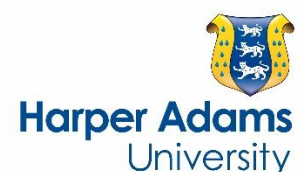# Rising CO2 from historical concentrations enhances the physiological performance of Brassica napus seedlings under optimal water supply but not under reduced water availability

by Faralli, M., Grove, I.G., Hare, M.C., Kettlewell, P.S. and Fiorani, F.

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1   **Rising CO$_2$ from historical concentrations enhances the physiological**

2   **performance of *Brassica napus* seedlings under optimal water supply but not**

3   **under reduced water availability.**

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11   **Running title**

12   Water stress at different [CO$_2$]

13   **Keyword index**

14   Canola, elevated CO$_2$, climate change, photosynthesis, stomatal conductance, shoot

15   biomass, leaf area, evapotranspiration

16   **Abstract**

17   The productivity of many important crops is significantly threatened by water

18   shortage and the elevated atmospheric CO$_2$ can significantly interact with

19   physiological processes and crop responses to drought. We examined the effects of

20   three different CO$_2$ concentrations (historical ~300 ppm, ambient ~400 ppm and

21   elevated ~700 ppm) on physiological traits of oilseed rape (*Brassica napus* L.)

22   seedlings subjected to well-watered and reduced water availability. Our data show i)

23  that, as expected, increasing $CO_2$ level positively modulates leaf photosynthetic

24  traits, leaf water-use efficiency and growth under non-stressed conditions although a

25  pronounced acclimation of photosynthesis to elevated $CO_2$ occurred; ii) that the

26  predicted elevated $CO_2$ concentration does not reduce total evapotranspiration under

27  drought when compared to the present (400 ppm) and historical (300 ppm)

28  concentrations because of a larger leaf area that does not buffer transpiration; iii) that

29  accordingly the physiological traits analysed decreased similarly under stress for all

30  $CO_2$ concentrations. Our data support the hypothesis that increasing $CO_2$

31  concentrations may not significantly counteract the negative effect of increasing

32  drought intensity on *Brassica napus* performance.

33  **Introduction**

34  The concentration of atmospheric $CO_2$ ($[CO_2]$) has been increasing steeply from

35  1958 (~316 ppm) to 2016 (>400 ppm) (NOAA Mauna Loa Observatory, Hawaii) and

36  the Intergovernmental Panel on Climate Change has predicted a $[CO_2]$ in 2050

37  between ~550 and ~700 ppm. From an agricultural point of view, the elevated $[CO_2]$

38  has been related to direct effects on crops' physiological processes (Leakey *et al.*

39  2012). Indeed, $C_3$ crops (Fitzgerald *et al.* 2016; O'Leary *et al.* 2015) and, to a lesser

40  extent $C_4$ crops (Ruiz-Vera *et al.* 2015), exhibited i) higher photosynthetic rates due

41  to an increased Rubisco carboxylation activity and a decreased oxygenation

42  reaction; ii) a lower stomatal conductance ($g_s$) following a depolarization of the

43  membrane potential of the guard cells accompanied by an increase of the outward

44  activity of $K^+$ channels and a subsequent increase in guard cell $Ca^{2+}$ concentration

45  (Leakey *et al.* 2012, Ainsworth & Rogers, 2007). These physiological changes have

46  been related to increasing biomass production and ameliorated responses of plants

47  to drought by an improvement of water-use efficiency at the canopy level (Leakey *et*

2

48 *al.* 2012). It is well-recognized that drought is the main factor affecting global crop

49 production and food security (Cattivelli *et al.* 2008) and climate change is predicted

50 to significantly increase the magnitude of water shortage and negatively affect the

51 yield of crops (Parmesan & Yohe, 2003). In this context, oilseed rape (OSR,

52 *Brassica napus* L.) is becoming one of the most popular oilseed crops in the world.

53 Drought periods over the most drought-sensitive phenological stages for OSR

54 (anthesis until mid-pod development) can lead up to 40% of seed yield losses (Faralli

55 *et al.* 2016).

56 It is therefore of pivotal importance to understand the physiological mechanisms that

57 relate atmospheric $CO_2$ to drought and to determine whether the expected

58 detrimental effects on crops following the increases in extreme environmental

59 conditions could be at least mitigated by increases in $CO_2$ concentrations. A large

60 amount of work showed positive effects of elevated atmospheric $CO_2$ concentration

61 on crop's drought tolerance and hence yield reduction mitigation (Baker *et al.* 1997;

62 Wall 2001; Qaderi *et al.* 2006; O'Leary *et al.* 2015; Fitzgerald *et al.* 2016). However,

63 experiments on OSR have shown that elevated $CO_2$ concentrations may not mitigate

64 the detrimental effects of increasing temperatures on yield (Frenck *et al.* 2011). In

65 addition, Gray *et al.* (2016) have recently shown in multi-year FACE experiments that

66 the improvements in physiological performance and yield of soybean from elevated

67 [$CO_2$] can be counteracted by severe drought conditions. Therefore we conducted

68 three experiments in the same controlled environment room at different $CO_2$

69 concentrations (~300 ppm – historical 1950/1960 [$CO_2$], $_h$[$CO_2$]; ~400 ppm – ambient

70 [$CO_2$], $_a$[$CO_2$]; ~700 ppm – elevated [$CO_2$], $_e$[$CO_2$]) and all three comparing the same

71 combinations of watering regimes. The aims were to i) explore the physiological

72 effects of these [$CO_2$] on winter OSR seedlings growth and development: ii) to

73  evaluate the interactions between the different $[CO_2]$ and drought;  iii) to characterize

74  and evaluate the  relationships between physiological traits involved in the response

75  of OSR to different $[CO_2]$ and drought.

76  **Materials and methods**

77  *Plant materials and experimental layout*

78  Winter oilseed rape (OSR, *Brassica napus* L.) seeds (cv. Excalibur, Dekalb, UK)

79  were sown on 8th January 2016 for Experiment I,  20th  January 2016 for Experiment

80  II, and  9th  February 2016 for Experiment III in individual pots (pot width 11 cm, pot

81  height 13 cm, total volume ~1500 cm$^3$) containing the same amount (650 g + 25g of

82  pot weight) of a peat, sand, and pumice plant cultivation substrate (SoMi 513,

83  Dachstauden; Hawita, Vechta, Germany) characterized with a water retention curve

84  as described  by Barboza-Barquero *et al.* (2015). Plants were germinated in a growth

85  chamber at 20°C day / 15°C night, 12 / 12 photoperiod, 60 % RH and ambinet $[CO_2]$.

