases the conversion of light energy into chemical energy for fixing carbon (Maxwell and Johnson, 2000; Baker and Oxborough, 2004). Thus, the combined effects of warmer and drier conditions expected in forest ecosystems can alter water and carbon balances of tree seedlings and reduce their survival.

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67   The availability of soil nitrogen is another important factor that can be altered by climate

68   change. Nitrogen is the most important mineral nutrient that regulates plant growth (Poorter et

69   al., 2012) and its natural inputs in forest ecosystems mainly depend on the activity of soil bacteria

70   (Brookshire et al., 2011). These microorganisms are extremely sensitive to changes in soil

71   temperature and moisture and, thus, the expected variations in climatic conditions can modify

72   their efficiency for mineralizing nitrogen (Brookshire et al., 2011; Chen et al., 2011). However,

73   the net effects of climate change on soil nitrogen are still controversial. This is because warming

74   may stimulate the metabolic activity of nitrogen-fixing bacteria and increase soil nitrogen

75   contents (Melillo et al., 2002; Brookshire et al., 2011; Butler et al., 2012), but reductions in soil

76   moisture content due to reduced water inputs are expected to cause the opposite effects (Borken

77   and Matzner, 2009; Chen et al., 2011). Instead, from the perspective of tree seedlings, the

78   combined effects of increasing temperatures and reduced precipitation levels can jeopardize their

79   ability to acquire nitrogen irrespective of whether its concentrations increase or decrease in the

80   future. This is because these climatic conditions can induce strong water deficits and decrease the

81   mobility of the mineral forms of nitrogen in the soil matrix, reducing the capability of plants to

82   uptake this resource through the roots (Rennenberg et al., 2009; He and Dijkstra, 2014). In

83   consequence, nitrogen contents and growth rates of tree seedlings in forest ecosystems are likely

84   to decrease with the advance of climate change.

85   This study assesses how climate change could affect the emergence, survival and performance

86   of recently emerged oak seedlings in a temperate forest of Mexico. For this, we performed a field

87   experiment in which temperature and precipitation were manipulated during the period of the

88   year in which the emergence of oak seedlings occurs, as these early life-cycle stages of oaks are

89   extremely sensitive to changing environmental conditions (Zavala-Chávez and García-Moya,

90   1996; Badano et al., 2011, 2015). In these ecosystems, adult oaks produce acorns (nut-type,

91   single-seeded fruits) in spring and release them in late summer, concurring with the second half

92   of the rainy season (Zavala-Chávez and García-Moya, 1996; González-Salvatierra et al., 2013;

93   Badano et al., 2015). As seeds of Mexican oaks are extremely recalcitrant (embryos die if seed

94   water content drops below 25-30%) and they require elevated hydration levels to germinate

95   (above 80% of seed water content), seedling emergence in the field occurs in short time after

96   acorns are released from parental trees (Zavala-Chávez and García-Moya, 1996; Zavala-Chávez

97   2008). The development of recently emerged oak seedlings mainly depends on the nutritional

98   reserves accumulated in their cotyledons, but they are extremely susceptible to die because of

99   thermal and water stress (Badano et al., 2009; González-Salvatierra et al., 2013). Thus, we

100   hypothesized that warmer and drier conditions expected in climate change scenarios will reduce

101   the emergence and survival of oak seedlings, also decreasing their overall performance. As

102   climate change can reduce nitrogen availability for plants, we also hypothesized that the content

103   of nitrogen in seedlings subjected to climate change conditions will be lower than those of

104   seedling growing under the current climate.

105

106   **2. Materials and Methods**

107   *2.1. Study area*

108   Our experiment was conducted at National Park "El Potosí", located in the westernmost section

109   of Sierra Madre Oriental (Sierra de Álvarez), state of San Luis Potosí, Mexico (include the file

110   "Pérez Ruiz et al. Experimental sites.kmz" here). Climate in this region is temperate (mean

111   annual temperature 18 °C) and up to 80% of rainfalls occurs between June and November

112   (annual precipitation 500-600 mm), but sparse precipitation events can happen until January

113   (Vargas-Márquez, 1997). Soils are shallow, with maximum depths of 10-15 cm, and vegetation is

114   composed by semi-deciduous oak forests. In these ecosystems, despite the shallowness of soils,

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115   tree roots penetrate the bedrock through small fissures, anchoring these organisms to the substrate

116   (Zavala-Chávez and García-Sánchez, 1999). As seedling emergence mainly occurs in the upper

117   section of the soil profile of the forest understory (Badano et al., 2015), we located the

118   experimental site beneath the canopy of a well-preserved oak forest dominated by *Quercus*

119   *crassifolia* Humb. & Bonpl., *Quercus eduardii* Trel., and *Quercus jonesii* Trel. (21°55'10'' N,

120   100°19'34'' W, 2123 m elevation).

121   To determine how much climate will change in the study site, we computed the difference

122   between current and future values of air temperature and precipitation using the geodatabases of

123   Fernández-Eguiarte et al. (2012, 2014). These geodatabases provide the values of these two

124   variables with a spatial resolution of 1-km^2 per pixel, where current values are monthly averages

125   that integrate data from 1902 to 2011 (Fernández-Eguiarte et al., 2012) and future values are

126   estimated for each month of the year with the HadGEM2-ES climate change model (Fernández-

127   Eguiarte et al., 2014). Future values of air temperature and precipitation are provided for the

128   period 2021-2040 and consider the four climate change scenarios (RCP2.6, RCP4.5, RCP6.0 and

129   RCP8.5) proposed in the latest report of IPCC (2013). The HadGEM2-ES model is commonly

130   used make climate change predictions across continental Mexico because it has lower deviations

131   than other models when past climatic conditions are estimated, and this reduces uncertainty about

132   the future climate (Fernández-Eguiarte et al., 2014). Because we focused on the earlier life-cycle

133   stages of oaks, differences between current and future values of these climatic conditions were

134   computed for the period of the year in which seedling emergence occurs, comprised between

135   October and February. These estimates indicated that, during the tree recruitment season, air

136   temperature in the study site will increase about 1.3-1.7° C and precipitation will decrease by 11-

137   18% (Table 1).

