1 **Nutrient Challenges in a Changing Atmosphere: Investigating Biomass Growth and**

2 **Mineral Concentration Changes in Soybean Plants under Elevated CO_2**

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23   **ABSTRACT**

24   Rising atmospheric CO_2 levels, projected to reach ~650 ppm by 2050, threaten the nutritional

25   value of food crops. This rise is expected to increase biomass yield in C_3 plants through

26   enhanced photosynthesis and water-use efficiency. However, elevated CO_2 (eCO2) reduces

27   protein, nitrogen, and essential minerals like zinc (Zn) and iron (Fe) in plant leaves and seeds,

28   posing a global nutrition risk. We conducted an experiment using Open Top Chambers to

29   examine the response of three soybean cultivars (Clark, Flyer, and Loda) to ambient (~410 ppm)

30   and eCO_2 (~610 ppm) conditions. These cultivars were selected due to their contrasting

31   responses to eCO_2. Measurements of physiological parameters (i.e., biomass, and nutrient

32   concentration) were taken at different growth stages. Our results showed that eCO_2 increased

33   carbon assimilation, leading to higher aboveground biomass and seed yield (through increased

34   seed number) while root biomass remained unchanged. eCO_2 also reduced stomatal conductance

35   and transpiration. There was a significant decrease in seed nutrient concentration at maturity,

36   particularly iron (Fe), phosphorous (P), potassium (K), and magnesium (Mg), in plants grown in

37   eCO_2. These findings suggest that increased yield, reduced transpiration, and unchanged root

38   biomass are key drivers of nutrient dilution in seeds under eCO_2.

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40   **KEYWORDS**

41   carbon dioxide, nutrient uptake, soybean, photosynthesis

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44 **INTRODUCTION**

45 Since the industrial revolution in the late 1800s anthropogenic emissions of carbon dioxide

46 (CO_2) have increased (Friedlingstein *et al.*, 2023; IPCC, 2023). Consequently, the concentration

47 of CO_2 in the Earth's atmosphere has reached unprecedented levels on a global scale

48 (Friedlingstein *et al.*, 2023). There are several studies providing evidence of increased leaf area

49 index, biomass and yield because of increasing atmospheric CO_2 concentrations (eCO_2) in C_3

50 plants (Ferris *et al.*, 1999; Dermody *et al.*, 2006; Digrado *et al.*, 2024). This increase in biomass

51 is caused by the enhanced rate of photosynthesis with simultaneous decrease in stomatal

52 conductance which drives the increase in water-use efficiency (WUE) in C_3 plants, resulting in a

53 "fertilization" effect (Drake *et al.*, 1997; Long *et al.*, 2006; Loladze, 2014; Myers *et al.*, 2014;

54 Ainsworth & Long, 2021). Consequently, the projected rise in eCO_2 to ~ 650 ppm by 2050 has

55 the potential to positively impact world food production and address the needs of a growing

56 population (Ciais *et al.*, 2014).

57 Several studies have shown that the increased plant growth due to eCO_2 is accompanied

58 by a significant reduction in protein, nitrogen and several other mineral nutrients in plant leaves

59 and seeds (Högy & Fangmeier, 2009; Loladze, 2014; Dietterich *et al.*, 2015; Myers *et al.*, 2017;

60 Soares *et al.*, 2019; Ainsworth & Long, 2021). Previous studies have shown that C_3 grains and

61 legumes, when cultivated in future eCO_2 conditions projected for 2050, have reduced zinc (Zn)

62 and iron (Fe) levels (Loladze, 2014; Myers *et al.*, 2014). The reported decrease in mineral

63 nutrition is of consequence, as much of the world population relies on C_3 grains and legumes for

64 their primary Zn and Fe intake (Tulchinsky, 2010; Myers *et al.*, 2014). This decrease in

65 micronutrients in C_3 grains and legumes has the potential therefore to increase essential

66   micronutrient deficiencies in developing and developed nations, impacting around 2 billion

67   individuals (Tulchinsky, 2010).

68   This change in micronutrients due to eCO_2 is associated with "hidden hunger", which is

69   defined as plant-based diets that meet caloric, but not nutritional needs (Kennedy, 2002; Welch

70   & Graham, 2005). Previous work has shown that plants grown in eCO_2 are adding a teaspoon of

71   total nonstructural carbohydrates (TNC) (about 5g of a starch-and-sugar mixture) per 100g of dry

72   plant mass (Loladze, 2014). The effect on TNC:protein and TNC:mineral ratios is similar to the

73   stoichiometric impact of adding a spoonful of carbohydrates to every 100g of dry plant tissue

74   (Loladze, 2014). To combat this "hidden hunger" (Kennedy, 2002; Welch & Graham, 2005) a

75   greater understanding of the physiological mechanisms responsible for reduced mineral

76   concentration in seeds and other plant organs under higher levels of eCO_2 is needed.

77   There are several hypothesized mechanisms associated with the reduction in nutrient

78   concentration in C_3 plants grown in eCO_2. This includes 1) a reduction in transpiration in leaves

79   and thus reduced bulk flow of nutrients (Mcgrath & Lobell, 2013); 2) an increase in the

80   carbohydrate and fiber content, leading to a mineral dilution in seeds and other plant parts

81   (Poorter *et al.*, 1997; Gifford *et al.*, 2000; Taub & Wang, 2008; Taub *et al.*, 2008; Chaturvedi *et*

82   *al.*, 2017); 3) a reduction in root mineral absorption due to changes in the structure of roots

83   (Beidler *et al.*, 2015) and an alteration in the expression of mineral transporters in root tissue

84   (Taub & Wang, 2008; Leakey *et al.*, 2009; Jauregui *et al.*, 2016), and 4); it has been

85   demonstrated that conditions reducing photorespiration, like high eCO_2, also restrict nitrate

86   uptake and assimilation (Searles & Bloom, 2003; Rachmilevitch *et al.*, 2004; Bloom *et al.*,

87   2010). It is important however, to note that there is a lack of empirical data supporting all these

88   hypotheses and that these hypothesized mechanisms are not mutually exclusive with one another.

89   Further work is needed to experimentally examine these hypotheses to determine the most likely

90   mechanisms contributing to reduced nutrient concentration.

91   Soybean (*Glycine max* L. Merr.) is an extensively cultivated crop and is a model legume

92   species that has notable phenotypic variation related to biomass accumulation and yield

93   responses to eCO_2 (Bishop *et al.*, 2015; Sanz☐Sáez *et al.*, 2017). Furthermore, soybean cultivars

94   that exhibited an increased yield response to eCO_2 levels also exhibited a notable decrease in

95   crucial mineral nutrients, specifically Fe and Zn, in their seeds (Loladze, 2014; Myers *et al.*,

96   2014; Bishop *et al.*, 2015; Aranjuelo *et al.*, 2015). The mechanisms associated with this

97   phenotypic variation in yield and mineral nutrient response to eCO_2 in soybeans are largely

98   unknown, however (Schmutz *et al.*, 2010; Myers *et al.*, 2014; Parvin *et al.*, 2019; Soares *et al.*,

99   2019). To begin investigating these unknowns, an experiment was conducted on the effects of

100   elevated CO_2 levels on soybean physiology and plant tissue mineral concentration. Results from

101   this work will help identify the physiological factors responsible for nutrient loss under high CO_2

102   conditions. Additionally, these findings can be used to help guide future molecular biology

103   experiments to test the transcriptomic responses associated with these physiological changes

104   playing an important role in developing molecular tools for creating more climate-resilient crops.

105

106   **MATERIALS AND METHODS**

107   ***Plant Material and Experimental Conditions***

108   Soybeans were grown at the USDA-ARS National Soil Dynamics Laboratory in Auburn, AL, in

109   open-top chambers (OTC). OTC consisted of a cylindrical, aluminum metal frame that was 3 m

110   wide and 2.4 m tall, with the bottom half covered with clear plastic, allowing the sunlight to

111   penetrate and reach the plants (Rogers *et al.*, 1983; Runion *et al.*, 2008). The double-walled

112   plastic chamber cover consisted of 2.5 cm of perforations in the inner plastic wall, allowing gas

113   distribution into the chamber. Two atmospheric eCO_2 were used across eight chambers, four at

114   ambient (~410 ppm) and four elevated (ambient + 200 ppm CO_2) during daylight hours. Three

115   soybean cultivars (Clark, Flyer, and Loda) were chosen based on their different yield and/or

116   nutrient accumulation responses under eCO_2: Loda had high strong yield response; Flyer showed

117   decreased Zn accumulation in seeds; and Clark showed no change in Zn in seeds (Myers *et al*.,

118   2014; Sanz□Sáez *et al.*, 2017; Soba *et al.*, 2020; Digrado *et al.*, 2024). Chambers were set up in

119   a randomized complete block design ($n = 4$), and each chamber contained four plants from each

120   of the three cultivars.