86  PAR was provided by neon lamps at ~200 µmol m$^{-2}$ s$^{-1}$ at shoot apex measured with

87  a LI-190R PAR sensor (LI-COR, Lincoln, Nebraska USA). Plants were watered daily

88  with rain water to maintain the optimum soil moisture conditions for plant germination

89  and growth (around 90% of available water content, AWC, according to a water

90  retention curve, as described in Barboza-Barquero *et al.*, 2015). Three seeds were

91  sown in each pot and for all the experiments two emerged seedlings were removed

92  after 4 days from emergence. At the 2nd leaf stage (GS 1.2 BBCH growth scale, 19th

93  January for Experiment I, 7th February for Experiment II and 25th February 2016 for

94  Experiment III) seedlings were placed in a controlled environment chamber (Jülich

95  Forschungszentrum, IBG-2 Plant Science, walk-in custom-made chamber) at 20°C

96  day / 15°C night, 16/8 photoperiod, 60% RH and a VPD of 0.94 kPa. PAR was

provided with HPI-T-plus lamps (400W/645 E40, Philips, Netherlands) at an average

of ~500 µmol m$^{-2}$ s$^{-1}$ at plant level with a minimum value of 460 µmol m$^{-2}$ s$^{-1}$ and a

maximum of 540 µmol m$^{-2}$ s$^{-1}$.

Each experiment consisted of five randomized blocks with two treatments (well-

watered - WW and droughted - WS) and six replicates in each block. Treatments

were applied when plants reached the 4$^{th}$ leaf stage based on visual observations

(GS 1.4, BBCH oilseed rape growth scale) (days after treatment 0, DAT 0 - 25$^{th}$

January for Experiment I, 12$^{th}$ February for Experiment II and 1$^{st}$ March 2016 for

Experiment III).

According to the soil retention curve of the SoMi substrate, target weight at "pot

capacity" (100% of available water content) was set to 1070 g of pot weight

(including 25 g of pot weight and 20 g of plant fresh weight at the 4th leaf stage ) and

the permanent wilting point was set at 600 g. Thus the total AWC of the pots was

accounted to be 425 ml. Hence, well-watered pots (WW, n=6) were weighed daily

with a MC1 balance (Sartorius, Göttingen, Germany) and then rewatered to avoid

any decrease in soil moisture according to the equation:

$$\text{Eqn (1)} \quad \text{Water to add} = \text{"pot capacity" weight} - \text{pot weight}$$

Watering was withheld to water stressed pots (WS) over the whole experimental

period (12 days). Pot saucers were used to avoid any water uptake. Total daily pot

evapotranspiration (ET) was calculated as the water to add for WW and the daily

reduction in pot weight for WS.

For each individual experiment, the chamber was set at a different [CO$_2$]: Experiment

I 400 ppm, $_a$[CO$_2$]; Experiment II 700 ppm, $_e$[CO$_2$]; and Experiment III 300 ppm,

$_h$[CO$_2$]. A respirator (Fig. S1) made up of a tube connected with a plastic mask on

one side (respirator) was developed and used in the growth chamber to avoid

122 undesired [$CO_2$] fluctuations due to experimenter breathing during data collection

123 and plant manipulation. The average [$CO_2$] for each experiment was 402.6 ± 10.1

124 (SD) for Experiment I, 700.9 ± 9.7 for Experiment II and 285.5 ± 14.3 for Experiment

125 III (Fig. S2)

126 *Growth and relative water content analysis*

127 On DAT 1, 4, 8 and 12, plants (n=6) from each treatment were harvested for all the

128 experiments. RWC was calculated according to Barr & Weatherley (1962) and was

129 determined as described by Faralli *et al.* (2015). Briefly, between 12:00 and 13:00,

130 one piece of leaf tissue of the fourth leaf (~2 $cm^2$) was collected using a scalpel for

131 each plant (n=6). These samples were collected in a central region of the lamina

132 between the main vein and the leaf margin avoiding lateral veins. The fresh weight

133 was then recorded ($F_W$) and the samples were soaked in distilled water in Petri

134 dishes in the dark and at ~4°C during four hours. The turgid weight ($T_W$) was

135 recorded, the dried disks (oven-dried at 80°C for 24 hours) were weighed the day

136 after, and the dry weight was recorded ($D_W$). RWC (%) was then calculated

137 according to:

138 $$\text{Eqn (2) } RWC(\%) = \frac{F_W - D_W}{T_W - D_W} \times 100$$

139 After that, leaf number (LN) was counted and plants were excised at the base of the

140 stem and leaf and stem fresh weight were recorded (Mettler Toledo XS 205 Dual

141 Range, Columbus, USA). Total leaf area (LA) was recorded with a leaf area meter

142 (LI-3100C area meter, Li-Cor, Lincoln, NE, USA). Samples were dried in an oven

143 (Nabertherm P330, Nabertherm GmbH Lilienthal/Bremen, Germany) over 24 hours

144 at 65°C and leaf, stem and total shoot dry weight were recorded. Specific leaf area

145 was calculated as: SLA (g $m^{-2}$) = leaf area / leaf DW.

*Gas-exchange*

147  Gas-exchange and chlorophyll fluorescence were simultaneously measured with a

148  Li-Cor 6400 (Li-Cor, Lincoln, NE, USA) with an integrated fluorescence leaf cuvette

149  (LI-6400-40; Li-Cor) on DAT 1, 4, 8 and 12 for all the experiments, between 09:00

150  and 12:00. To minimize potential leaf position and developmental age effects, all the

151  gas-exchange measurements were taken on the $4^{th}$ fully expanded leaf of six

152  randomly selected plants for each treatment. In the Li-Cor cuvette, all the parameters

153  (leaf $CO_2$ assimilation at saturating light - $A_{max}$, stomatal conductance - $g_s$ and the

154  ratio between sub-stomatal $CO_2$ and the ambient [$CO_2$] - $C_i/C_a$) were collected at 400

155  ppm $CO_2$ (Experiment I), 700 ppm $CO_2$ (Experiment II) and 300 ppm $CO_2$

156  (Experiment III). Cuvette temperature was maintained at 25°C, photosynthetically

157  active photon flux density (PPFD) was 1200 µmol m$^{-2}$ s$^{-1}$ (saturating PPFD for OSR

158  seedlings, previously evaluated by light curves, Fig. S3), with a 10:90 blue:red light

159  and a flow rate of 300 µmol s$^{-1}$. Intrinsic water-use efficiency was calculated as

160  $A_{max}/g_s$. The ratio ($A_{max}/g_s$) is considered to be more reliable than the water-use

161  efficiency calculated from $CO_2$ assimilation to transpiration rate, since it is not

162  affected by the VPD inside the leaf chamber (Webster *et al.* 2016). The data were

163  collected after the leaf achieved a steady-state. The data were collected after the

164  leaf achieved a steady-state, and minimum fluorescence ($F_s$) and maximum

165  fluorescence ($F_m'$) were recorded by ensuring a light-saturating pulse of 8000 µmol

166  m$^{-2}$ s$^{-1}$. The actual photochemical efficiency of the photosystem II ($\Delta F/F_m'$) was

167  calculated according to Baker (2008) as follow:

168  $$\text{Eqn (3)} \quad \Delta F/F_m' = \frac{F_m' - F_s}{F_m'}$$

169  and the electron transport rate as:

170 $$\text{Eqn (4) } J = \Delta F/Fm' \text{ x PPFD x } \alpha \text{ x } \beta$$

171 *Statistical analysis*

172 Available water content and daily evapotranspiration data from the three experiments

173 are daily means (n=6) ± standard error (SE). Carbon dioxide treatments are means

174 (n=1440) ± standard deviation (SD). With the exception of $[CO_2]$, the parameters