Climate change scenario	Air temperature (net change)	Precipitation (percent change)
RCP2.6	+1.7 °C	-18%
RCP4.5	+1.2 °C	-14%
RCP6.0	+1.3 °C	-11%
RCP8.5	+1.3 °C	-16%

Table 1. Short term changes (period 2021-2040) in air temperature and precipitation predicted for the study site during the oak recruitment season (October-February). Predictions were performed with the HadGEM2-ES model considering the four climate change scenarios (RCP2.6, RCP4.5, RCP6.0 and RCP8.5) proposed in the latest IPCC report.

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139 *2.2. Experimental design*

140 The field experiment was carried out between 17 October 2015 and 27 February 2016,

141 comprising the entire period in which oak seedlings emerge and their early establishment occurs.

142 For this, we draw a quadrant of 50 x 50 m in the study site and randomly selected 20 point-sites

143 within it on September 2015, taking care of maintaining a minimum distance of 5 m among them.

144 Leaf litter and rocks were removed in a radius of 3 m around each of these sites. The half of the

145 sites were randomly assigned to climate change simulation plots (hereafter, CCS plots) where we

146 manipulated temperature and precipitation, while the other half of the sites were assigned to

147 control plots that were maintained under the current environmental conditions. To manipulate

148 temperature in CCS plots we used hexagonal open-top chambers (Marion et al., 1997), which

149 were built with sheets of transparent acrylic (3 mm thick, wavelength transmission 280-750 nm).

150 The resulting open-top chambers were 0.50 m tall, 1.5 m wide at the open-top, and 2.08 m wide

151 at the base (Fig. 1A). Previous studies indicate that this open-top chamber design can increase air

152 temperature by 1-3 °C relative to the external environment, without affecting light transmission

153 within the chambers (Dabros and Fyles, 2010; Aragón-Gastélum et al., 2014). Precipitation

154 within CCS plots was reduced with rainout shelters (Yahdjian and Sala, 2002), which consisted

7

155   in five U-shaped channels of transparent polycarbonate (1.5 mm thick, 10 cm wide, 3 m length)

156   equidistantly arranged on metallic frames in order to cover 15% of the open-top of chambers

157   (Fig. 1A). Before the experiment, we tested whether rainout shelters reduced precipitation within

158   the ranges predicted for the study site (see above). These trials indicated that these shelters

159   reduced precipitation by 10-20% at each rainfall event. Control plots, on the other hand, were

160   fenced with wire mesh to prevent the access of granivorous and herbivorous vertebrates, such as

161   mice, squirrels and birds (Fig. 1B).

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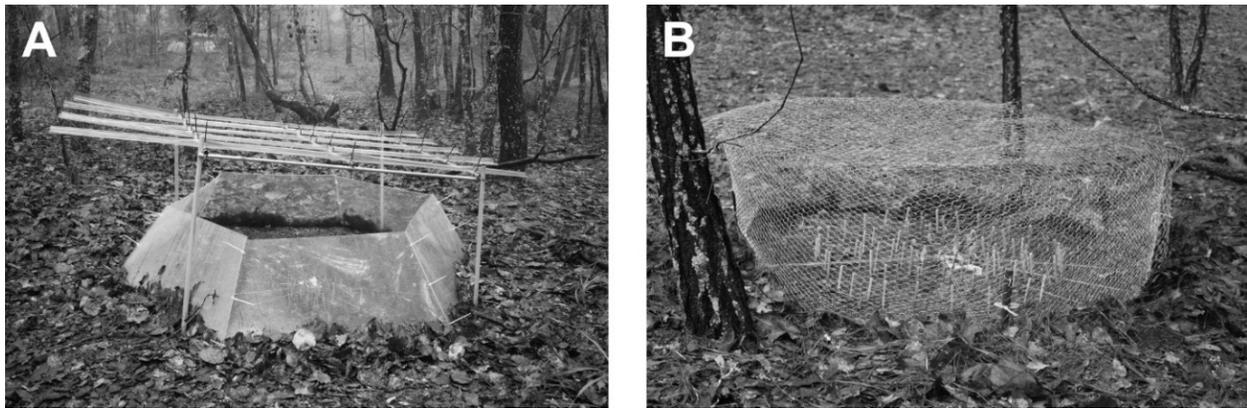
Fig. 1. Climate change simulation plots (CCS) consisting in open-top chambers covered with rainout shelters (A) and control plots fenced with wire mesh (B).

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165   Oak seedlings were developed in the field by sowing acorns within the former experimental

166   plots. We only included two dominant oaks of the study site (*Q. crassifolia* and *Q. eduardii*)

167   because the third species (*Q. jonesii*) did not produce acorns in 2015. To gather acorns of *Q.*

168   *crassifolia* and *Q. eduardii*, we marked ten trees of each species with developing fruits on 09

169   August 2015 and removed leaf litter beneath their canopies. On 27 September 2015, after acorns

170   were released from parental trees, we collected 100 mature fruits beneath the canopy of each of

171   them. Because acorns quickly lose viability due to desiccation or infestation by insects, they were

172   taken to the laboratory and the float test was used to separate viable and unviable acorns (Gribko

173   and Jones, 1995). For this, we removed acorn cupules and placed the nuts in 20 L containers

174   filled with water. After 2 h in water, we assumed that sunken acorns were viable and floating

175   acorns were unviable (Gribko and Jones, 1995). This resulted in more than 600 viable acorns of

176   each species, which were stored at 5° C to prevent viability loss due to desiccation.