121   Seeds were inoculated with commercial *Bradyrhizobium japonicum* (N-dure, Verdesian

122   Inc., Cary, NC, https://vlsci.com/products/n-dure/) and germinated in a greenhouse on May 6,

123   2021. On May 10, 2021, seedlings were transplanted into 20-liter black containers filled with soil

124   from the E.V. Smith Research Station (Shorter, AL). The soil is classified as sandy loam,

125   consisting of 23.6% silt and clay, 76.4% sand, 3.2% clay, and 20.4% silt with a pH of 6.1.

126   Immediately following transplanting, containers were placed in the OTC. Following soil test

127   recommendations from Auburn University Soil Testing Laboratory, 1 gram of potash was

128   applied to each container and 2 grams of Miracle Gro to ensure sufficient nutrient availability.

129   Plants were watered daily with a drip tape irrigation system that applied 1.9 liters of water every

130   other day for the first four weeks and every day afterwards to avoid drought stress.

131

132   ***Leaf Gas Exchange, Crop Growth, and Harvest Measurements***

133   Leaf CO_2 assimilation (A) and stomatal conductance (g_s) were measured using an infrared gas

134   analyzer (LI-6800, LI-COR Biosciences, Lincoln, NE). Measurements were conducted during

135   midday hours (10:00 a.m.–2:00 p.m.) on the most recently fully expanded leaf located at the top

136   of the canopy. These measurements were taken 34 days after planting (DAP) on June 9 (V5

137   vegetative stage) and 91 DAP (full seed, R6 developmental stage) (Fehr *et al.*, 1971) on August

138   5. To ensure accuracy, the light intensity and temperature inside the leaf cuvette were adjusted to

139   match the ambient conditions. The LI-6800 was used to measure the ambient light intensity. The

140   relative humidity within the leaf cuvette was maintained at 60–70% and the concentration of CO_2

141   inside the cuvette was set to match that of the ambient or elevated OTC conditions. Gas

142   exchange measurements were averaged from two plants per cultivar per OTC per time point.

143   Biomass sampling occurred 70 DAP (pod filling, R5 developmental stage) on July 15th

144   and 126 DAP (maturity, R8 developmental stage) on September 9th. At the pod-filling stage (70

145   DAP) seed count were measured immediately at harvest. Both aboveground biomass (shoots,

146   leaves, and pods) and belowground biomass (roots) were collected at 70 DAP. The samples were

147   oven-dried for at least 72 hours at 60 °C and then weighed. At maturity (126 DAP), aboveground

148   biomass (stems and pods) was collected. These samples were also dried for 72 h at 60 °C and

149   then weighed. Biomass measurements were averaged from two plants per cultivar per OTC per

150   time point. Harvest index (HI) (HI=seed yield/ (seed yield + aboveground biomass) was also

151   calculated for both 70 and 126 DAP harvest.

152   Nutrient analysis was also completed at 70 and 126 DAP. Aboveground (leaf, stem, and

153   pod) and belowground (root) samples were analyzed at 70 DAP, and aboveground (stem and

154   pods) samples were analyzed at 126 DAP. Dried tissue samples were weighted and ground. The

155   samples were sent to Waters Agricultural Laboratory, Inc. in Camilla, GA, for nutrient

156   concentration analysis. Macronutrient concentrations (%) of nitrogen (N), phosphorus (P),

157   potassium (K), magnesium (Mg), sulfur (S), calcium (Ca), and micronutrient concentrations

158   (ppm) of boron (B), iron (Fe), zinc (Zn), manganese (Mn) and copper (Cu) were determined

159   using inductively coupled plasma mass spectroscopy (ICP-MS). Samples were averaged from

160   two plants per cultivar per OTC at both time points. Nutrient concentration of aboveground

161   biomass samples was calculated as the sum across leaf, stem and pod tissues. Nutrient uptake for

162   macronutrients (g/total biomass per tissue per plant) and micronutrients (mg/total biomass per

163   tissue per plant) was calculated from nutrient concentration and biomass data. The percent

164   change in measurements for elevated vs ambient CO_2 was calculated as ((elevated-

165   ambient)/elevated) *100.

166

167   ***Statistical Analysis of physiological data and data visualization***

168   Statistical analysis of the physiological data (gas exchange, biomass, and nutrient analysis) was

169   conducted using a mixed model procedure of SAS (Littell *et al.*, 1996). The cultivars and CO_2

170   treatments were considered fixed effects, while blocks were considered random effects. When

171   the fixed effect of cultivar, CO_2 or their interaction was significant, Tukey's honest significant

172   differences post-hoc test was performed to compare means (LSMEANS, SAS 9.4, SAS Institute,

173   Cary, NC, USA). Plots were made using ggplot2 in R (v.3.4.4; Wickham *et al.* 2016), and

174   Microsoft® Excel.

175

176   **RESULTS**

177   ***Gas exchange parameters were impacted by the eCO₂ treatment***

178   At 34 DAP, midday stomatal conductance (g_s) (mol H_2O m^{-2} s^{-1}) significantly decreased in plants

179   grown in eCO₂ when averaged across all cultivars (Fig. 1A). Midday stomatal conductance (g_s)

180   decreased by 76.3% in Clark, 82.4% in Flyer, and 73.5% in Loda but main effect of cultivar was

181   not significant (Fig. 1A). At 34 DAP midday carbon assimilation (A) (μmol CO_2 m^{-2} s^{-1})

182   significantly increased in plants grown in eCO_2 when averaged across all cultivars but main

183   effect of cultivar was not significant (Fig. 1B). Specifically, A increased by 18.6% in Clark, 9.4%

184   in Flyer, and 15% in Loda (Fig. 1B). A similar trend was observed at 91 DAP as g_s was

185   significantly decreased under eCO_2 when averaged across all cultivars (Fig. 1C) with both eCO_2

186   and cultivar as significant main effects. g_s was decreased by 15.4%, 31%, and 15% in Clark,

187   Flyer and Loda, respectively, under eCO_2 (Fig. 1C). At 91 DAP, A significantly increased in

188   plants grown in eCO_2 when averaged across all cultivars (Fig. 1D). A was increased by 6.4%,

189   18.1% and 19 % in Clark, Flyer and Loda, respectively and had significant cultivar main effect

190   (Fig. 1D). There was also a trend for a CO_2 by cultivar interaction ($p = 0.07$) at 91 DAP; Flyer

191   and Loda had a significant increase in photosynthesis under eCO_2, while Clark did not (Fig. 1D).

192

193   ***Biomass of aboveground but not belowground plant tissues increased under eCO_2 conditions***

194   At 70 DAP aboveground biomass was significantly increased under eCO_2 levels when averaged

195   across all cultivars, but there was no significant interaction between cultivar and CO_2 (Fig. 2A,

196   Table S1a). Aboveground biomass increased by 19.43%, 20.28% and 22.46% in Clark, Flyer and

197   Loda, respectively. When averaged across all cultivars leaf biomass was also significantly

198   increased under eCO_2 (Fig. 3A). There was also a significant cultivar main effect for leaf

199   biomass with Loda having lower biomass than Clark and Flyer (Fig. 3A). At 70 DAP, root

200   biomass was affected only by cultivar; Loda had significantly lower root biomass than both

201   Clark and Flyer (Fig. 3B Table S1a).

202   At 70 DAP seed yield increased under eCO_2 when averaged across cultivars (Fig. 2C,

203   Table S1a). There was also a significant cultivar effect on seed yield; Loda had significantly

204 higher seed yield than Clark and Flyer, regardless of treatment (Fig. 2C). Per seed dry weight

205 was not affected by elevated CO_2 and varied significantly only with cultivar at 70 DAP (Fig.

206 S1A). Per seed dry weight was significantly higher for Loda than other cultivars (Fig. S1A). At

207 70 DAP total seed number was higher under eCO_2 and was also significantly affected by cultivar

208 (Fig. S2A). Total seed number was significantly higher in Flyer than Clark, regardless of CO_2

209 treatment (Fig. S2A). There was no effect of CO_2 on HI at 70 DAP; however, a significant

210 cultivar main effect was present (Fig. S3A). Loda had significantly higher HI than other cultivars

211 regardless of CO_2 treatments.

212   At 126 DAP, aboveground biomass (pod and stem) was significantly increased under

213 eCO_2 when averaged across cultivars (Fig. 2B, Table S1b). Flyer (21.5%) and Clark (22.39%)

214 showed a significant increase and Loda (19.72%) showed a marginally significant increase in

215 aboveground biomass under eCO_2 compared to ambient CO_2 (Fig. 2B, Table S1b). Seed yield

216 had a 14.46% increase in plants grown in eCO_2 when averaged across cultivars (Fig. 2D, Table

217 S1b). Per seed dry weight only varied significantly with cultivar (Fig. S1B). Clark and Loda had

218 significantly higher per seed dry weight than Flyer regardless of CO_2 level (Fig. S1B). Total seed

219 number significantly increased in plants grown in eCO_2 when averaged across all cultivars (Fig.

