

Nitrogen and Water Demands for Maximum Growth of *Solanum tuberosum* under Doubled CO₂: Interaction with Phosphorus Based on the Demands

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10 **Abstract**

11 Crop growth promotion utilizing elevated carbon dioxide concentrations (e[CO₂]) may
12 be limited by soil nutrient availability. Although numerous studies have suggested the
13 importance of nitrogen (N) for the promotion of growth under e[CO₂], N requirement
14 for maximum plant growth is rarely examined. We have found that increase in potato
15 (*Solanum tuberosum* L.) biomass depends on phosphorus (P) availability under doubled
16 [CO₂] conditions. To address whether the N requirement for maximum growth under
17 e[CO₂] is dependent on P supply or not in potatoes, we quantified potato growth and
18 water consumption in response to five N supply rates at low P (LP) and high P (HP)
19 conditions. A pot experiment was conducted in controlled-environment chambers with
20 ambient CO₂ concentrations (a[CO₂]) and an e[CO₂] level of double a[CO₂]. Foliar
21 critical N concentration per area (critical [N]_{area}), the minimum N requirement for 90%

22 maximum plant growth, was similar (1.43 g N m^{-2}) regardless of $[\text{CO}_2]$ conditions under
23 LP. Under HP, however, the critical $[\text{N}]_{\text{area}}$ increased under $e[\text{CO}_2]$ conditions (1.65 g
24 N m^{-2}) compared with $a[\text{CO}_2]$ conditions (1.52 g N m^{-2}). Water use did not change with
25 $e[\text{CO}_2]$ under HP conditions, whereas it decreased with $e[\text{CO}_2]$ under LP conditions
26 despite the increase in biomass owing to higher water-use efficiency (WUE). Although
27 WUE with $e[\text{CO}_2]$ or HP was independent of N supply, biomass increment with $e[\text{CO}_2]$
28 or HP depended on N supply. We concluded that the N and water required by potato
29 plants under $e[\text{CO}_2]$ would be dependent on P supply. Although under HP, $e[\text{CO}_2]$
30 increased N but not water required to obtain maximum growth during the early growth
31 stage, N demand was unchanged and water demand decreased by $e[\text{CO}_2]$ under LP
32 conditions, probably owing to growth limited by P availability.

33 **Keywords**

34 elevated CO_2 , nitrogen, phosphorus, *Solanum tuberosum* L., plant growth, water-use
35 efficiency

36 **1. Introduction**

37 Atmospheric carbon dioxide concentration ($[\text{CO}_2]$) has increased to over 400 ppm at
38 present and is predicted to keep rising in the future (IPCC, 2014). Regardless of global
39 warming due to elevated $[\text{CO}_2]$ ($e[\text{CO}_2]$), $e[\text{CO}_2]$ can efficiently promote crop growth,
40 known as the CO_2 -fertilization effect, especially in C3 plants (Kimball, 1983; Kimball,
41 2016). Photosynthesis in C3 crops will directly benefit from an increase in atmospheric
42 $[\text{CO}_2]$ because Rubisco activity is not currently CO_2 -saturated, thus $e[\text{CO}_2]$ can
43 competitively inhibit the oxygenation reaction of the enzyme (Drake et al., 1997;
44 Lemonnier and Ainsworth, 2018). However, when crops are grown under $e[\text{CO}_2]$ in the
45 long term, down-regulation of photosynthetic capacity, or photosynthetic acclimation
46 to an $e[\text{CO}_2]$ environment is frequently reported (Ainsworth and Long, 2005; Rogers
47 and Humphries, 2000; Stitt and Krapp, 1999), which in turn inhibits the CO_2 -

48 fertilization effect. Down-regulation of photosynthesis under e[CO₂] is often
49 attributable to insufficient sink capacity to use or store carbohydrates (Lemonnier and
50 Ainsworth, 2018). Further, the accumulation of carbohydrates (e.g., starch) in source
51 leaves could directly lead to the inhibition of photosynthesis (Ainsworth and Bush,
52 2011; Stitt, 1991). Potato (*Solanum tuberosum* L.) is the most important non-grain crop
53 in the world (Raymundo et al., 2018). It has great potential for increased growth in an
54 e[CO₂] environment because of its large sink capacity, which has generally been
55 proposed as a critical factor for maximizing plant production under e[CO₂] conditions
56 (Marschner, 1995).