175 inside the growth chamber (i.e. RH, temperature, light and VPD) were very similar or

176 identical during the three experiments. Also, since the growth conditions of the three

177 experiments were very similar and no morphological differences were recorded

178 between the three sets of plant material, a hypothetical time-specific effect affecting

179 the $CO_2$ treatments was not considered a possibility. Therefore all the physiological

180 traits were analysed with a two-way ANOVA ($CO_2$ x watering regimes, Figure S4) in

181 GenStat 17th Edition (VSN International, UK). However, since the three experiments

182 were formally independent, the homogeneity of variance for each two-way ANOVA

183 was checked by visually examining the distribution histogram of the residuals and

184 the fitted vs. residuals plot. While the distribution was acceptable for all the traits and

185 DAT, in five cases (RWC DAT 12, Leaf Area DAT 8, Amax DAT 12, $A_{max}/g_s$ DAT 1)

186 the fitted vs. residuals plot showed heteroscedasticity. These data were transformed

187 to a natural logarithmic scale and in all cases the heteroscedasticity was reduced

188 and the distribution was improved. Subsequently the log-transformed data were

189 subjected to two-way ANOVA and the *P*-values for all the interactions were

190 recorded. In all the cases logarithmic transformation had a negligible effect on *P*-

191 values suggesting that the data heteroscedasticity was not significantly affecting the

192 data. Therefore, due to the above evidence, the data are statistically analysed and

193 presented in their original form without logarithmic transformation and thus including

194    [CO$_2$] factor in the ANOVA model. The two-way ANOVA outputs are presented in

195    Supplementary Fig. 4.

196    **Results**

197    *Total plant evapotranspiration and pot available water content*

198    Total plant ET of WW was similar at each [CO$_2$], increasing from ~50 mL at DAT 1 to

199    ~130 mL at DAT 7 (Fig. 1A). From DAT 8, while $_h$[CO$_2$] maintained total  ET at ~ 180

200    mL, the plants grown under $_a$[CO$_2$], slightly decreased the ET from ~160 mL to ~120

201    mL. On the contrary, $_e$[CO$_2$], steadily maintained a significant lower ET value from

202    ~100 ml at DAT 8 to ~60 mL at DAT 12. Therefore, pot AWC of WW plants from DAT

203    8 to DAT 12 was higher in $_e$[CO$_2$] conditions than under $_a$[CO$_2$] and, to a greater

204    extent, $_h$[CO$_2$] (Fig. 1B). WS treatment decreased both ET and AWC. Under WS

205    conditions, $_e$[CO$_2$] plants showed a significantly higher ET when compared to $_a$[CO$_2$]

206    and $_h$[CO$_2$] but only until DAT4 (Fig. 1C). From DAT 5, similar decreases in ET were

207    recorded between [CO$_2$]. Therefore, pot AWC of WS plants was lower under $_e$[CO$_2$]

208    than under $_a$[CO$_2$] and $_h$[CO$_2$], although PWP was reached similarly for all the [CO$_2$]

209    between DAT 7 and DAT 8 (Fig. 1D).

210    *Growth analysis*

211    Leaf number (LN) significantly increased over time in WW plants grown at all the

212    [CO$_2$] (from 4 leaves at DAT 1 to 7.5-8 leaves at DAT 12) and no significant

213    differences were found between the different [CO$_2$] treatments (Fig. 2A). WS

214    significantly decreased LN compared to the WW plants when grown at $_h$[CO$_2$] and

215    $_a$[CO$_2$] since DAT 4 (Fig. 2B). However $_e$[CO$_2$] showed  a reduction in LN under WS

216    only from DAT 8.

217 Over the twelve days of the experiments, WW plants grown in $_a[CO_2]$ had a shoot

218 DW (Fig. 2C) that was significantly higher than that of the plants grown in $_h[CO_2]$ by

219 34% on average (Fig. 2C) ($P<0.001$ for DAT 4, 8 and 12). Increases in shoot DW of

220 20% on average were observed in plants grown at $_e[CO_2]$ (Fig. 2C) with respect to

221 the plants grown under $_a[CO_2]$. Significant reductions in shoot DW were observed

222 under WS on DAT 8 and DAT 12 at all the $[CO_2]$ when compared to the WW plants

223 (Fig. 2D). However, WS plants grown under $_e[CO_2]$ exhibited a higher DW compared

224 to $_h[CO_2]$ and $_a[CO_2]$ by 42% and 33% on DAT 4, 40% and 26% on DAT 8 and 37%

225 and 27% on DAT 12, respectively .

226 Leaf area (LA) of WW plants grown under $_a[CO_2]$ significantly increased with time

227 from 190 $cm^2$ on DAT 1 to 800 $cm^2$ on DAT 12 (Fig. 2E). Under $_h[CO_2]$, WW plants

228 exhibited a similar significant increase in LA that was however lower than that of the

229 plants grown at $_a[CO_2]$ (Fig. 2E) (from 190 $cm^2$ on DAT 1 to 590 $cm^2$ on DAT 12).

230 $_e[CO_2]$ stimulated LA of WW plants leading to an average LA of 1190 $cm^2$ on DAT 12

231 (Fig. 2E). For DAT 4 and DAT 8, however, no significant differences in LA were

232 found between plants grown at $_e[CO_2]$ and $_a[CO_2]$. WS had a significant effect on LA

233 at all the $[CO_2]$ and the decrease was significant since DAT 4 in $_e[CO_2]$, $_a[CO_2]$ and

234 $_h[CO_2]$-grown plants (Fig. 2F).

235 Specific leaf area (SLA) decreased over time on WW plants grown at all the $[CO_2]$

236 and WS significantly decreased SLA values at all the $[CO_2]$ ($P<0.001$ since DAT 4)

237 (Fig. 2G and H).

238 *Plant water status*

239 The leaf RWC of WW plants fluctuated from 90% to 91% on average and no

240 significant differences were observed between the three $[CO_2]$ (Table 1). Under WS

241 conditions, leaf RWC significantly decreased from DAT 4 at all the $[CO_2]$ ($P<0.001$ at

242 all the DAT). RWC of $_e$[CO$_2$] plants under WS conditions was significantly higher

243 (RWC 70%, *P*=0.016) than $_a$[CO$_2$] (RWC 62%) and $_h$[CO$_2$] (RWC 67%) at DAT 8.

244 However, since the drop in RWC was similar for all the other DAT, RWC values were

245 not overall significantly different under WS between [CO$_2$] leading to very similar

246 values at DAT 12.

247 *Gas-exchange*

248 WW plants grown under $_a$[CO$_2$] showed an average A$_{max}$ of 26 µmol m$^{-2}$ s$^{-1}$ that was

249 22% lower than that of the plants grown in $_e$[CO$_2$] (Fig. 3A). On the contrary, $_a$[CO$_2$]

250 exhibited an increase in A$_{max}$ by 12% on average with respect to the $_h$[CO$_2$] (Fig. 3A).

251 Plants under WS conditions exhibited a reduction in A$_{max}$ compared to the WW

252 plants that was significant from DAT 4 for all the [CO$_2$] (*P*<0.001) (Fig. 3B).