220 S2B). There was also a significant main effect of cultivar on total seed number at 126 DAP, with

221 Flyer having higher seed numbers than the other cultivars (Fig. S2B). There was significant

222 reduction in HI under eCO_2 with CO_2 and cultivar as significant main effect (Fig. S3B).

223

224 ***Nutrient concentration in different plant tissues changed under eCO_2 conditions***

225 *Macronutrients*

226   The concentration of macronutrients (N, P, K, Mg, Ca, and S) was assessed at the 70 DAP and

227   126 DAP. At 70 DAP nutrient concentration was measured in root, seed, and aboveground

228   biomass (combination of leaf, pod and stem). At 126 DAP, macronutrient concentration was

229   measured in stems and seeds only. At 70 DAP the concentration of macronutrients in seeds

230   varied significantly only with cultivar, except for S which had both cultivar and CO_2 as

231   significant main effects (Fig. 4A; Table S2). Additionally, N concentration had a significant CO_2

232   x cultivar interaction (Fig. 4A). N had a decrease of 1.1% in Clark and 2.4% in Flyer, and an

233   increase of 1.5% in Loda in eCO_2. Leaf macronutrient concentration did not change with eCO_2

234   or cultivar (Table S2). In roots no macronutrient concentration showed a significant change with

235   eCO_2 except for a significant increase in Mg (5.7-13.4%), a significant decrease in S (5-21%)

236   and a marginally significant decrease in K ($p = 0.07$; 7.2-38%) when averaged across cultivars

237   (Table S2). In aboveground tissues N, Mg and Ca varied significantly with cultivar. K was

238   significantly reduced with eCO_2 while P and S varied significantly by both eCO_2 and cultivar

239   (Table S2). Nutrient uptake of macronutrients increased in leaves (6.3%- 38.43%) under eCO_2 at

240   70 DAP when averaged across all cultivars and was also significantly impacted by cultivar

241   regardless of CO_2 treatment (Fig. S4A; Table S4). The leaves of cultivar Loda showed

242   significantly higher uptake of Mg, Ca, and S, whereas Flyer had a significant increase in Ca and

243   S uptake under eCO_2 (Fig. S4A; Table S4). At 70 DAP nutrient uptake of only N and Mg were

244   significantly increased by eCO_2 when averaged across cultivars in roots (Fig. S4B; Table S4),

245   while Ca ($p = 0.052$) showed a marginally significant increase in nutrient uptake in eCO_2

246   averaged across all cultivars (Fig. S4B; Table S4). Uptake of all macronutrients in roots varied

247   significantly with cultivar at 70DAP except for Cu ($p = 0.081$) (Fig. S4B; Table S4).

248   At 126 DAP all seed macronutrients showed a significant main effect of cultivar (Fig.

249   4B; Table S3). There was significant decrease caused by eCO_2 for P, K, and Mg when averaged

250   across all cultivars (Fig 4B). Reduction in P, K, and Mg concentrations in seed under eCO_2

251   varied from 0.7% - 10.6% across cultivars. There was a moderately significant reduction in Ca (p

252    $= 0.051$) in plants grown in eCO_2 when averaged across cultivars (Fig. 4B). There was also a

253   moderately significant CO_2 x cultivar interaction for Mg ($p = 0.056$) (Fig. 4B; Table S3). Mg

254   decreased in Flyer significantly with eCO_2 (Fig. 4B). In aboveground tissue macronutrients such

255   as P, Ca and S varied significantly by cultivar (Table S3). None of the macronutrients in

256   aboveground biomass showed significant change with eCO_2 (Table S3). At 126 DAP seeds

257   showed an increase (4.1% - 154.2%) for uptake of all macronutrients under eCO_2, with Cu

258   having a moderately significant increase ($p = 0056$). Mg and Ca also varied significantly with

259   cultivar as significant main effect (Fig. 6; Table S4).

260

261   *Micronutrients*

262   The concentration of micronutrients (B, Zn, Mn, Fe, and Cu) was also measured 70 and 126

263   DAP. At 70 DAP nutrient concentration was measured in root, seed, and aboveground biomass

264   and at 126 DAP nutrient concentration was measured in stems and seeds only. All seed

265   micronutrients at 70 DAP showed a significant main effect of cultivar, and Zn, B, and Cu were

266   significantly decreased by eCO_2 when averaged across cultivars (Fig. 5A; Table S2). Mn had a

267   slight increase in eCO_2 when averaged across cultivars ($p = 0.077$). There was also a significant

268   CO_2 x cultivar interaction for Zn and Cu in seeds at 70 DAP (Fig. 5A). The concentration of Zn

269   in Clark was reduced by 15.7% in eCO_2, and Cu in Loda was reduced by 14.8% under eCO_2. The

270   concentration of B was significantly reduced in Clark under eCO_2 by 23.1% (Fig. 5A; Table S2).

271   At 70 DAP B, Zn and Cu were the only micronutrients which varied in aboveground tissue with

272   eCO$_2$ as significant main effect (Table S2). None of the micronutrients in leaf tissue changed

273   significantly with eCO$_2$ or cultivar (Table S2). Concentration of Zn, Fe and Mn in roots varied

274   with cultivar as significant main effect whereas concentration of B and Cu did not show

275   significant change with eCO$_2$ or cultivar (Table S2).

276   Nutrient uptake for micronutrients was also measured at 70 DAP. Nutrient uptake of

277   micronutrients was significantly increased by eCO$_2$ in leaves at 70 DAP, with the except of Zn

278   and Fe (Fig. S4A; Table 4). Only B and Cu had a significant cultivar main effect, with Mn

279   having a moderate cultivar main effect ($p = 0.065$) (Fig. S4A; Table S4). B showed a significant

280   increase in Loda and Cu showed a significant increase in Flyer under eCO$_2$ (Fig. S4A; Table S4).

281   The micronutrient uptake in root tissue at 70 DAP varied with cultivar as significant main effect

282   except Fe ($p = 0.055$) and Cu ($p = 0.081$) which had marginally significant effect of cultivar (Fig.

283   S4B; Table S4). There was no significant impact of eCO$_2$ on nutrient uptake of micronutrients in

284   roots at 70 DAP (Fig. S4B; Table S4).

285   At 126 DAP, seed concentrations of Fe, B, and Cu significantly decreased under eCO$_2$

286   when averaged across cultivars (Fig. 5B; Table S3). Additionally, there was a significant effect

287   of cultivar on Zn, Fe, B, and Cu levels. Both Fe and Cu concentrations were reduced across all

288   cultivars, with Fe decreasing by 11.7 in Clark, 2.5% in Flyer, and 2.4% in Loda, and Cu

289   decreasing by 7.7% in Clark, 6.3% in Flyer, and 14.4% in Loda. Zn concentrations were reduced

290   only in Clark (11.04%) and Loda (6.35%) under eCO$_2$. B also showed a marginally significant

291   CO$_2$ x cultivar interaction ($p = 0.057$), with B concentrations significantly reduced in Clark

292   (42%), Flyer (27%), and Loda (32%) under eCO$_2$. Although there was no significant effect of

293   CO$_2$ or cultivar on Mn concentration, there was a general decrease in Mn concentration with

294   eCO_2 when averaged across cultivars ($p = 0.078$). B was the only micronutrient that decreased in

295   aboveground tissue with both eCO_2 and cultivar as significant main effects (Fig. 6; Table S3.

296   Uptake of all micronutrients except B increased in seeds at 126 DAP under eCO_2 (Fig. 6).

297   Reduction in B uptake was significantly impacted by both CO_2 and cultivar and B was decreased

298   in eCO_2. Finally, Zn uptake was significantly impacted by cultivar only (Fig. 6; Table S3).

299

300   **DISCUSSION**

301   The aim of this study was to explore the physiological mechanisms underlying plant nutrient

302   responses to eCO_2 levels. We selected soybean, a key C_3 commodity crop and a model for

303   legume species, due to its significant role in ecosystem services through atmospheric nitrogen

304   fixation via symbiotic relationships with microorganisms (Schmutz *et al.*, 2010). Our research

305   focused on three soybean cultivars—Clark, Flyer, and Loda—chosen for their contrasting

306   phenotypes under eCO_2 conditions (Myers *et al.*, 2014; Sanz☐Sáez *et al.*, 2017). Flyer and Loda

307   were known to show reduction in Zn concentration (Myers *et al.*, 2014). Loda was previously

308   documented to show increased yield and Clark was documented to be non-responsive under

309   eCO_2 (Bishop *et al.*, 2015). This approach allowed us to investigate how different cultivars

310   respond to increased CO_2 and the resulting impacts on nutrient uptake and assimilation.