177   After acorn collection, we weekly visited the study site to assess the moment in which natural

178   emergence of oak seedlings started in the field. This occurred on 14 October 2015 and, at this

179   date, we randomly selected 200 viable acorns of each oak species and placed them in water

180   during 48 h. We did this because acorns require to reach their maximum hydration capacity to

181   germinate (Zavala-Chávez and García-Moya, 1996; Zavala-Chávez, 2008). These acorns were

182   sowed on 17 October 2015. For this, we randomly assigned five controls and five CCS plots to

183   each oak species and sowed 20 acorns within them. Acorns were sowed 2 cm depth to mimic the

184   behavior of scatter-hoarding mice, which are their main secondary dispersers in North American

185   oak forests (Smallwood et al., 2001). Sowing positions were marked with small wood stakes and

186   the emergence of aerial shoots was monitored every seven days until 27 February 2016. At each

187   monitoring date, we also recorded whether emerged seedlings were alive. Seedlings were

188   assumed to have died when their stems and apical buds were completely withered (González-

189   Salvatierra et al., 2013). We also recorded the dates of leaf unfolding on each seedling because, if

190   climate change alters their phenology, leaf age would be required to control for effects of time in

191   the analyses of foliar functional traits described below. To avoid pseudo-replication, leaf ages

192   (i.e., number of days elapsed after leaf unfolding) were averaged across leaves of each oak

193   seedling.

194

195   *2.3. Climate and soil conditions*

196   To determine the extent of the microclimatic changes induced with open-top chambers and

197   rainout shelters, as compared to current climate, we randomly selected three plots of each

198   treatment (controls and CCS plots) and installed automatized sensors to measure temperature and

199   relative humidity of the air, soil temperature and precipitation. Temperature and relative humidity

200   of the air were measured with dual dataloggers fixed on wood stakes 20 cm above ground

201   (HOBO U23-Pro-V2, Onset Computer Corporation, USA), while soil temperature was measured

202   with dataloggers buried 5 cm depth (HOBO Pendant-UA-002, Onset Computer Corporation,

203   USA). In this way, temperature was measured in the environments in which acorns germinate

204   (belowground) and oak seedlings develop (aboveground). These dataloggers were programed to

205   measure climatic variables every hour during the entire experiment. Precipitation was measured

206   with automatized pluviometers programed to record this variable at each rainfall event (HOBO S-

207   RGB-M002, Onset Computer Corporation, USA). All these sensors were arranged at the center

208   of each plot taking care of avoiding interference among them.

209   We also measured soil volumetric water content during the entire experiment. For this, we

210   randomly selected five experimental plots of each climate treatment and marked them. In these

211   plots, we performed six point-measures every week using a time-domain reflectometer

212   (FieldScout TDR 300, Spectrum Technologies, USA) and these values were averaged per plot at

213   each measurement date. This latter variable was measured at 3.8 cm depth, corresponding to the

214   environment in which acorns were sowed and can influence seedling emergence, and 12.0 cm

215   depth, corresponding to the environment from which the roots of emerged seedlings can uptake

216   water and nutrients.

217   To assess whether the climate change induction alters soil nitrogen content, we measured the

218   concentrations of nitrate (NO_3^-) and ammonium (NH_4^+) in controls and CCS plots. We focused

219   on NO_3^- and NH_4^+ because these are the mineral forms of nitrogen that plants can use (Fitter,

220   1997). Concentrations of NO_3^- and NH_4^+ were measured on the five plots of each climate

221   treatment that we selected to perform water content measures. Nevertheless, as measuring NO_3^-

222   and NH_4^+ required to extract soil samples and this could damage oak seedlings, these

223   measurements were only conducted at the beginning (before climate manipulation) and at the end

224   (after climate manipulation) of the experiment. At both times, four topsoil cores (5 cm depth, 2

225   cm width) were collected within each experimental unit and they were pooled in a mixed sample.

226   This sample was meshed (2 mm mesh) and its NO_3^- content was determined in the field with

227   portable ion-selective meters (LAQUATwin NO_3^- meter B-742, Horiba, Japan). This procedure

228   was repeated three times for each soil sample and NO_3^- readings were averaged. The remaining

229   fraction of each soil sample was placed in a hermetic plastic bag and stored in ice to reduce the

230   metabolic activity of soil microorganism. After arriving to the laboratory (about 3 h after soil

231   collection), these soil samples were spread on aluminum sheets and dried in an air-forced stove at

232   40° C until their weight remained constant (about 48 h). Once samples were fully dry, we took 5

233   g of soil and NH_4^+ was extracted with 2 M potassium chloride solution. These extracts were

234   treated with Berthelot's reagent and read in a UV-visible spectrophotometer (Genesys 10S,

235   Thermo Fisher Scientific). This procedure was repeated three times for each soil sample and

236   NH_4^+ values were averaged.

237

238   *2.4. Oak seedling responses*

239   At the end of the experiment, ungerminated acorns were recovered from the experimental plots

240   and taken to the laboratory to determine their viability. For this, we made a small incision on the

241   pericarp of acorns and incubated them in 1% triphenyltetrazolium chloride solution during 24 h

242   in dark. If seed embryos are alive, the colorless tetrazolium salt reacts with the hydrogen released

243   by active dehydrogenase enzymes and this generates formazan, a non-diffusible red dye (Bewley

244   et al., 2013). As no recovered acorn was viable (i.e., the embryos were not red-stained), we

245   assumed that no additional germination would occur after the experiment was finished. These

246   data were used to compare the proportion of germinated acorns and surviving seedlings between

247   controls and CCS plots. At this time, we also measured the length of the aerial shoots of all

248   surviving seedlings (i.e., length from the ground to the apical bud) with a digital caliper. These

249   data were used to estimate their net growth rates as the ratio between the length of the aerial

250   shoot and the age of each seedling (i.e., the number of days elapsed after shoot emergence) and

251   these values were compared between climatic treatments.

252   We later assessed a series of foliar functional responses on three surviving seedlings from each

253   experimental plot to determine whether climate change can affect their performance. All these

254   seedlings had at least three mature leaves without symptoms of wilting or insect damage, which

255   were marked and numbered with small paper tags. In the field, we used a portable fluorometer

256   (MINI-PAM II, Heinz Walz, Germany) to assess foliar temperature and photosynthetic photon

257   flux density (*PPFD*) on each leaf, and estimated the effective quantum yield (ϕ_{PSII}) by measuring

258   their chlorophyll fluorescence. Values of ϕ_{PSII} were computed as $(F'm - Ft) / F'm$, where F_t is

259   the fluorescence emitted by leaves under steady-state illumination and F'_m is their maximum

260   fluorescence when a saturating pulse of actinic light (≈ 2500 µmol photon m^{-2} s^{-1}, in our case) is

261   superimposed on the environmental light level (Maxwell and Johnson, 2000). These

262   measurements were conducted between 10:00 and 12:00 h because, after midday, the elevated

263   levels of light could mask the effects of other environmental factors on photosynthetic quantum

264   yield (Franco and Lüttge, 2002). Values of leaf temperature, *PPFD* and ϕ_{PSII} were averaged

265   across leaves belonging to the same seedling to avoid pseudo-replication.