57 It has been frequently observed that nitrogen (N) deficiency accelerates down-
58 regulation of photosynthesis with e[CO₂] (Pettersson and McDonald, 1994; Stitt and
59 Krapp, 1999), because N-deficiency will limit growth and activity of sink tissues.
60 Previous studies suggested that crop growth under e[CO₂] conditions was inhibited by
61 N deficiency (Ainsworth and Long, 2005; Reich et al., 2014). Furthermore, low N
62 supply was also reported to accelerate senescence that is also induced under e[CO₂]
63 (Agüera and de la Haba, 2018; Aoyama et al., 2014). Therefore, increasing the N supply
64 may be an efficient means to enhance maximum growth in an e[CO₂] environment by
65 inhibiting accelerated senescence. However, despite the fact that there are numerous
66 studies on potato response to N supply (Mokrani et al., 2018; Vos, 1997; Vos and Van
67 der Putten, 1998) and e[CO₂] (Finnan et al., 2005; Lahijani et al., 2018; Miglietta et al.,
68 1998; Schapendonk et al., 2000), the interaction between [CO₂] and N on potato plants
69 has not been examined. Furthermore, how e[CO₂] affects the N requirement for the
70 maximum growth in potato plants is also unclear. To address the issue, we used critical
71 nitrogen concentration (critical [N]), defined as the minimum [N] the crop requires to
72 reach 90% of its maximum growth (Chisholm et al., 1981; Conroy, 1992), to evaluate
73 N status in plants. Critical [N] reportedly became lower under e[CO₂] in cotton and
74 wheat (Rogers et al., 1993), which indicates that N demand by crop plants needs to be
75 reassessed for rising CO₂ concentrations in the future. Therefore, we attempted to

76 quantify the minimum N supply for the maximum biomass as well as foliar critical [N]
77 in potato plants for $e[\text{CO}_2]$.

78 Another major factor that limits plant growth is water (Pugnaire et al., 1999).
79 Terrestrial plants acquire CO_2 and simultaneously lose water via stomata. Stomatal
80 closure increases water-use efficiency (WUE) owing to reduced water loss at the
81 expense of CO_2 acquisition, commonly resulting in growth reduction. However, $e[\text{CO}_2]$
82 actually allows increased WUE without growth reduction, especially in C3 plants
83 (Brouder and Volenec, 2008). An important question is how $e[\text{CO}_2]$ can affect water
84 demand of crop plants, as approximately 70% of the freshwater consumed globally is
85 used in agriculture (Clarke and King, 2004).

86 Our previous study showed that maximum growth of potato plants could be enhanced
87 by 1.5-fold under doubled $[\text{CO}_2]$ without additional water demand because of higher
88 WUE (Yi et al., 2019). However, increases in biomass and WUE in potato plants under
89 $e[\text{CO}_2]$ conditions depend on phosphorus (P) nutrition (Yi et al., 2019). As phosphate
90 rock, from which phosphate fertilizers are made, is a finite and non-renewable resource
91 that may be exhausted in the near future (Vaccari, 2009), crops may be grown in an
92 $e[\text{CO}_2]$ and P-deficient condition. Therefore, how $e[\text{CO}_2]$ and P nutrition affect N and
93 water demands in potato plants should be clarified to manage N fertilizer and water
94 supply in the future.