253 WW plants grown under $_a$[CO$_2$] exhibited a $g_s$ of 0.67 mol mm$^{-2}$ s$^{-1}$ on average,

254 showing a decreasing slope over the last days of the experiment (Fig. 3C). With

255 respect to the $_a$[CO$_2$], a 7% increase on average in $g_s$ was observed in $_h$[CO$_2$]-grown

256 plants (Fig. 3C), without showing any $g_s$ reduction over time. On the contrary, $g_s$ was

257 significantly reduced over time in $_e$[CO$_2$] plants and by 40% on average with respect

258 to the $_a$[CO$_2$] (Fig. 3C). WS conditions reduced $g_s$ by 64%, 55% and 40% under

259 $_h$[CO$_2$], $_a$[CO$_2$] and $_e$[CO$_2$] respectively (Fig. 3D).

260 WW plants grown under $_h$[CO$_2$] and $_a$[CO$_2$] showed a relatively stable A$_{max}$/$g_s$ over

261 time with an average value of 31 µmol mol$^{-1}$ and 40 µmol mol$^{-1}$ respectively (Fig.

262 3E). On the contrary, $_e$[CO$_2$]-grown plants showed a significant increase in time from

263 DAT 1 to DAT 12 leading to an average value of 90 µmol mol$^{-1}$ (Fig. 3E). WS plants

264 had higher A$_{max}$/$g_s$ values with respect to the WW ones, with an average of 52, 76

265 and 95 µmol mol$^{-1}$ for $_h$[CO$_2$], $_a$[CO$_2$] and $_e$[CO$_2$]-grown plants respectively (Fig. 3F).

266 Under WW conditions, ETR values of $_e$[CO$_2$]-grown plants was on average higher

267 (190 µmol e$^{-1}$ m$^{-2}$ s$^{-1}$) than $_h$[CO$_2$] and $_a$[CO$_2$] (177 and 182 190 µmol e$^{-1}$ m$^{-2}$ s$^{-1}$

268 respectively) until DAT 8.  Conversely, on DAT 12, similar ETR values were recorded

269 between $_h$[CO$_2$] and $_a$[CO$_2$] (171 and 164 µmol e$^{-1}$ m$^{-2}$ s$^{-1}$ respectively) that were

270 lower compared to the $_e$[CO$_2$] (154 µmol e$^{-1}$ m$^{-2}$ s$^{-1}$). Under WS, declines in ETR

271 values were evident for all the [CO$_2$] and although similar values were recorded at

272 DAT 12, the reduction was less pronounced under $_e$[CO$_2$] at DAT 8.

**Discussion**

*[CO$_2$] influenced biomass accumulation and leaf gas-exchange under WW*

*conditions*

276 In our experiments, $_e$[CO$_2$] at 700 ppm stimulated A$_{max}$ by ~22% (in line with the

277 findings of Qaderi *et al.* (2006)) and $_h$[CO$_2$] reduced A$_{max}$ by ~12% when both

278 compared to $_a$[CO$_2$] under WW conditions. The increase under $_e$[CO$_2$] found in OSR

279 seedlings was however lower than in other C$_3$ crops such as durum wheat (~37%;

280 Aranjuelo *et al.* 2015) and rice (~40%, Vu *et al.* 1997) and in our experiments at DAT

281 12 the A$_{max}$ of $_e$[CO$_2$] plants was similar to that of $_a$[CO$_2$]. This suggests strong

282 photosynthetic acclimation potentially due to the increased LA of plants at $_e$[CO$_2$] that

283 lead to a reduction in Rubisco activity due to a depression in area-based leaf N

284 content. In addition, the concomitant decrease in ETR and A$_{max}$ that occurred under

285 $_e$[CO$_2$] but not under $_h$[CO$_2$]  and $_a$[CO$_2$] at DAT 12 can be also an indication of

286 signals derived from increases in sucrose transport to guard cells (Outlaw, 2003),

287 thylakoid membrane damage (Haworth *et al.* 2016) and/or increase in soluble sugar

288 in the apoplast that reduced the mesophyll signal (Haworth *et al.* 2016).

289 The initial enhanced A$_{max}$ following the increasing [CO$_2$] was accompanied by larger

290 LA and shoot DW, consistent with previous work in OSR (Qaderi *et al.* 2006). This

291  report however is the first showing differences in $A_{max}$, LA and shoot DW between

292  $_a$[$CO_2$] and $_h$[$CO_2$]-grown OSR. It is not clear whether increased $A_{max}$ following high

293  [$CO_2$] can enhance sink activity but carbohydrate translocation can be reduced when

294  sink size is limited (Arp, 1991). A FACE experiment with spring canola grown at 500

295  ppm [$CO_2$] showed no significant increases in yield compared to the ambient

296  concentration-grown plots (Franzaring *et al.* 2008) probably due to limited carbon

297  storage structures, therefore a potential small sink size (Reekie *et al.* 1998). Our

298  data however indicate that OSR was potentially source-limited in the first years of its

299  agricultural production (~1960) and that rising [$CO_2$] may have played a role at

300  boosting the steady increase in OSR seed yield seen in recent years (Berry and

301  Spink, 2006).

302  It has been proposed that the larger plant size that can be achieved at elevated $CO_2$

303  is related to higher water use (hence transpiration) (Xu *et al.* 2013; Polley *et al.* 2008;

304  Nowak *et al.* 2004). Our results show that in OSR seedlings grown under WW

305  conditions the standardized transpiration over time for LA is increased under $_a$[$CO_2$]

306  (1106 ± 105 mmol $H_2O$) when compared to the $_h$[$CO_2$] (713 ± 105 mmol $H_2O$), but

307  not under $_e$[$CO_2$] (852 ± 105 mmol $H_2O$) suggesting that the short-term acclimation to

308  $_e$[$CO_2$] is highly beneficial in the WW plants even if the LA is dramatically increased.

309  In addition, ET data show that from DAT 8, $_e$[$CO_2$] plants are subjected to a

310  significant reduction in water loss, potentially due to the larger leaf area that

311  attenuates soil evaporation. This confirms that, although plant water loss and LA are

312  not linearly correlated, larger LA can buffer the greater areas devoted to transpiration

313  when self-shading and/or elevated leaf-area index threshold are reached under non-

314  stressed conditions.

315  *[$CO_2$] did not affect the whole-plant response under WS conditions.*

316 In our experiments, RWC of WS plants was on average 89, 80, 66 and 54%, that

317 reflects a good range of dehydration. When RWC was decreased to 66% (DAT 8), $g_s$

318 was completely inhibited at all the $[CO_2]$, in line with previous findings (Albert *et al.*

319 2012). These data suggest application of mild stress at DAT 4, severe stress at DAT

320 8 and terminal stress at DAT 12. We hypothesized that by maintaining low $g_s$,

321 elevated atmospheric $[CO_2]$ may ameliorate plant water status under stress by

322 preserving soil water content (Leakey *et al.* 2012). Polley *et al.* (1999) suggested that

323 the effects of elevated $[CO_2]$ on osmotic adjustment are minimal, indicating that the

324 maintenance of high plant water status in plants grown at elevated $[CO_2]$ under

325 stress is a result of stomatal control. However, and contrary to expectation, in our

326 experiments no differences were found in RWC between $[CO_2]$ treatments. In our

327 experiments the larger leaf area produced under $_e[CO_2]$ led to a similar transpiration

328 rate standardized for the LA (~200 mmol $m^{-2}s^{-1}$ for all the $[CO_2]$ under stress) thus

329 offsetting the water-loss reduction following the $_e[CO_2]$-induced stomatal closure.