266   Tagged leaves were later harvested, stored in hermetic plastic bags and placed in ice to reduce

267   water loss. These leaves were weighed immediately after they arrived at the laboratory (about 3 h

268   after harvesting) to determine their fresh biomass (*FB*). After that, leaves were dried in an air-

269   forced stove at 60° C until their dry biomass (*DB*) remained constant (about 24-36 h). Values of

270   *FB* and *DB* were determined using an analytical balance with resolution of 0.1 mg (Cubis,

271   Sartorius, Germany) and used to estimate the percent water content (*PWC*) of leaves as

272   (*FB* − *DB*) / *FB*. These values were averaged across the leaves of each seedling and used as

273   surrogate of their water status at the moment of harvesting (Gond et al., 1999). Further, to assess

274   whether climate change can affect the carbon and nitrogen balance of recently emerged oak

275   seedlings, dried leaves were ground in a ball mill (MM200, Retsch, Germany) and processed to

276   determine the percent contents of carbon and nitrogen with an elemental analyzer (ECS 4010,

277   Costech, USA). Because of their small size, leaves collected on each seedling were pooled in a

278   single sample to reach the minimum biomass (0.8 g) required for these analyses.

279

280   *2.5. Statistical analysis*

281   We used repeated measures ANOVAs to compare all environmental variables between climate

282   treatments. For these analyses, values of air and soil temperature, as well as values of relative

283   humidity of the air, were averaged at each week of the experiment for each datalogger (n = 3 per

284   climate treatment; repeated measures = 19 weeks). On the other hand, precipitation recorded by

285   each pluviometer was accumulated for every week of the experiment (n = 3 per climate

286   treatment) but, as ANOVAs does not admit groups with zero variance (Zar, 2010), we excluded

287   those weeks with no rainfalls from this analysis (repeated measures = 11 weeks). Comparisons of

288   soil water contents at 3.8 and 12.0 cm depth were conducted with the values measured every

289   week of the experiment (n = 5 per climate treatment; repeated measures = 19 weeks), while soil

290 concentrations of NO_3^- and NH_4^+ were compared by only including the two dates in which these

291 variables were measured (n = 5 per climate treatment; repeated measures = 2 dates).

292   The proportion of germinated acorns and surviving seedlings were compared between climate

293 treatments and oak species with two-way factorial ANOVA (2 climate treatments x 2 oak species

294 = 4 treatments in total; n = 5 per treatment). The identity of oaks was included in these analyses

295 because responses to climate change may vary between species. The same analyses were used to

296 compare the age of seedlings, their net growth rates and leaf ages, *PWC*, foliar temperature,

297 *PPFD*, ϕ_{PSII}, and foliar contents of nitrogen and carbon. The values of all these variables were

298 averaged across seedlings from the same experimental unit to avoid pseudo-replication. All

299 datasets that support the former analyses were deposited in Mendeley Data

300 (http://dx.doi.org/10.17632/bsvxh88ypz.1).

301

302 **3. Results**

303 *3.1. Climate and soil conditions*

304 Air temperature was higher in CCS plots than in controls across the entire experiment, while the

305 converse occurred with precipitation (Table 2). Differences in air temperature between treatments

306 increased from the beginning to the end of the experiment; CCS plots were about 1.3° C warmer

307 than controls until 18 December 2015, but this difference increased up to 1.5° C after that date

308 (Fig. 2A). Precipitation was 10-11% lower in CCS plots than in controls on those weeks where

309 rainfalls surpassed 10 mm, while CCS plots received 14-15% less precipitation on those weeks

310 where rainfalls were below that value (Fig. 1B).

Environmental variable	Climate treatment	Time (weeks)	Interaction climate treatment-time
Air temperature	$F_{(4,1)} = 853.759$*	$F_{(18,72)} = 5178.385$*	$F_{(18,72)} = 3.163$*

Precipitation	$F_{(1,4)} = 339.846*$	$F_{(8,32)} = 10846.486*$	$F_{(8,32)} = 31.435*$
Soil temperature	$F_{(1,4)} = 5310.901*$	$F_{(18,72)} = 1482.021*$	$F_{(18,72)} = 0.229$
Air relative humidity	$F_{(1,4)} = 1076.431*$	$F_{(18,72)} = 14174.897*$	$F_{(18,72)} = 109.919*$
Soil water content-3.8 cm depth	$F_{(1,8)} = 180.184*$	$F_{(18,144)} = 1722.143*$	$F_{(18,144)} = 32.258*$
Soil water content-12.0 cm depth	$F_{(1,8)} = 743.359*$	$F_{(18,144)} = 1522.408*$	$F_{(18,144)} = 35.600*$
Soil nitrate concentration	$F_{(1,8)} = 181.203*$	$F_{(1,8)} = 562.389*$	$F_{(1,8)} = 299.475*$
Soil ammonium concentration	$F_{(1,8)} = 405.474*$	$F_{(1,8)} = 1111.181*$	$F_{(1,8)} = 577.643*$