311

312   *Increased carbon assimilation and WUE under eCO_2 resulted in increased yield due to increase*

313   *in seed number*

314   The eCO2 treatments significantly enhanced *A* and reduced g_s in soybean plants, leading to

315   improved WUE (Fig. 1). At both 34 and 91 DAP, *A* increased notably across all cultivars, with

316   Flyer and Loda showing the most significant enhancements under eCO_2 conditions (Fig. 1B-1D).

317   This increase in *A* was accompanied by a substantial reduction in g_s, indicating improved WUE

318   (Fig. 1A-1C). The increased photosynthetic efficiency under eCO_2 conditions contributed to

319   greater biomass accumulation in aboveground plant tissues (Fig. 2A) at 70, as well as at 126

320   DAP (Fig. 2B).

321   Furthermore, eCO_2 led to a significant increase in total seed yield, primarily driven by an

322   increase in seed number rather than individual seed weight (Fig. 2C-D; Fig. S1-S2). This

323   suggests that the enhanced yield observed under eCO_2 can be attributed to the increased number

324   of seeds produced per plant, highlighting the role of improved carbon assimilation and WUE in

325   driving yield increases in soybean. The findings underscore the potential of eCO_2 to enhance

326   crop productivity through physiological adaptations that boost photosynthesis and optimize water

327   usage, resulting in more seeds and total yield per plant (Fig. 1; Fig. 2B). Our results align with

328   previously reported studies showing increased *A* and WUE resulting in stimulated increased

329   biomass and yield in soybean (Ferris *et al.*, 1999). Previous work also found increased seed yield

330   under eCO_2 was attributable to increased pod number and total seed number per plant, causing an

331   increase in total seed yield per plant (Rogers *et al.*, 1984; Li *et al.*, 2013).

332

333   *Reduced seed nutrient concentration was observed when plants were grown in eCO_2*

334   The concentrations of macronutrients like P, K, and Mg, as well as micronutrients such as Fe, B,

335   and Cu were significantly decreased in seeds at maturity (126 DAP) in soybean plants grown in

336   eCO_2 (Fig. 4-5 and Table S2-S3). There was also a moderately significant reduction in Ca and

337   Mn concentrations in seeds under eCO_2 at maturity (Fig. 4-5). Mg is essential for the functioning

338   of ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) and chlorophyll (McGrath &

339   Lobell 2013), and the increased carbon assimilation associated with eCO_2 levels during the 70

340   DAP could potentially explain the reduced Mg concentration in seeds. Previous studies have also

341   observed a similar reduction in Cu concentration under eCO_2, while Mg has been previously

342   shown to increase in eCO_2 in fresh edible soybean varieties (Li *et al.*, 2018). Taken together,

343   findings from this study suggest that the observed increase in seed yield, driven by the higher

344   number of seeds, might contribute to the observed mineral dilution across the total seeds

345   produced by each plant. Similar results have been seen previously where increased yield either

346   due to eCO_2 or breeding has resulted in reduced P, K, Mg, Fe, B, Zn, and Cu concentration

347   (Monasterio & Graham 2000; Mcgrath & Lobell 2013; Myers *et al.*, 2014).

348

349   *Cultivar-specific responses to eCO_2 were observed*

350   eCO_2 significantly increased seed yield in Loda, primarily due to an increased number of seeds

351   rather than individual seed weight (Fig. S3-S4). This increase in yield was associated with a

352   substantial rise in *A* and a notable reduction in g_s, leading to improved WUE (Fig. 1A and 1C).

353   Despite the higher yield, Loda exhibited a consistent reduction in both macro- and micronutrient

354   concentrations at 70 and 126 DAP. Previous studies have demonstrated a strong yield increase

355   for Loda under eCO_2 (Bishop *et al.*, 2015; Sanz-Sáez *et al.*, 2017; Digrado *et al.*, 2024). This

356   trend was evident across multiple growth stages, highlighting the challenge of nutrient dilution in

357   high-yielding cultivars under future atmospheric conditions.

358   Clark showed a negative percent change in nutrient concentration in seeds at 126 DAP

359   for macronutrients like P, K, Mg, S, and Ca, and micronutrients such as Zn, Fe, B, Mn, and Cu.

360   It also showed increased seed yield with eCO_2 (Fig. 2D; Fig. 4-5; Table S2, S3). Flyer exhibited

361   an increase in aboveground biomass at both 70 and 126 DAP (Fig. 2A and 2B), along with a

362   notable reduction in key nutrients such as P, K, and Mg in seeds at 126 DAP (Fig. 4-5).

363   However, we observed almost no change in Zn concentration at 70 DAP and a slight positive

364   percent change in Zn concentration in Flyer at 126 DAP (Fig. 4-5).

365   Previously, Clark has been shown to be non-responsive to eCO▢ in terms of yield and

366   seed nutrient concentration, and Flyer has been known to show a significant reduction in Zn

367   concentration (Myers *et al*., 2014; Bishop *et al*., 2015). A study by Bishop et al., (2015)

368   examining the impact of eCO_2 on nine cultivars of soybean (including Clark) reported a

369   significant impact of genotype by year interaction on seed yield, however. Previous studies have

370   also shown the complexity of yield and nutrient concentration responses in soybean cultivars

371   grown under different environmental stresses, geographical locations, and during different years

372   (Köhler *et al.*, 2019; Digrado *et al.*, 2024). For example, a recent study found that when soybean

373   plants were grown in eCO_2 and elevated temperature there no observed yield gains or nutrient

374   losses (Köhler *et al.*, 2019). This indicates that the observed cultivar-specific responses observed

375   in our study may differ from previous studies due to genotype x environmental interactions. This

376   is an important consideration for future work investigating cultivar-specific impacts of eCO_2 on

377   nutrient concentration in soybean.

378

379   *Lack of response of root biomass with eCO_2 may be contributing to decreases in nutrient*

380   *concentration in seeds*

381   Our study reports an increase in seed yield and aboveground biomass at both 70 DAP and 126

382   DAP (Fig. 4 and Table S1). Leaf biomass at 70 DAP increased with eCO_2 in all genotypes (Fig.

383   3A). Leaf biomass and nutrient uptake of all nutrients except Zn and Fe at 70 DAP increased

384   with eCO_2 in all genotypes (Fig. 3A; Fig. S4A). Although the effect of the cultivar on nutrient

385   uptake by leaves was significant for most nutrients, and marginally significant for Mn (p =

386   0.065) it was not significant for Zn and Fe. Under eCO_2, the uptake of all nutrients, except for Zn

387   and Fe, increased significantly when averaged across all cultivars (Fig. S4A; Table S4).

388   However, root biomass measured at 70 DAP (Fig. 3B) did not show any change under eCO_2.

389   Additionally, we observed increases in the uptake of all macro- and micronutrients in seeds

390   (except for a decrease in B) and leaves (except Zn and Fe) under eCO_2 (Fig. 6; Fig. S4A), In

391   roots however, but, there was no significant response in nutrient uptake, except for increase N,

392   Mg, and Ca ($p = 0.052$) to eCO_2 (Fig. S4B).

393   Root architecture, morphology, and physiology play a primary role in water and nutrient

394   acquisition from the soil (Wang *et al*., 2006) and could therefore play an important role in

395   nutrient concentration in seeds. Previous work has found that eCO_2 increased root biomass in

396   non-nodulating soybeans (Rogers *et al.*, 1992), while Van Vuuren et al., (1997) found eCO_2

397   delayed root development in spring wheat. We hypothesize that if eCO_2 results in delayed root

398   development during growth stages associated with pod filling and nutrient accumulation in seeds

399   (Bender *et al*., 2015), it could lead to reduced nutrient absorption and transport by roots. It is of

400   note that while our study was done in pots, we used 20-liter pots which have been shown to be of

401   sufficient size to not impact root biomass in soybean (Ainsworth *et al.*, 2002; Poorter *et al.*,

402   2012). Future work focusing on soybean root biomass changes under eCO_2 in field conditions is

403   needed to better understand how root biomass changes under eCO_2 and the impact this may have

404   on nutrient accumulation in seeds.

405

406   **CONCLUSION**

407   Overall, findings of this study enhance our understanding of how eCO_2 impacts the nutrient

408   concentration in soybean seeds. By measuring physiological parameters such as carbon

409   assimilation (A) and stomatal conductance (g_s), and the biomass of individual tissues like leaf,

410   seed, stem, and root we highlighted the role of g_s and nutrient uptake and transport, particularly

411   in roots. Our work supports the claim that eCO_2 leads to a reduction in micronutrient

412   concentrations in seeds and there is significant cultivar variation in this response. Furthermore,

413   we observed likely genotype x-environment interactions in cultivar-specific responses to eCO_2,

414   namely in the change in Zn and other nutrients in Clark which was previously hypothesized to be

415   non-responsive to eCO_2. We propose that an increased number of seeds, rather than increased per

416   seed weight, contributes to the reduction in seed nutrient concentration due to mineral dilution.