330 Recently Gray *et al.* (2016) showed that, at field level, elevated atmospheric $CO_2$

331 concentration (from 550 to 585 ppm) does not always conserve water in soil

332 following reduction in transpiration rate. This leads to non-significant benefits in

333 terms of soybean yield when drought progressively increases. Since the relation

334 between the increase in LA and transpiration in the field is not linear due to the self-

335 shading effect that occur when large LA are reached, Gray *et al.* (2016)

336 hypothesised that in wet years (large baseline LA), higher atmospheric $[CO_2]$  may

337 significantly reduce crop WU, consistently with our WW data on OSR (as explained

338 above). However this may not occur in dry years, where increases in LA may only

339 lead to higher plant water loss, similarly to our findings on WS. To confirm that, in our

340 experiments $A_{max}$-to-$g_s$ curves show that in the first part of soil drying, $g_s$ is reduced

341 more than $A_{max}$ leading to higher $A_{max}/g_s$ values for all the $[CO_2]$ treatments (Fig. 4A).

342 However, despite the curve plateau being higher at $_e[CO_2]$, the shape of the $A_{max}$-to-

343 $g_s$ curves were similar for all the $[CO_2]$, possibly because the increased LA resulted

344 in similar detrimental effects on $A_{max}$ and RWC (Fig. 4B), thus similarly reducing $g_s$

345 (Fig. 4C), between the $[CO_2]$ under stress. This hypothesis is confirmed by ET data

346 where $_e[CO_2]$ plants exhibited a very high ET from DAT 1 to DAT 4 due to higher LA

347 and LN that lead to fast use of the water resources available. Therefore, although

348 leaf traits collected with gas-exchange system (e.g $A_{max}$ and $A_{max}/g_s$) were higher on

349 DAT 4 (therefore under a moderate stress) under $_e[CO_2]$, increasing drought intensity

350 leads to similar detrimental effects on OSR physiology regardless of the $[CO_2]$  at

351 which they are grown.

352 *Conclusions*

353 The work presented here shows improvements in the physiological performance of

354 winter OSR seedlings in elevated $[CO_2]$, mainly by stimulating photosynthesis and

355 thus increasing leaf area in line with work focusing in other major food crops. Strong

356 differences were found between plants grown at 300 and 400 ppm suggesting that

357 OSR may have been significantly source-limited in the first years of its agricultural

358 production (~1960). Increasing the $[CO_2]$ decreased leaf stomatal conductance and

359 improve leaf water-use efficiency but no significant beneficial effects were found

360 under drought due to the larger leaf area gained over the first few days of stress

361 (when water was available) that offsets the elevated $[CO_2]$-induced stomatal closure

362 leading to similar total ET between the $[CO_2]$ treatments. Our data are in line with

363 recent findings in field-grown soybean, and shows this behaviour on another crop

364 species and over a much broader range of $[CO_2]$.

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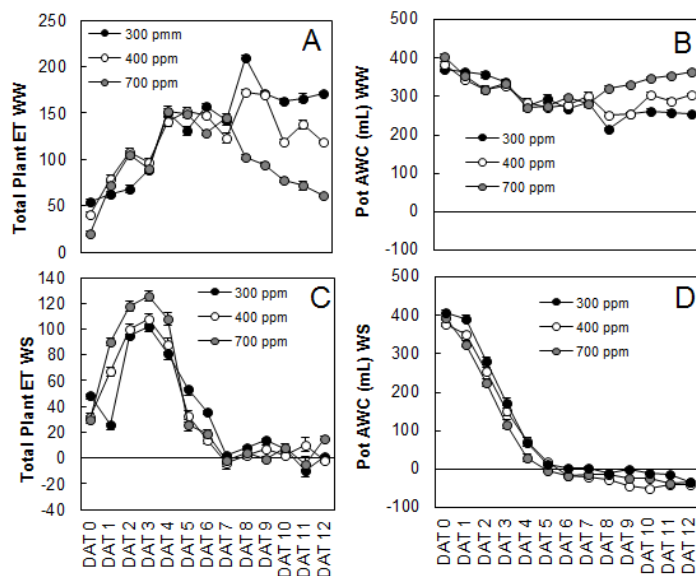
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Figure 1. Development of daily total plant evapotranspiration (ET, mL) under well-
watered (WW, A) and droughted conditions (WS, C) and development of pot
available water content (AWC, mL) under well-watered (WW, B) and droughted
conditions (WS, D) of  winter oilseed rape plants grown at historical (300 ppm),
ambient (400 ppm) and elevated (700 ppm) atmospheric $CO_2$ concentration. DAT=
days after treatment application. Data were collected daily with a balance. Data are
means (n=6) ± SE.

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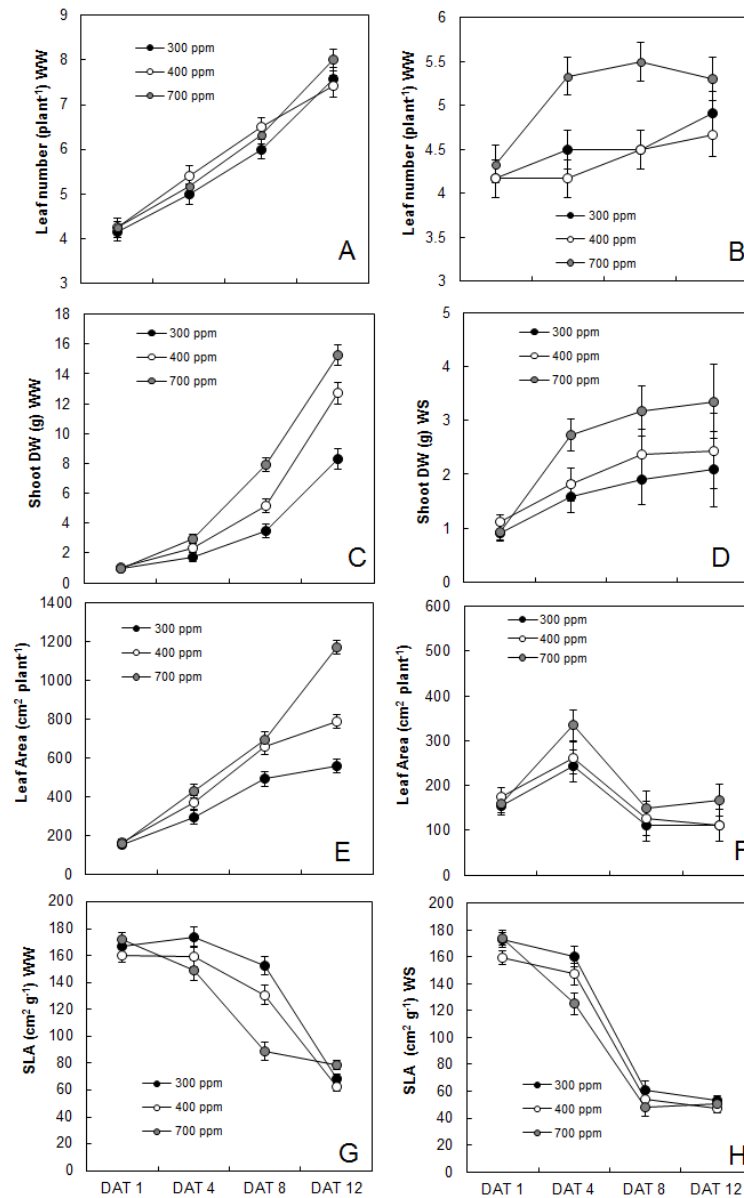
Figure 2. Development of leaf number (A, B), shoot dry weight (C, D; DW, g), total leaf area (E, F; LA, cm$^2$ plant$^{-1}$) and specific leaf area (G, H; SLA cm$^2$ g$^{-1}$) of winter oilseed rape plants grown at historical (300 ppm), ambient (400 ppm) and elevated (700 ppm) atmospheric $CO_2$ concentration under well-watered (WW; A, C, E, G) and water-stress (WS; B, D, F, H) conditions. Data were collected at days after treatment application (DAT) 1, 4, 8 and 12. Data are means