Table 2. Results of repeated measures ANOVA addressed to compare climatic variables and soil features between climate treatments. The table shows the F-values obtained for each factor (climate treatments and time) and their interaction. Significant effects on each response variable are indicated with an asterisk (critical $\alpha = 0.05$).
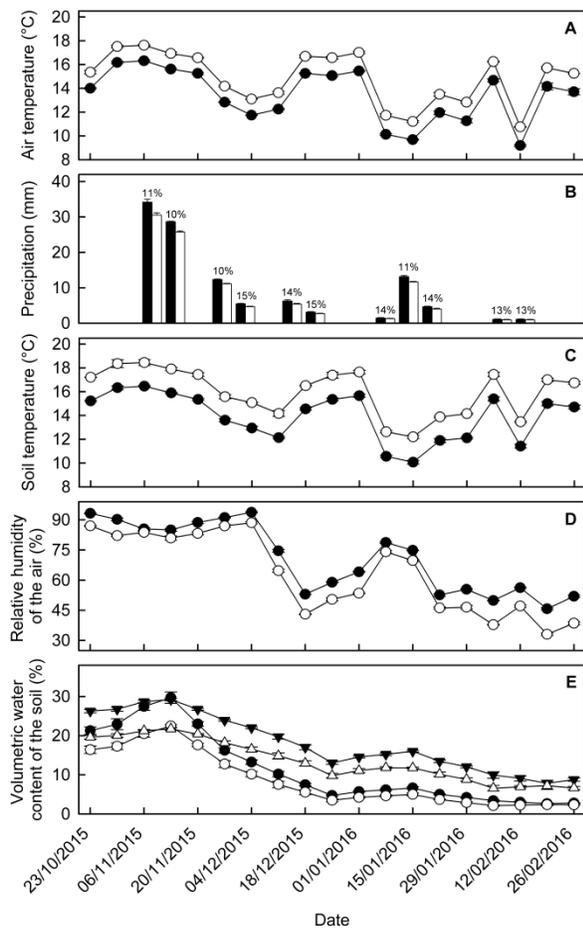
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Fig. 2. Average values (± 95% C.I.) of air temperature (A), precipitation (B), soil temperature (C), relative humidity of the air (D), and soil water content (E) at each week of the experiment in controls (black symbols) and CCS plots (white symbols). Numbers above precipitation bars indicate the percent difference between climate treatments. Soil water content is reported at 3.8 cm (circles) and 12.0 cm depth (triangles).

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312

313   Soil temperature, relative humidity of the air and soil water contents also differed between

314   climate treatments (Table 2). Soil temperature within CCS plots was about 2.0° C higher than in

315   controls and this difference was sustained along the entire experiment (Fig. 2C). Air relative

316   humidity was always lower in CCS plots than in controls, but these differences decreased in rainy

317   weeks (Fig. 2D). Water contents at both, 3.8 cm and 12.0 cm depth, were lower in CCS plots that

318   in controls and these differences increased when rainfalls decreased (January-February; Fig. 2E).

319   Soil concentrations of NO_3^- and NH_4^+ differed between climate treatments and sampling dates

320   (Table 2). At the beginning of the experiment (i.e., before climate manipulation was applied),

321   there were no differences in the concentrations of NO_3^- (Fig. 3A) or NH_4^+ (Fig. 3B) between

322   plots assigned to controls and CCS. However, concentrations of both mineral nutrients were

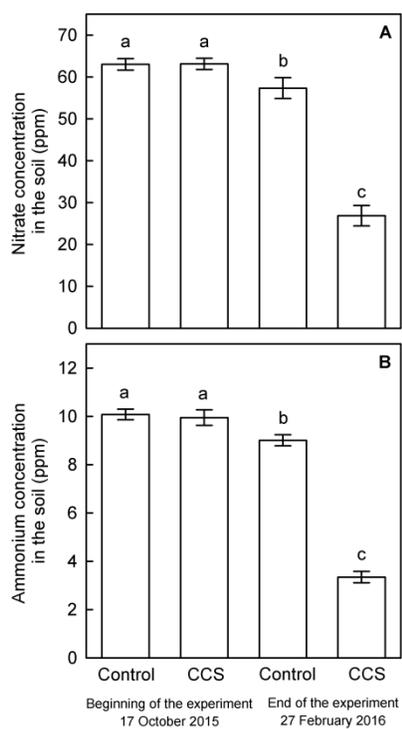323   lower in CCS plots than in controls by the end of the experiment (Fig. 3).

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Fig. 3. Soil concentrations (average ± 95% C.I.) of nitrate (A) and ammonium (B) in controls and CCS plots at the beginning (17 October 2015) and at the end of the experiment (27 February 2016). Different letters above the bars indicate significant differences between treatments (Tukey tests critical α = 0.05).

326

327   *3.2. Seedling responses*

328   The proportion of emerged and surviving seedlings differed between climate treatments

329   (emergence: $F_{(1,16)}$ = 18.086, p = 0.006; survival: $F_{(1,16)}$ = 50.585, p < 0.001) and oak species

330   (emergence: $F_{(1,16)}$ = 60.409, p < 0.001; survival: $F_{(1,16)}$ = 152.669, p < 0.001). Interactive effects

331   of these two factors (climate treatment x oak species) were also found on both response variables

332   (emergence: $F_{(1,16)}$ = 7.763, p = 0.013; survival: $F_{(1,16)}$ = 108.411, p < 0.001). Emergence of *Q.*

333   *crassifolia* seedlings was lower in CCS plots than in controls, while emergence of *Q. eduardii*

334   seedlings did not differed between climate treatments (Fig. 4A). However, in both controls and

335   CCS plots, the proportion of emerged seedlings was higher for *Q. eduardii* than for *Q. crassifolia*

336   (Fig. 4A). The proportion of surviving seedlings in controls did not differ between oak species,

337   but seedlings of *Q. eduardii* had higher survival than those of *Q. crassifolia* in CCS plots (Fig.

338   4B).

339   Age of surviving seedlings neither differed between climate treatments nor oak species,

340   averaging 102 days across all experimental units (data not shown). Conversely, shoot growth

341   rates differed between climate treatments ($F_{(1,16)}$ = 346.225, p < 0.001) and oak species ($F_{(1,16)}$ =

342   9.181, p = 0.008), but no interactive effects between these two factors were found ($F_{(1,16)}$ = 0.113,

343   p = 0.741). This analysis indicated that seedlings of both, *Q. eduardii* and *Q. crassifolia*, had

344   higher shoot growth rates in controls than in CCS plots (Fig. 4C).