417   Additionally, eCO_2 resulted in increased A and increased biomass of leaves, stems, and seeds but

418   did not impact root biomass. The root nutrient uptake of only N, Mg and Ca increased

419   significantly under eCO_2. This suggests that roots could not compensate for the increase in A by

420   enhancing nutrient absorption, uptake and transport, leading to reduced nutrient concentration in

421   seeds. Future research should aim to enhance our understanding of how nutrient transport and

422   absorption by roots are affected under elevated CO_2 conditions.

423

424   **ACKNOWLEDGEMENTS**

425

427

428   **CONFLICT OF INTEREST STATEMENT**

429   The authors declare no conflicts of interest.

430

431   **DATA AVAILABILITY STATEMENT**

432   Data from this research will be made available upon request.

433

434

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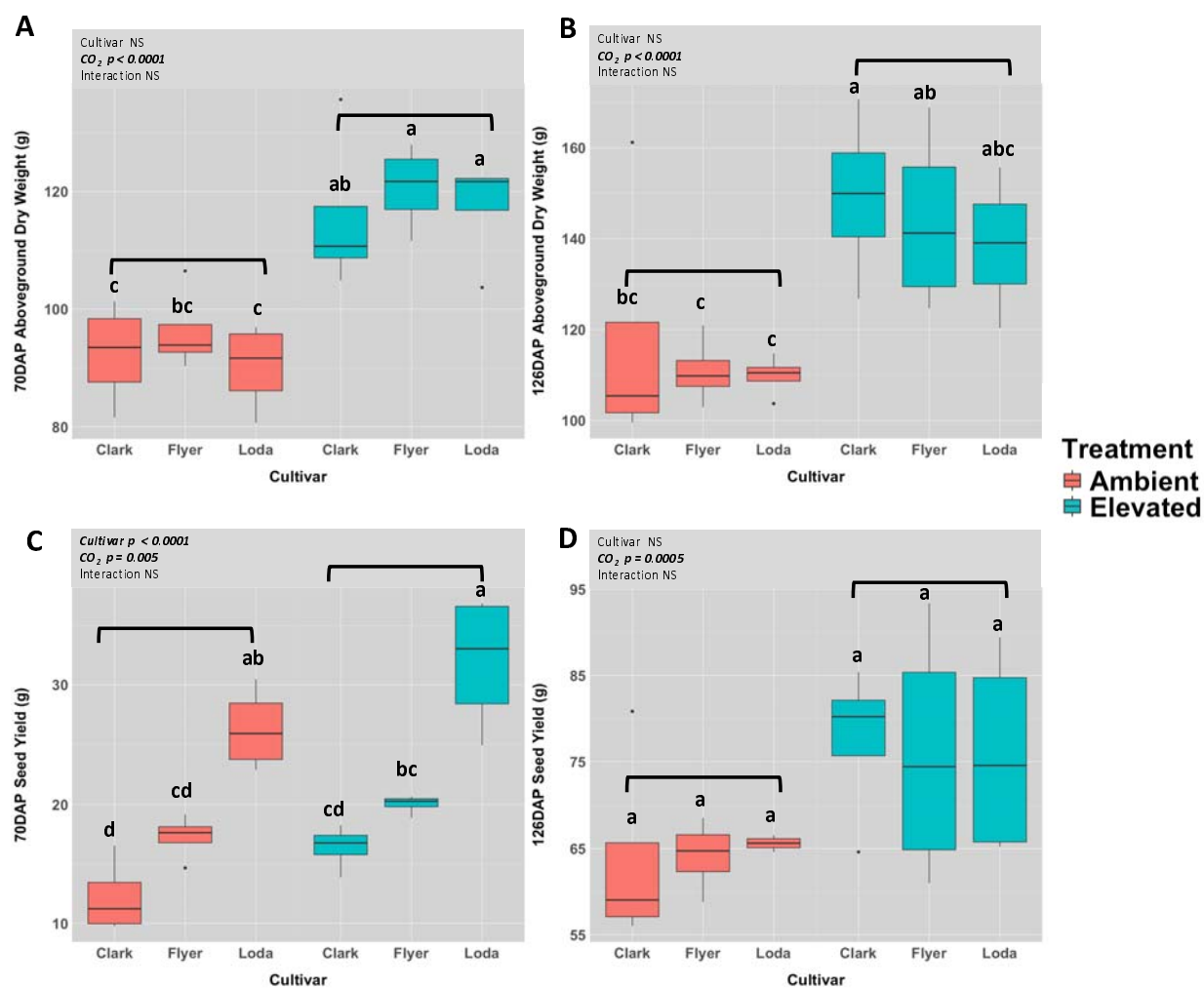
590   **Figure Legends**

591   **Figure 1. The effect of eCO_2 on (A) stomatal conductance (g_s) and (B) carbon assimilation**

592   **(A) rate during 34 DAP and (C) stomatal conductance (g_s) and (D) carbon assimilation rate**

593   **(A) 91 DAP time points.** Different letters indicate significant differences across treatments.

594   Brackets represent significance ($p < 0.05$) of main effect of CO_2 from the two-way ANOVA and

595   are provided for clarity. NS represents no significance. Results of main effect statistics are placed

596   in the upper corners.

597
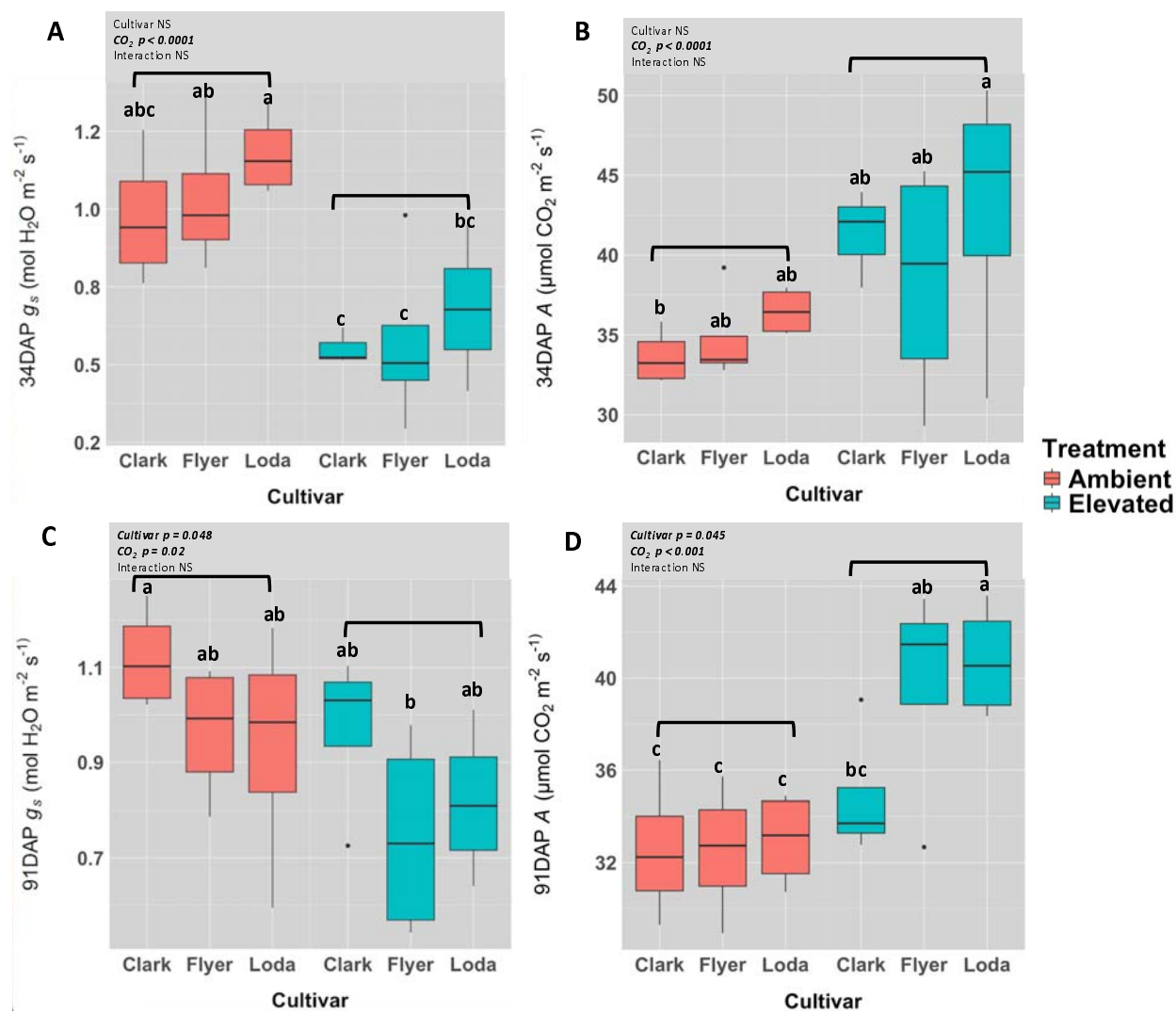
Figure 2. The effect of eCO_2 on aboveground biomass and total seed yield. at (A) 70 DAP (B) 126 DAP and on seed yield at (C) 70 DAP and (D) 126 DAP. Different letters indicate significant differences across treatments. Brackets represent significance ($p < 0.05$) of main effect of CO_2 from the two-way ANOVA and are provided for clarity. Results of main effect statistics are placed in the upper corners. NS represents no significance.
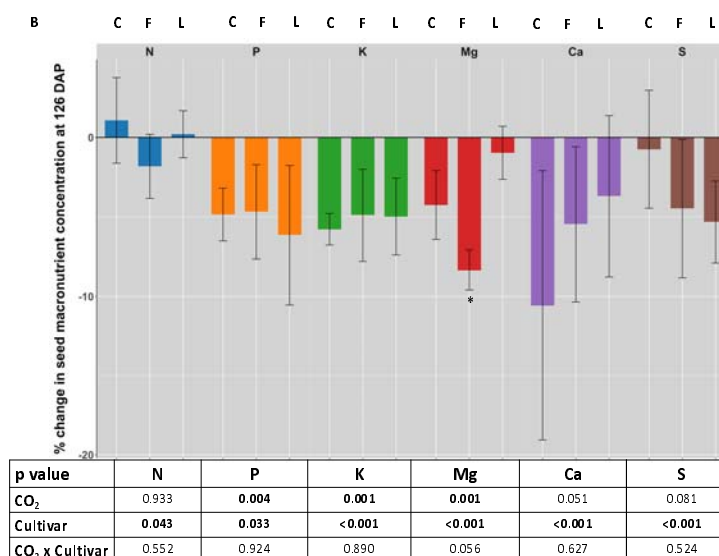
605 **Figure 3. The effect of eCO₂ on (A) leaf dry weight and (B) root dry weight at 70 DAP.** Leaf