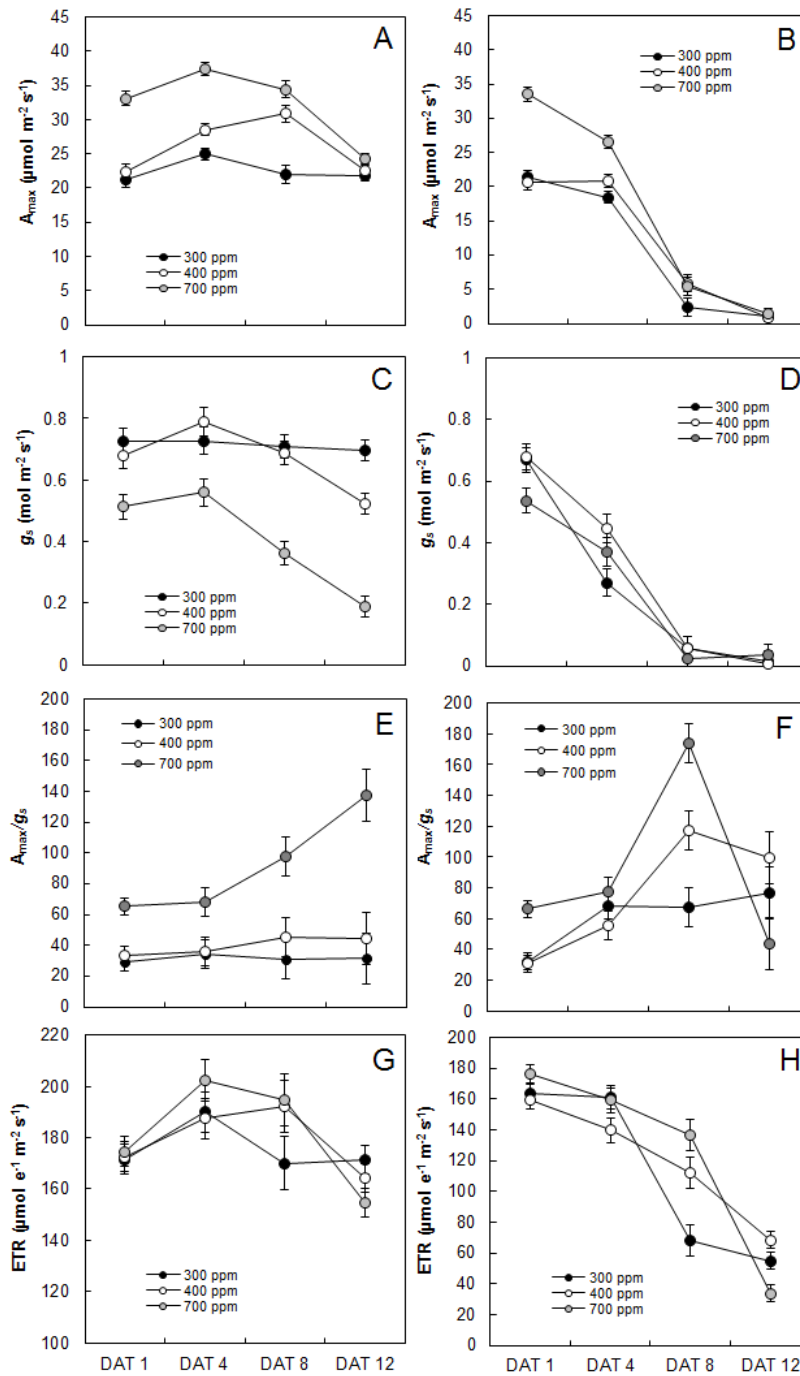Figure 3. Development of carbon assimilation rate at saturating light (A,B; $A_{max}$), stomatal conductance (C, D; $g_s$), intrinsic water-use efficiency (E, F; $A_{max}/g_s$) and electron transport rate (G, H; ETR) of winter oilseed rape plants grown at historical (300 ppm), ambient (400 ppm) and elevated (700 ppm) atmospheric $CO_2$ concentration under well-watered (WW; A, C, E) and water-stress (WS; B, D, F) conditions. Data were collected at days after treatment application (DAT) 1, 4, 8 and 12. Data are means (n=6) ± SE.

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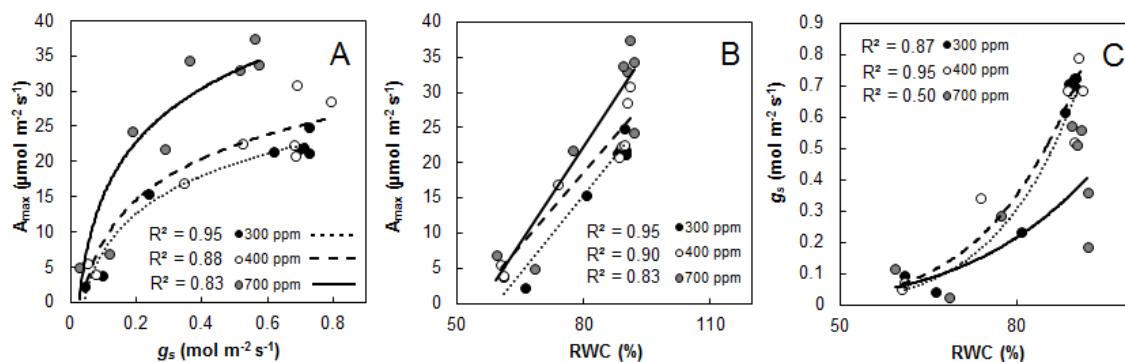
Figure 4. Correlations between A) $CO_2$ assimilation rate at saturating light ($A_{max}$) and stomatal conductance ($g_s$),B) $A_{max}$ and leaf relative water content (RWC) and C) $g_s$ and RWC, of winter oilseed rape plants grown at 300 ppm (black symbols), 400 ppm (grey symbols) and 700 ppm white symbols) atmospheric $CO_2$ concentration under well-watered (WW) and water-stress (WS) conditions Data points are means (n=6). Lines were fitted using exponential curve for A, linear regression for B and polynomial regression for C.