345
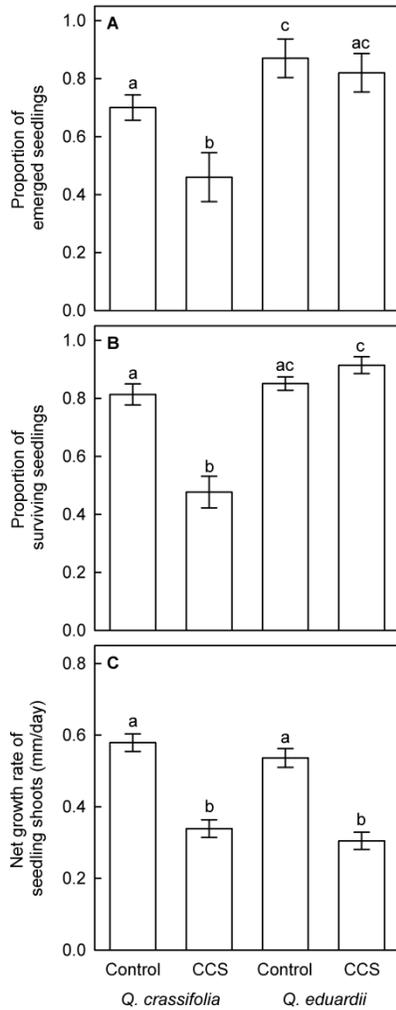
346

Fig. 4. Proportion (average ± 95% C.I.) of emerged seedlings (A) and surviving seedlings (B) of *Q. crassifolia* and *Q. eduardii* in controls and CCS plots. The figure also shows the average (± 95% C.I.) net growth rates of seedling shoots (C) in both climate treatments. Different letters above the bars indicate significant differences between treatments (Tukey tests critical α = 0.05).

347

348   Age of seedling leaves differed between climate treatments ($F_{(1,16)} = 38.267$, $p < 0.001$) and

349   oak species ($F_{(1,16)} = 8.152$, $p = 0.011$), and significant effects were also found for the interaction

350   of these two factors ($F_{(1,16)} = 5.060$, $p = 0.039$). In this case, leaf unfolding occurred latter in *Q.*

351   *crassifolia* seedlings than in seedlings from all the other treatments (Fig. 5A). For this reason,

352   leaf age was included as continuous concomitant variable in the analyses comparing the other

353   foliar functional traits (i.e., we used ANCOVA instead of ANOVA). Values of *PWC* were not

354   affected by leaf age, but significant differences were found between climate treatments and oak

355   species (Table 3). Leaves of both oak species had lower *PWC* in CCS plots than in controls and

356   these differences were higher for *Q. crassifolia* than for *Q. eduardii* (Fig. 5B). Neither leaf age

357   nor oak species affected foliar temperatures (Table 3), but both oak species had higher foliar

358   temperatures CCS plots than in controls (Fig. 5C).

Environmental variable	Leaf age	Climate treatment	Oak species	Interaction climate treatment-oak species
Leaf percent water content	$F_{(1,15)} = 1.501$	$F_{(1,15)} = 60.208*$	$F_{(1,15)} = 10.332*$	$F_{(1,15)} = 2.299$
Foliar temperature	$F_{(1,15)} = 0.285$	$F_{(1,15)} = 32.095*$	$F_{(1,15)} = 1.331$	$F_{(1,15)} = 1.061$
ϕ_{PSII}	$F_{(1,15)} = 1.397$	$F_{(1,15)} = 115.490*$	$F_{(1,15)} = 0.693$	$F_{(1,15)} = 60.504*$
Total foliar nitrogen	$F_{(1,15)} = 0.640$	$F_{(1,15)} = 142.040*$	$F_{(1,15)} = 40.424*$	$F_{(1,15)} = 48.803*$
Total foliar carbon	$F_{(1,15)} = 0.034$	$F_{(1,15)} = 501.276*$	$F_{(1,15)} = 60.225*$	$F_{(1,15)} = 37.492*$

Table 3. Results of ANCOVA comparing leaf traits and associated variables between climate treatments and oak species. The table shows the F-values obtained for each factor (climate treatments and time) and their interaction. Significant effects on each response variable are indicated with an asterisk (critical $\alpha = 0.05$). In these analyses, leaf age was included as concomitant continuous variable.

359

360   Values of *PPFD* neither differed between climate treatments nor oak species (data not shown),

361   indicating that leaves of all seedlings were exposed to similar levels of light (578.606 μmol m^{-2} s^{-1}

362    $\pm$ 6.643 95% I.C.) during the chlorophyll fluorescence measurements. Values of ϕ_{PSII} were not

363   affected by leaf age and they did not differ between oak species (Table 3). However, seedlings

364   both of oak species displayed higher ϕ_{PSII} values in controls than CCS plots (Table 3; Fig. 5D).