606 biomass per plant of each cultivar at 70 DAP in ambient and eCO₂. Different letters indicate

607 significant differences across treatments. Brackets represent significance ($p < 0.05$) of main

608 effect of CO_2 from the two-way ANOVA and are provided for clarity. Results of main effect

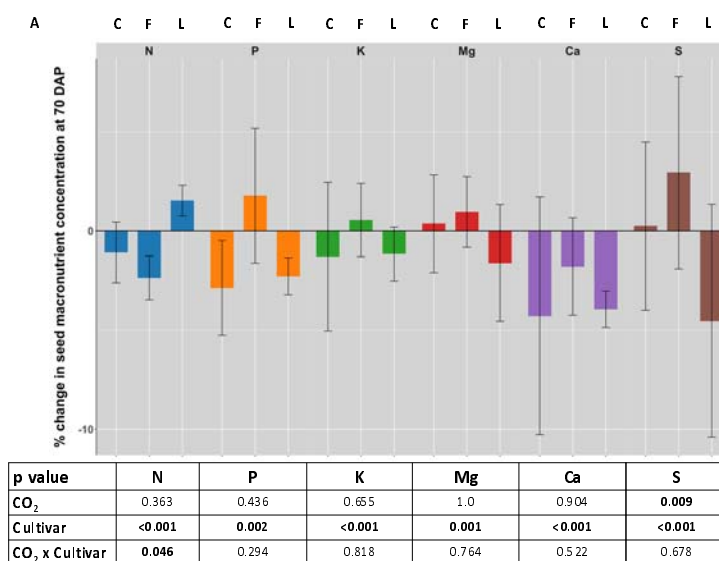609 statistics are placed in the upper corners. NS represents no significance.
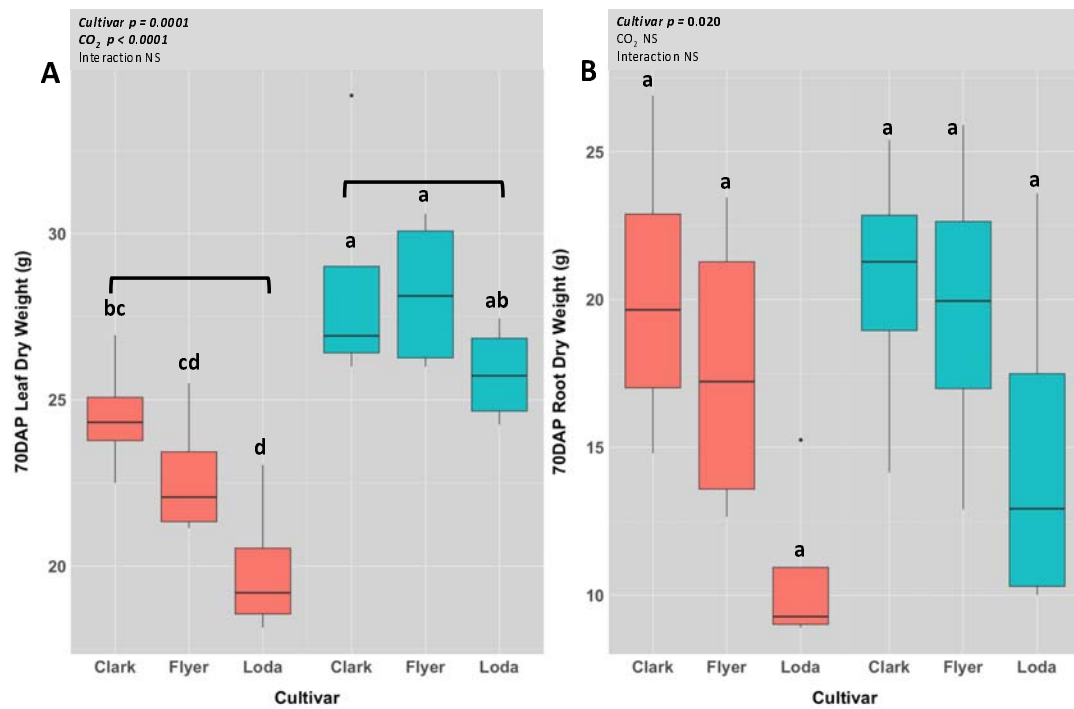
610

611

612 **Figure 4. Percent change (%) at eCO$_2$ versus ambient CO$_2$ of seed macronutrient**

613 **concentration in Clark (C), Flyer (F) and Loda (L).** (A) The percent change in macronutrient

614 (N, P, K, Mg, Ca, and S) concentration in seed, during (A) 70 DAP and (B) 126 DAP. Percent

615 change was calculated as ((elevated-ambient)/elevated) *100. Asterisks indicate significant

616 differences ($p < 0.05$) between elevated and ambient measured/absolute values (for values see

617 Table S2-3). The p values are from two-way ANOVA of the absolute values. Results of main

618 effect statistics are placed in the table below the figure.

p value	N	P	K	Mg	Ca	S
CO$_2$	0.363	0.436	0.655	1.0	0.904	0.009
Cultivar	<0.001	0.002	<0.001	0.001	<0.001	<0.001
CO$_2$ x Cultivar	0.046	0.294	0.818	0.764	0.522	0.678

p value	N	P	K	Mg	Ca	S
CO$_2$	0.933	0.004	0.001	0.001	0.051	0.081
Cultivar	0.043	0.033	<0.001	<0.001	<0.001	<0.001
CO$_2$ x Cultivar	0.552	0.924	0.890	0.056	0.627	0.524

619

620 **Figure 5. Percent change (%) at eCO$_2$ versus ambient CO$_2$ of the seed micronutrient**

621 **concentration in Clark (C), Flyer (F) and Loda (L).** The percent change in micronutrient (Zn,

622 Fe, B, Mn, and Cu) concentration in seed at (A) 70 DAP and (B) 126 DAP. Percent change was

623 calculated as ((elevated-ambient)/elevated) *100. Asterisks indicate significant differences ($p <$