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| [CO$_2$] | WW | WS | ANOVA Factor | P-values |
|---|---|---|---|---|
| | RWC (%) DAT 1 | | | |
| 300 ppm | 89.9 | 88.9 | CO$_2$ | 0.064 |
| 400 ppm | 89.0 | 89.0 | Watering regimes | 0.802 |
| 700 ppm | 90.1 | 90.0 | CO$_2$ x Watering regimes | 0.869 |
| | RWC (%) DAT 4 | | | |
| 300 ppm | 89.5 | 82.7 | CO$_2$ | 0.182 |
| 400 ppm | 90.2 | 76.6 | Watering regimes | <.001* |
| 700 ppm | 90.8 | 81.6 | CO$_2$ x Watering regimes | 0.120 |
| | RWC (%) DAT 8 | | | |
| 300 ppm | 88.7 | 67.7 | CO$_2$ | 0.016* |
| 400 ppm | 90.9 | 61.9 | Watering regimes | <.001* |
| 700 ppm | 91.8 | 69.2 | CO$_2$ x Watering regimes | 0.018* |
| | RWC (%) DAT 12 | | | |
| 300 ppm | 89.9 | 56.2 | CO$_2$ | 0.501 |
| 400 ppm | 89.6 | 55.6 | Watering regimes | <.001* |
| 700 ppm | 91.8 | 52.3 | CO$_2$ x Watering regimes | 0.065 |

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531 Table 1. Relative water content data (RWC,%) of winter oilseed rape plants grown at
532 historical (300 ppm), ambient (400 ppm) and elevated (700 ppm) atmospheric CO$_2$
533 concentration under well-watered (WW) and water-stress (WS) conditions. Data
534 were collected at days after treatment application (DAT) 1, 4, 8 and 12. Data are
535 means (n=6) ± SE.

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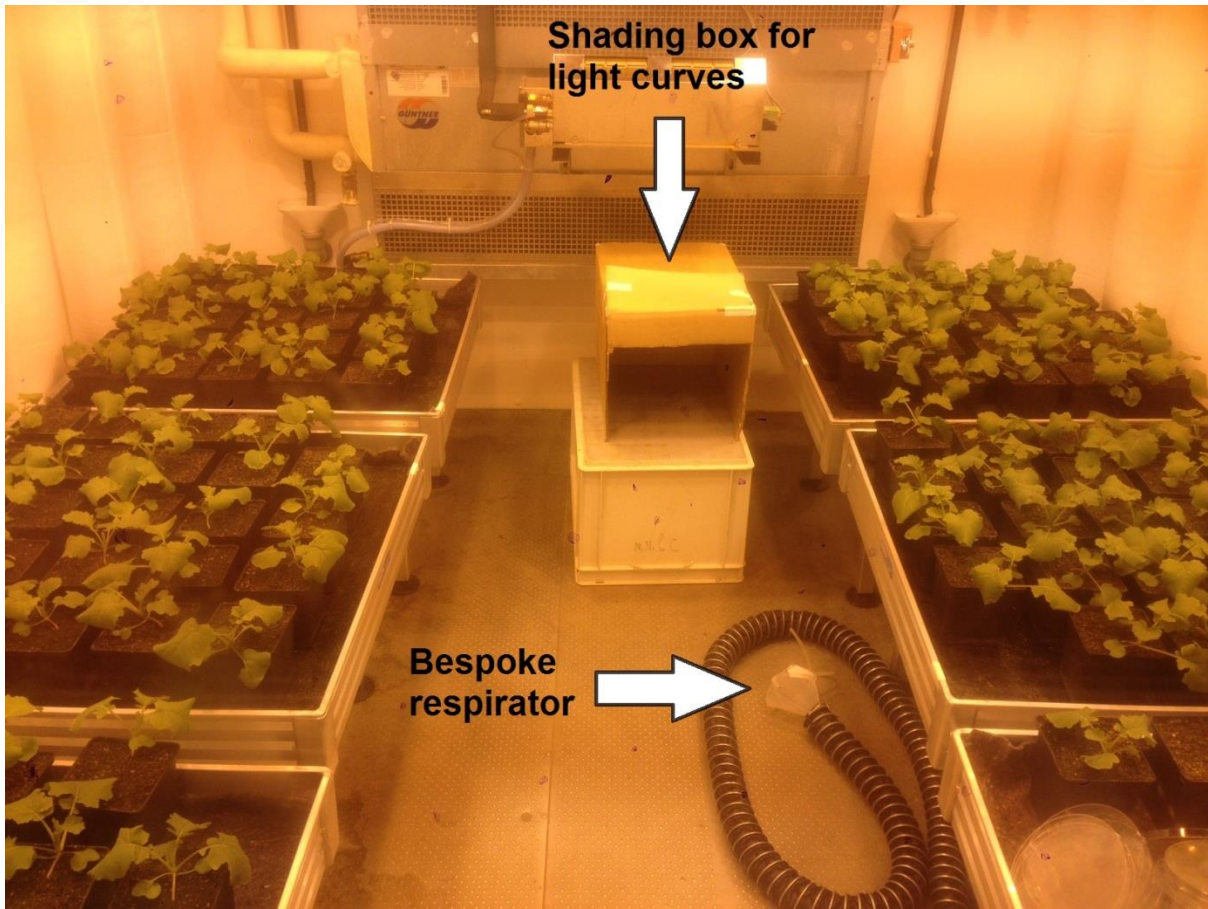
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552     Supplementary Figure 1. Example of experimental room with the bespoke respirator
553          and the cardboard box used to cover plants before the light curve protocol.
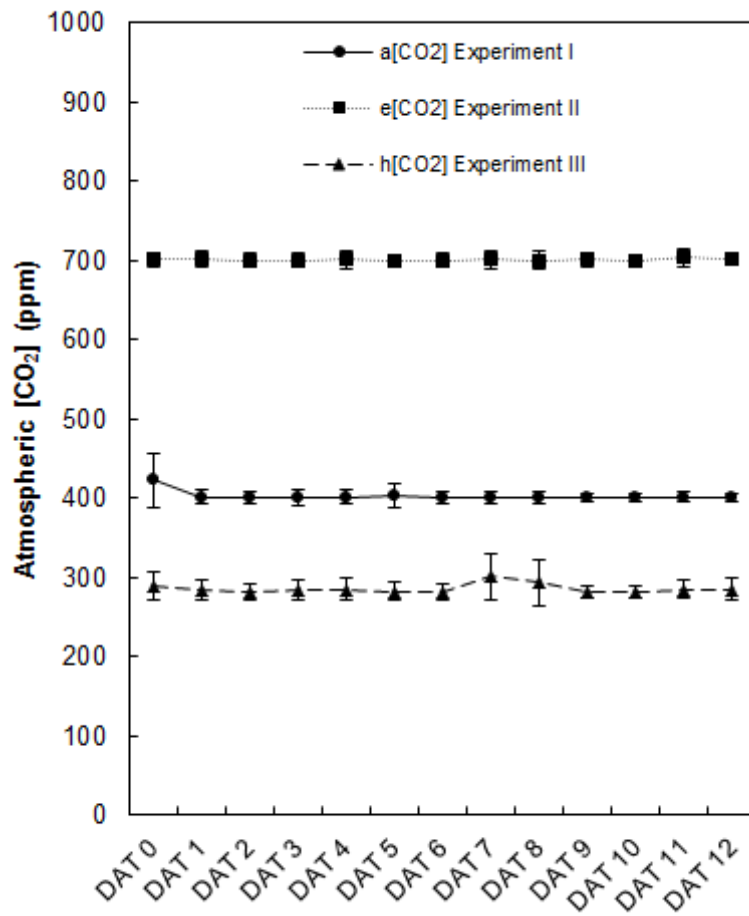
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Supplementary Figure 2. Development of atmospheric [CO$_2$] over Experiment I
($_a$[CO$_2$]), Experiment II ($_e$[CO$_2$]) and Experiment II ($_h$[CO$_2$]). Data points are means
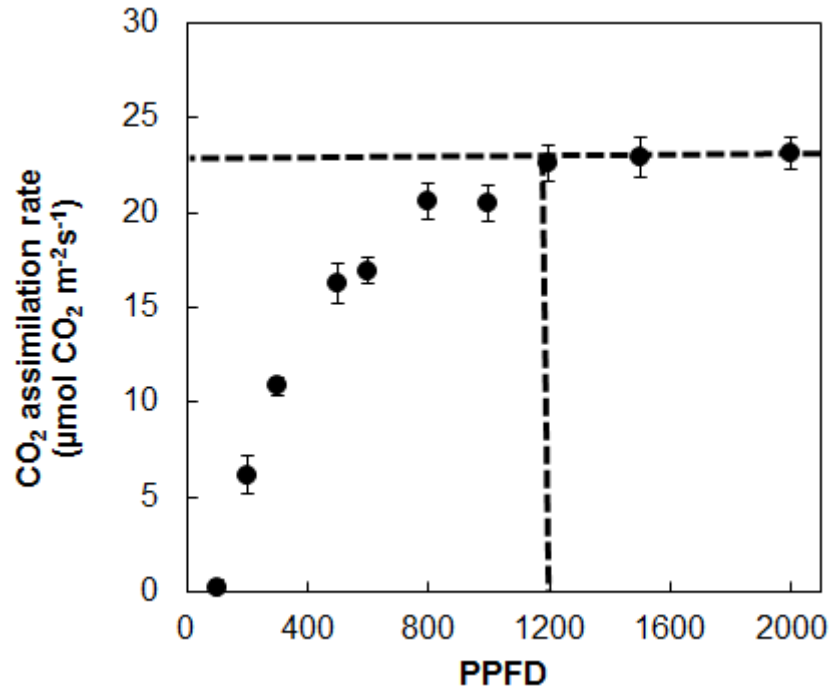(n=1440) ± standard deviation (SD).