365

Fig. 5. Foliar functional traits (average ± 95% C.I.) of *Q. crassifolia* and *Q. eduardii* seedlings from controls and CCS plots, including age of leaves (A), leaf percent water content (B), foliar temperature (C), effective quantum yield of photosystem II (D), foliar content of nitrogen (E) and foliar content of carbon (F). Different letters above the bars indicate significant differences between treatments (Tukey tests critical $\alpha = 0.05$).
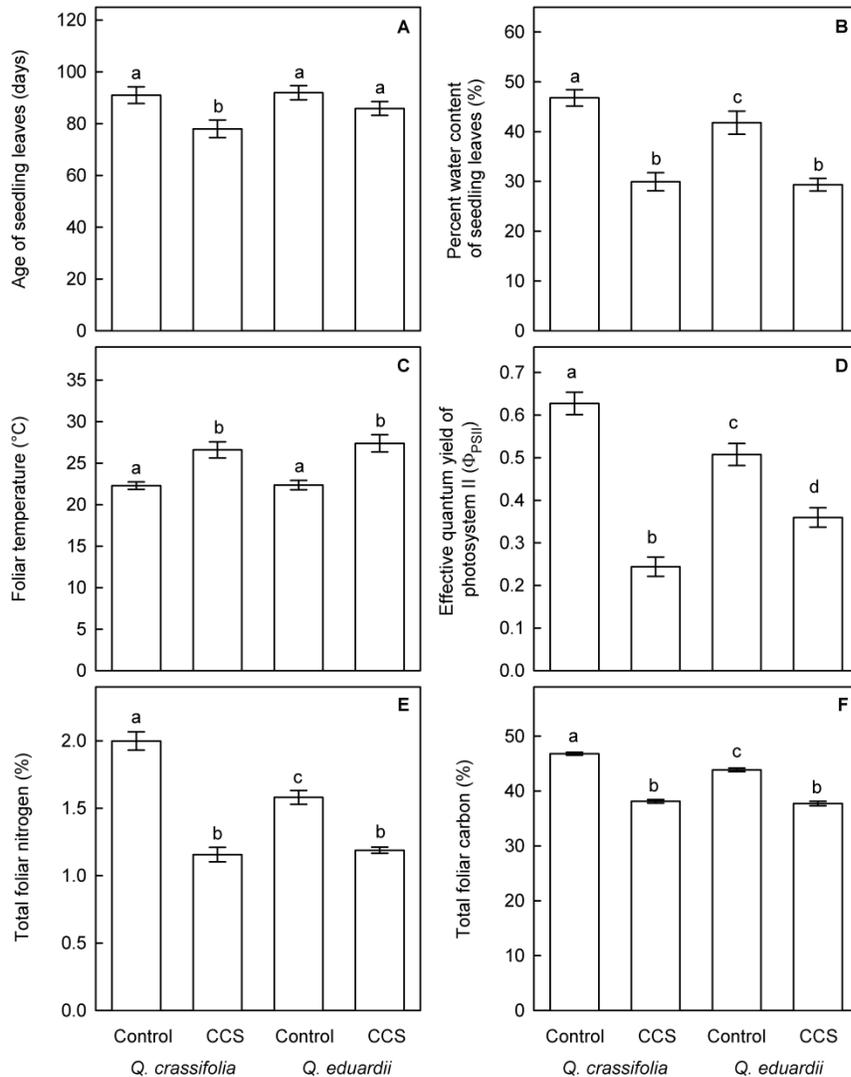
No effects of leaf age were found on foliar contents of nitrogen and carbon, but values of these two variables differed between climate treatments and oak species (Table 3). Both oak species had higher contents of foliar nitrogen (Fig. 5E) and foliar carbon (Fig. 5F) in controls than in

20

371   CCS plots, and these differences were larger in seedlings of *Q. crassifolia* than in seedlings of *Q.*

372   *eduardii*.

373

374   **4. Discussion**

375   Our study indicates that increasing temperature and drought will impair the development of

376   recently emerged oak seedlings in the field and, as far as we are aware, this is the first

377   experimental study addressing these issues in temperate forests of North America. Indeed, as

378   open-top chambers and rainout shelters properly simulated the conditions of temperature and

379   rainfall expected in the short term (2021-2040), our experimental results suggest that these

380   negative impacts of climate change would occur much sooner than predicted by other studies,

381   which have used species distribution models to simulate the future distribution of tree species and

382   proposed that climate change effects on these forests will be evident after the middle of this

383   century (Gómez-Mendoza and Arriaga, 2007; Mclaughlin and Zavaleta, 2012). The induction of

384   higher temperature and lower rainfall in the field also modified other environmental variables that

385   can impair the development of oak seedlings, and it is important to note that the future shifts in

386   the values of these variables are not directly predicted by climate change models. For instance,

387   we observed an overall decrease of air relative humidity within CCS plots, which can be

388   attributed to the direct inverse relationship between the values of this variable and air temperature

389   (Hardwick-Jones et al., 2010). Further, higher soil temperatures resulting from warming could

390   have increased soil water evaporation within CCS plots (Parlange et al., 1998) and this, together

391   with the smaller water inputs due to rainout shelters, may be responsible for the lower soil water

392   contents recorded in these experimental units. In this way, the climatic changes that will occur

393   during the next decades in temperate forest due to the increased emission of greenhouse gases

394   may reduce the recruitment rates of oaks in these ecosystems. Nevertheless, our results also

21

395   indicated that the two oak species included in this study somewhat differed in their responses to

396   climate change.

397   Emergence and survival of *Q. crassifolia* seedlings were lower in CCS plots than in controls,

398   while these differences were not found for *Q. eduardii*. This suggests that the early life-cycle

399   stages of *Q. crassifolia* would be more sensitive to climate change than those of *Q. eduardii*.

400   Because acorn of most oak species quickly lose viability when external factors enhance seed

401   water loss (Zavala-Chávez, 2008; Joët et al., 2013), the higher soil temperatures induced in CCS

402   plots may have accelerated acorn desiccation and, consequently, this may have reduced the

403   emergence of *Q. crassifolia* seedlings. Further, as oak seeds require elevated hydration levels to

404   stimulate the development of their embryos (Zavala-Chávez and García-Moya, 1996), the lower

405   soil moisture contents in CCS plots may have prevented that the acorns of *Q. crassifolia* reached

406   the water contents they needed to germinate. Thus, the combined effects of higher temperature

407   and reduced rainfall may be responsible of the lower proportion of emerged seedlings of this oak

408   species in CCS plots.

409   The lower survival of recently emerged *Q. crassifolia* seedlings in CCS plots can also be

410   attributed to the combination of increased warming and drought. Several authors have reported

411   that, under the current climatic conditions, oak seedlings quickly wilt if they face thermal and/or

412   water stress during their recruitment season, which drastically increases their mortality in the

413   field (Rey-Benayas et al., 2005; González-Salvatierra et al., 2013; Montes-Hernández and López-

414   Barrera, 2013; Badano et al., 2011, 2015). In our study site, climate change models predict that

415   these two stress factors will intensify in the near future, and our experimental results indicate that

416   such increased environmental harshness will reduce the survival of *Q. crassifolia* seedlings.

417   However, neither emergence nor survival of *Q. eduardii* seedlings were reduced within CCS

418   plots, as compared to controls, suggesting that this oak species may be able to deal with climate

419   change.