624 0.05) from the two-way ANOVA between elevated and ambient measured/absolute values (see
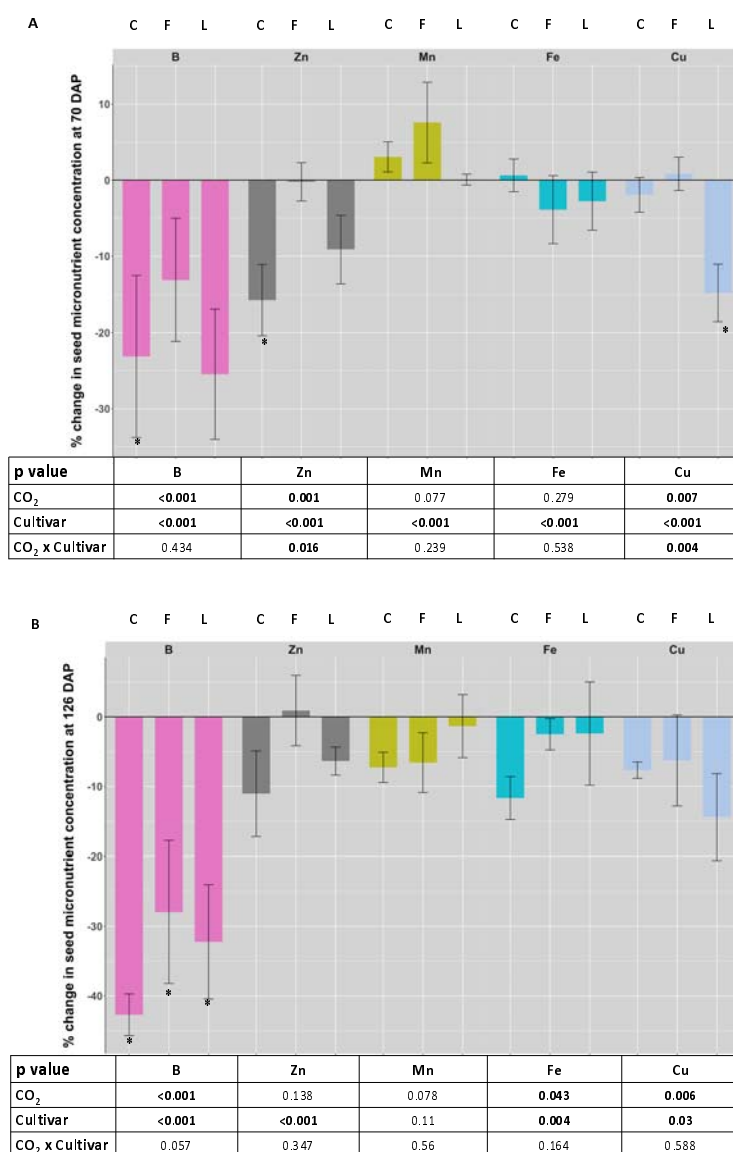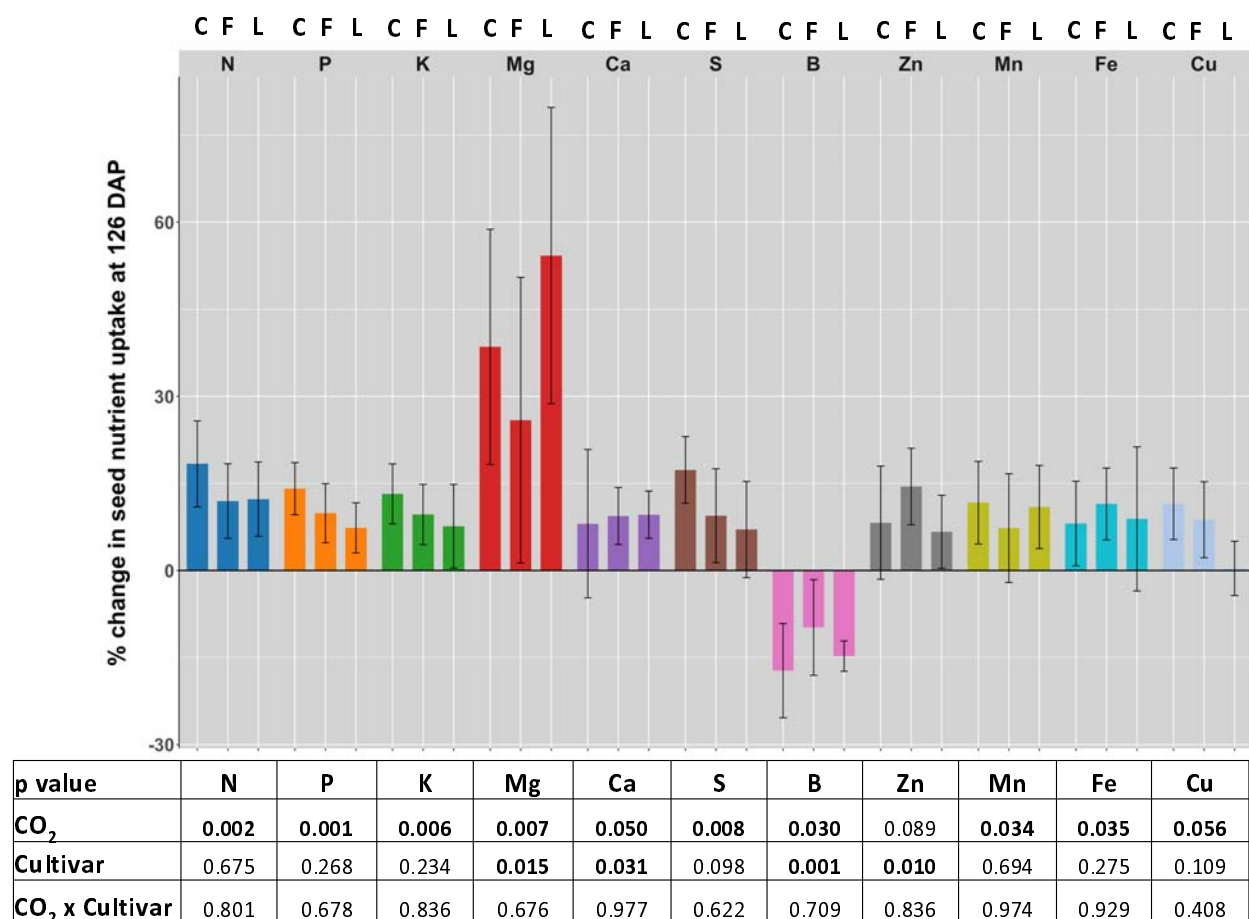
625 Table S2-3).

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Figure 6. Percent change (%) at eCO$_2$ versus ambient CO$_2$ of the seed nutrient uptake in Clark (C), Flyer (F) and Loda (L). The percent change in macro (g/total biomass per tissue per plant) and micro (mg/total biomass per tissue per plant) nutrient uptake (macronutrients: N, P, K, Mg, Ca, S; micronutrients: B, Zn, Mn, Fe and Cu) in seed at 126 DAP. Percent change was calculated as ((elevated-ambient)/elevated) *100. Asterisks indicate significant differences between elevated and ambient measured/absolute values (see Table S4). The *p* values are from two-way ANOVA of the absolute values.

p value	N	P	K	Mg	Ca	S	B	Zn	Mn	Fe	Cu
CO$_2$	0.002	0.001	0.006	0.007	0.050	0.008	0.030	0.089	0.034	0.035	0.056
Cultivar	0.675	0.268	0.234	0.015	0.031	0.098	0.001	0.010	0.694	0.275	0.109
CO$_2$ x Cultivar	0.801	0.678	0.836	0.676	0.977	0.622	0.709	0.836	0.974	0.929	0.408

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Supplemental Figures

Figure S1. The effect of eCO$_2$ on per seed dry weight (A) 70 DAP (B) 126 DAP. Per seed dry weight of each cultivar at 70 DAP in ambient and eCO$_2$. Different letters indicate significant differences across treatments. Brackets represent significance ($p < 0.05$) of main effect of CO$_2$ from the two-way ANOVA and are provided for clarity. Results of main effect statistics are placed in the upper corners. NS represents no significance.