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Supplementary Figure 3. Light-response curves for oilseed rape seedlings. The curves were plotted on the third fully expanded leaf before Experiment I. A Li-Cor 6400 was used supplemented with a LI-6400-40 cuvette. $CO_2$ assimilation rates were collected at 100, 200, 300, 500, 600, 800, 1000, 1200, 1500 and 2000 PPFD. Cuvette temperature was maintained at 25°C, the flow rate was 300 µmol s$^{-1}$, $CO_2$ ($C_a$) was 400 ppm and the light source was maintained at 10:90 blue-red light. Data points are means ± SEM ($n$=6) with the exception of 200, 500 and 800 PPFD where $n$=3.

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586 Supplementary Figure 4. Analysis of variance (*P*-values) for [$CO_2$], watering regimes and
587 their interactive effects on plant dry-weight (shoot DW), Leaf area, Leaf number, Specific
588 Leaf Area, $CO_2$ assimilation rate at saturating light ($A_{max}$), stomatal conductance ($g_s$) and
589 water use efficiency ($A_{max}/g_s$) of winter oilseed rape plants grown at 300ppm, 400ppm and
590 700ppm atmospheric $CO_2$ concentration under well-watered (WW) and water-stress (WS)
591 conditions. Values significance is highlighted with asterisks (*P*<0.05)

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| ANOVA Factor | *Shoot DW* | | | |
|---|---|---|---|---|
| | DAT 1 | DAT 4 | DAT 8 | DAT 12 |
| | *P*-values | | | |
| $CO_2$ | 0.219 | <.001* | <.001* | <.001* |
| Watering regimes | 0.662 | 0.053 | <.001* | <.001* |
| $CO_2$ x Watering regimes | 0.829 | 0.358 | <.001* | <.001* |
| **ANOVA Factor** | *Leaf Area* | | | |
| | DAT 1 | DAT 4 | DAT 8 | DAT 12 |
| | *P*-values | | | |
| $CO_2$ | 0.333 | <.001* | <.001* | <.001* |
| Watering regimes | 0.367 | <.001* | <.001* | <.001* |
| $CO_2$ x Watering regimes | 0.953 | 0.052 | 0.001* | <.001* |
| **ANOVA Factor** | *Leaf number* | | | |
| | DAT 1 | DAT 4 | DAT 8 | DAT 12 |
| | *P*-values | | | |
| $CO_2$ | 0.232 | <.001 | <.001* | <.001* |
| Watering regimes | 1 | 0.016* | <.001* | <.001* |
| $CO_2$ x Watering regimes | 0.929 | 0.097 | 0.005* | 0.066 |
| **ANOVA Factor** | *Specific Leaf Area* | | | |
| | DAT 1 | DAT 4 | DAT 8 | DAT 12 |
| | *P*-values | | | |
| $CO_2$ | 0.15 | <.001* | <.001* | <.001* |
| Watering regimes | 0.302 | 0.003* | <.001* | <.001* |
| $CO_2$ x Watering regimes | 0.682 | 0.277 | <.001* | 0.45 |
| **ANOVA Factor** | *$A_{max}$* | | | |
| | DAT 1 | DAT 4 | DAT 8 | DAT 12 |
| | *P*-values | | | |
| $CO_2$ | <.001* | <.001* | <.001* | 0.008* |
| Watering regimes | 0.445 | <.001* | <.001* | <.001* |
| $CO_2$ x Watering regimes | 0.043* | 0.063 | <.001* | 0.071 |
| **ANOVA Factor** | *$g_s$* | | | |
| | DAT 1 | DAT 4 | DAT 8 | DAT 12 |
| | *P*-values | | | |
| $CO_2$ | <.001* | <.001* | <.001* | <.001* |
| Watering regimes | 0.254 | <.001* | <.001* | <.001* |
| $CO_2$ x Watering regimes | 0.71 | <.001* | <.001* | <.001* |
| **ANOVA Factor** | *$A_{max}/g_s$* | | | |
| | DAT 1 | DAT 4 | DAT 8 | DAT 12 |
| | *P*-values | | | |
| $CO_2$ | <.001* | <.001* | <.001* | <.001* |
| Watering regimes | 0.505 | <.001* | <.001* | 0.956 |
| $CO_2$ x Watering regimes | 0.536 | 0.062 | 0.003* | <.001* |

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