420   These differences in seedling emergence and survival between *Q. crassifolia* and *Q. eduardii*

421   could be attributed to differential capabilities they have for recruiting in contrasting habitats. In

422   temperate oak forest of Mexico, *Q. crassifolia* is considered a late successional species whose

423   seedlings mainly recruit in shaded understory habitats and, although they have an elevated

424   competitive ability for soil resources in comparison with seedlings of other tree species, they do

425   not tolerate elevated temperatures or water shortage (Castillo-Lara et al., 2008). Instead, even

426   when *Q. eduardii* is also a conspicuous species within these forest, this oak is considered a

427   pioneer tree because it colonizes disturbed open areas, where temperature and drought are much

428   higher than beneath forest canopies (Alfonso-Corrado et al., 2007). Thus, lack of effects of

429   climate change on the emergence and survival of *Q. eduardii* seedlings may be due to adaptions

430   they have for establishing in harsh environments.

431   Despite the higher emergence and survival of *Q. eduardii* seedling in CCS plots, as compared

432   with those of *Q. crassifolia*, our results suggest that climate change will negatively affect the

433   development of the early life-cycle stages in both species. For instance, aboveground growth of

434   these seedlings was about twice higher in controls than in CCS plots. This contrasts with the

435   results of experiments that have manipulated temperature in cold and temperate forests, which

436   have indicated that warming can hasten aerial shoot development of tree seedlings (Danby and

437   Hik, 2007; Xu et al., 2012; Piper et al., 2013). Nevertheless, experiments conducted in arid and

438   semi-dry environments have reported that the occurrence of drought events during the plant

439   recruitment season can strongly impair the growth rates of tree seedling (Valladares and Sánchez-

440   Gómez, 2006; Mclaughlin and Zavaleta, 2012). Thus, the lower growth rates of oak seedlings in

441   CCS plots could be due to this latter factor, as the environmental variables measured in these

442   experimental units suggested that the combined effect of increased temperatures and reduced

443   rainfalls generated strong soil water deficits.

444   Climate manipulation within CCS plots also induced other detrimental effects on oak

445   seedlings, including reduced leaf water content (*PWC*), lower carbon accumulation in their

446   leaves, and diminished photosynthetic quantum yield (ϕ_{PSII}). The lower *PWC* of seedlings in CCS

447   plots can be linked with their higher foliar temperatures, which may have increased water loss

448   through transpiration (Haldimann and Feller, 2004; Rodriguez-Dominguez et al., 2016). Further,

449   the lower air relative humidity within CCS plots may have reduced the boundary vapor layer of

450   leaves, also enhancing foliar transpiration (Martin et al., 1999; Will et al., 2013). Since leaf

451   dehydration induces stomatal closure (Brodribb and Holbrook, 2003), these lower *PWC* could

452   have led to reduced gas exchange and this may partially explain why seedlings from CCS plots

453   had lower carbon contents than those from the controls. The reduced values of ϕ_{PSII} in these

454   seedlings can also be related with their lower carbon contents, as thermal and/or water stress

455   decrease ϕ_{PSII} in plants (Xu and Zhou, 2006; González-Salvatierra et al., 2013; Aragón-Gastélum

456   et al., 2014; Badano et al., 2015) and causes energetic deficits for fixing carbon through the

457   Calvin cycle (Baker and Oxborough 2004; Xu and Zhou, 2006). Therefore, our results suggest

458   that climate change can also impair the photosynthetic efficiency of oak seedlings.

459   The lower contents of NO_3^- and NH_4^+ in the soil CCS plots concur with the results of other

460   experiments conducted in forest ecosystems, which showed that climate change may cause

461   nitrogen deficits for plants (Rennenberg et al., 2009; Chen et al., 2011; Roa-Fuentes et al., 2015).

462   Decreases in soil nitrogen content under climate change conditions have been mainly attributed to

463   the detrimental effects of water deficits on the metabolic activity of nitrogen-fixing bacteria,

464   rather than increasing temperatures (Borken and Matzner, 2009; Chen et al., 2011). This

24

465   decreased availability of nitrogen in the soil may explain the lower foliar nitrogen contents of

466   seedlings that grew within CCS plots. However, is also important to note that the lower soil

467   moisture in these plots may have reduced the mobility of this mineral nutrient in the soil matrix

468   and, consequently, impaired its uptake through the roots of seedlings. These effects of drought on

469   the activity of nitrogen-fixing organism and/or the mobility of nitrogen in the soil matrix, may

470   explain the lower growth rates of oak seedlings in CCS plots, as nitrogen is a key nutrient that

471   regulates plant development (Rennenberg et al., 2009; He and Dijkstra, 2014).

472   Overall, this study indicates that climate change conditions that will occur across temperate

473   forest of North America have the potential to reduce the recruitment of oak species. Under the

474   current climate, elevated temperatures and drought events that occur during the growing season

475   of oaks constitute important ecological barriers for their establishment in temperate forest

476   (Zavala-Chávez and García-Moya, 1996; González-Salvatierra et al., 2013; Montes-Hernández

477   and López-Barrera, 2013; Badano et al., 2009, 2011, 2015), and our results reveal that the

478   strengthening of these stress factors will impair even more the development of recently emerged

479   oak seedlings. This, in turn, may have strong detrimental effects on the natural regeneration

480   processes that regulate the maintenance of these forests, and this can threat the provisioning of

481   ecosystem services that support human well-being.

482

483   **5. Conclusion**

484   Our results indicate that the advance of climate can reduce the emergence, establishment, growth

485   and physiological performance of oak seedlings in temperate forest. Nevertheless, the differences

486   in seedling emergence and survival that we observed between the two oaks included in this study

487   suggest that some species could be more susceptible to climate change (e.g., *Q. crassifolia*) than

488   others (e.g., *Q. eduardii*). On this issue, extensive field experiments are still required to identify

489 what tree species are vulnerable to climate change and what species will be able to cope with

490 warming and drought. This would allow to focus conservation actions on those species highly

491 sensitive to climate change, promoting their assisted migration towards zones climatically

492 suitable for their development and hence ensure their prevalence. Conversely, those species with

493 elevated resilience to climate change could be used for developing sustainable forestry and/or

494 addressing restoration programs to recover forests in degraded areas.

495

496 **Statement of authorship**

497 CLPR and EIB conceptualized the study, designed the field experiment, gathered the data and

498 performed the satistical analyses; JPRO, PDS, JF, DD and JAFC contributed with the

499 measurement of seedling variables and foliar traits in the field and the laboratoy; all authors

500 contributed interpret the results and co-wrote the article.

501

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