Figure S2. The effect of eCO$_2$ on seed number at (A) 70 DAP and (B) 126 DAP. Seed number per plant of each cultivar at 70 DAP in ambient and eCO$_2$. Different letters indicate significant differences across treatments. Brackets represent significance ($p < 0.05$) of main effect of CO$_2$ from the two-way ANOVA and are provided for clarity. Results of main effect statistics are placed in the upper corners. NS represents no significance.
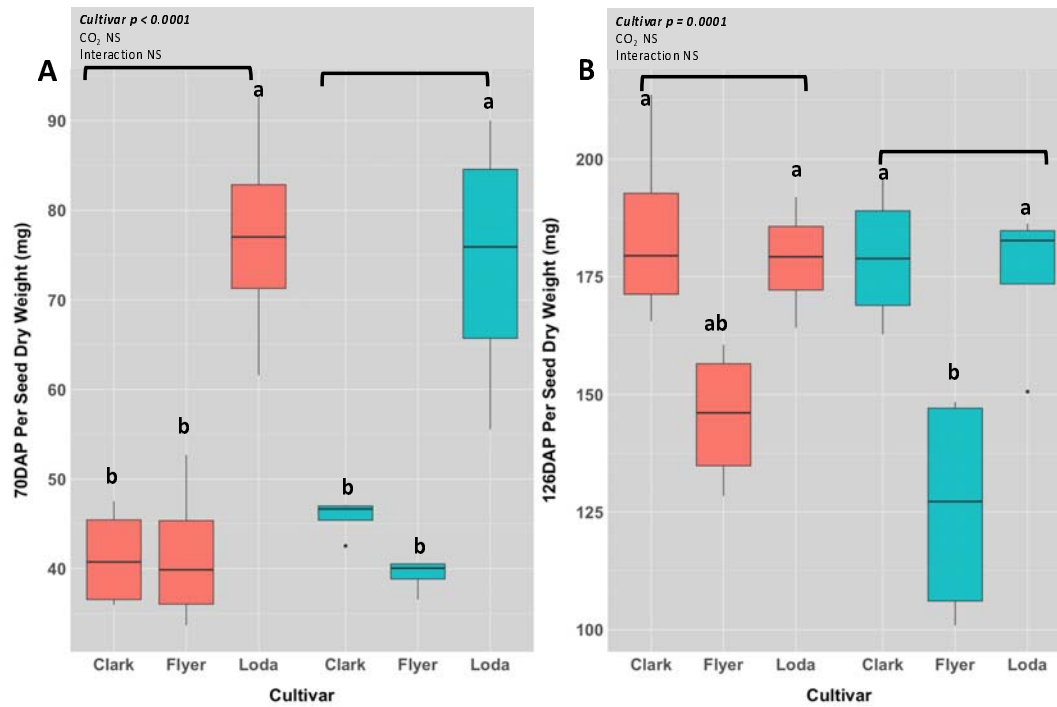
Figure S3. The effect of eCO$_2$ on harvest index at (A) 70 DAP and (B) 126 DAP. Harvest index per plant of each cultivar at 70 DAP in ambient and eCO$_2$. Different letters indicate significant differences across treatments. Brackets represent significance ($p < 0.05$) of main effect of CO$_2$ from the two-way ANOVA and are provided for clarity. Results of main effect statistics are placed in the upper corners. NS represents no significance.
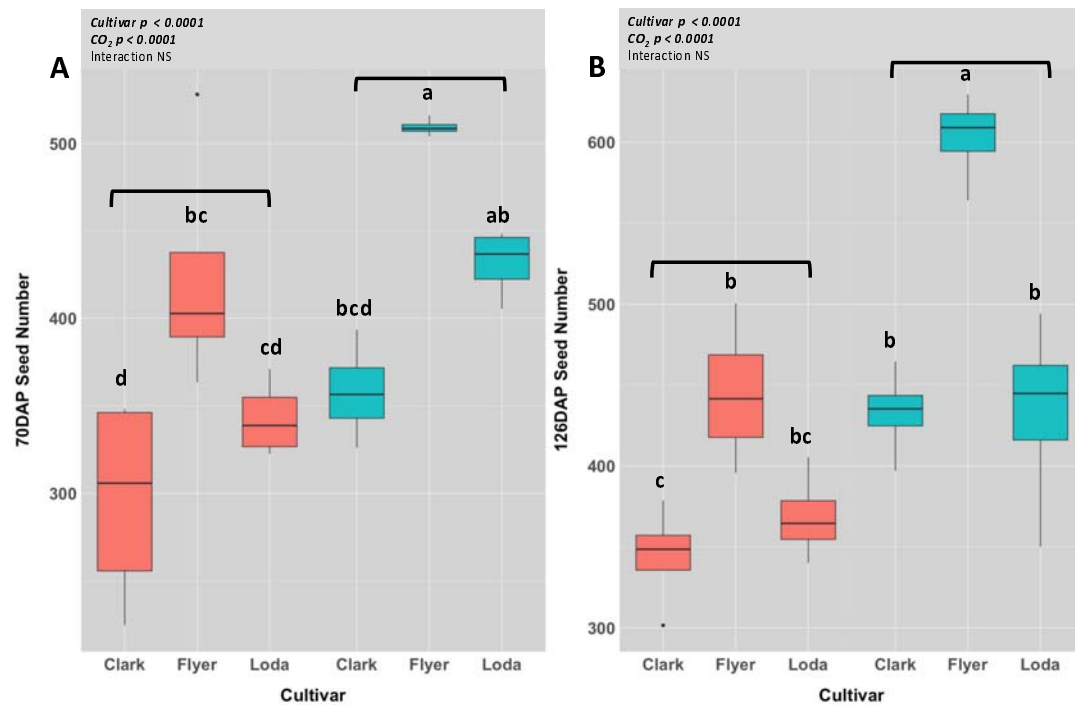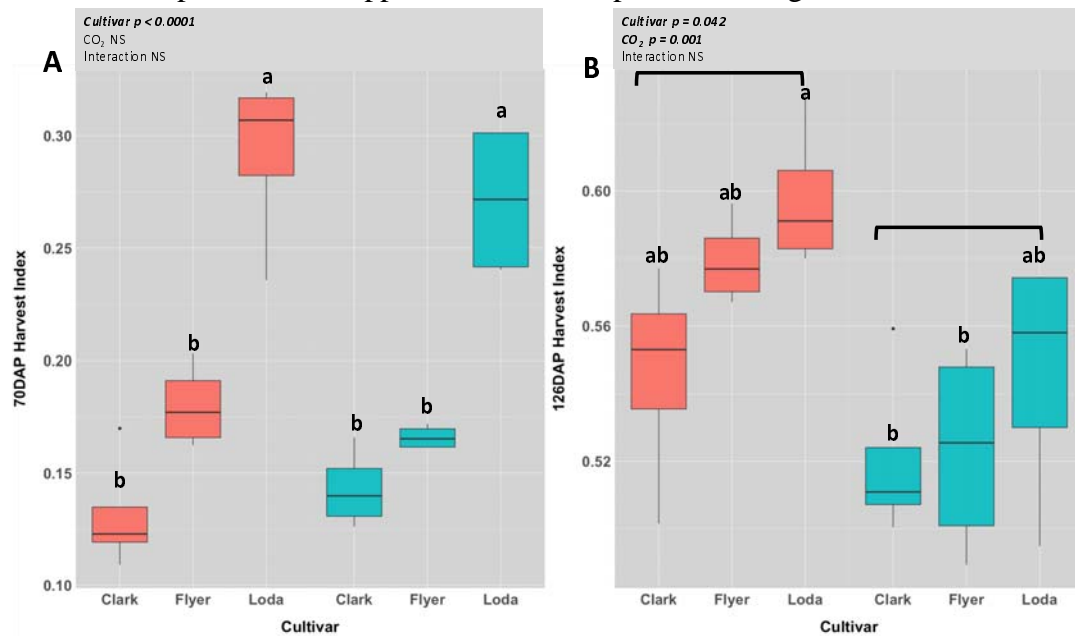
Figure S4. Percent change (%) at eCO$_2$ versus ambient CO$_2$ of the root and leaf nutrient uptake in Clark (C), Flyer (F) and Loda (L). The percent change in macro- (g/total biomass per tissue per plant) and micronutrient (mg/total biomass per tissue per plant) uptake (macronutrients: N, P, K, Mg, Ca, S; micronutrients: B, Zn, Mn, Fe and Cu) in (A) leaf and (B) root tissue at 70 DAP. Percent change was calculated as ((elevated-ambient)/elevated) *100. Asterisks indicate significant differences between elevated and ambient measured/absolute values (see Table S4). The *p* values are from two-way ANOVA of the absolute values.

p value	N	P	K	Mg	Ca	S	B	Zn	Mn	Fe	Cu
CO$_2$	<0.001	0.004	0.008	<0.001	<.0001	<.0001	<.0001	0.093	0.007	0.285	<0.001
Cultivar	0.002	0.013	0.021	0.004	0.001	0.003	0.009	0.160	0.065	0.564	0.011
CO$_2$ x Cultivar	0.887	0.479	0.826	0.182	0.070	0.802	0.056	0.139	0.435	0.631	0.418

p value	N	P	K	Mg	Ca	S	B	Zn	Mn	Fe	Cu
CO$_2$	0.040	0.738	0.579	0.026	0.052	0.975	0.113	0.292	0.471	0.594	0.747
Cultivar	0.045	0.011	0.001	0.007	0.033	0.003	0.020	0.004	0.014	0.055	0.081
CO$_2$ x Cultivar	0.465	0.401	0.066	0.841	0.515	0.501	0.441	0.460	0.733	0.716	0.387