95 In the present study, we examined the effects of $[\text{CO}_2]$ and P supply as well as N
96 supply on biomass production and water economy in potato plants. The aim of this
97 study was to quantify the growth response of potato plants to N supply under different
98 $[\text{CO}_2]$ conditions and P supply rates to clarify how much N and water are required for
99 maximum growth under each $[\text{CO}_2]$ condition and P supply. The following questions
100 were addressed in the present research: 1) how much N is required to achieve maximum
101 biomass accumulation in potato plants, and whether it is altered by $e[\text{CO}_2]$ and P
102 nutrition, and 2) how $e[\text{CO}_2]$ and P nutrition affect water consumption by potato plants
103 to reach maximum biomass under varying N supply rates?

104 2. Materials and Methods

105 2.1. Experimental design and growth conditions

106 A pot experiment was carried out in controlled-environment chambers (LPH-410
107 SPC, Nippon Medical & Chemical Instruments Co., Ltd., Japan) with the following
108 conditions settings: light intensity, $400 \mu\text{mol m}^{-2} \text{s}^{-1}$; relative humidity, 60 %;
109 temperature, 25 and 17 °C, day and night, respectively; and photoperiod, 14 and 10 h,
110 day and night, respectively. The CO₂ concentrations were controlled at approximately
111 400 ppm for a[CO₂] and 800 ppm for e[CO₂]. The plants and CO₂ concentrations were
112 switched weekly between the two chambers to minimize any potential chamber effects.
113 A CO₂ recorder (TR-76Ui, T&D Inc., Japan) was placed inside each chamber to monitor
114 practical conditions ([CO₂], temperature, and relative humidity) in the chambers every
115 5 min ([Supplementary Figure S1](#)).

116 Naturally sprouted potato tubers ('Irish Cobbler') were transplanted into 1-L pots
117 (diameter, 11.3 cm; depth, 14 cm; one plant per pot) filled with 580 g of dry andosol.
118 Before transplanting, potassium (1.6 g K₂O kg⁻¹ dry soil) was uniformly mixed with the
119 soil in the form of potassium chloride (60.0 % K₂O). Calcium superphosphate (17.5%
120 P₂O₅) was uniformly mixed with the soil to control phosphorus rates at 0.3 (low
121 phosphorus, LP) and 3 (high phosphorus, HP) g P kg⁻¹ dry soil. Urea (46.0 % N) was
122 uniformly mixed with the soil to control N supply rates at 0, 0.2, 0.4, 0.8, or 1.6 g N kg⁻¹
123 of dry soil (hereafter, these treatments are designated as N0, N0.2, N0.4, N0.8, and
124 N1.6, respectively). Soil water condition was kept at approximately 80 % (w/w) by
125 weighing pots and supplementing water. The experiment was organized following a
126 factorial design (two CO₂ concentrations × two P supply rates × five N supply rates)
127 with four biological replicates.

128 2.2. Measurement of water use

129 A transparent plastic film was used to cover each pot to prevent water loss through
130 soil evaporation. Because the pots used had no holes in the bottom, leaching was not
131 considered. We weighed the pots at the day of planting and then from 8 days after
132 planting, weighed every 2-3 days until 15 days after transplanting and then every day,
133 before watering, until harvest. A decrease in pot weight was regarded as water
134 consumption through transpiration, and the amount of water loss by transpiration was
135 provided to each pot. Considering plant keep growing along with the time, extra water
136 was provided to keep soil water condition at about 80% (w/w) by estimating the plant
137 weight according to water-use efficiency in potato plants examined in our previous
138 study (Yi et al., 2019). The pot weight and the amount of water given to each pot were
139 recorded throughout the growth period. Water use during the growth period was
140 calculated from cumulative transpiration. WUE was calculated as total plant
141 biomass/water use according to Jones (2004).

142 **2.3. Stomatal conductance and stomatal density**

143 One day before harvest, stomatal conductance of the youngest fully expanded leaf
144 was measured on the adaxial surface between 8:00 and 12:00 in the morning with a leaf
145 porometer (SC-1, Decagon Devices Inc., USA). Immediately after the measurement of
146 stomatal conductance, the same leaves were coated with nail polish; next, imprints were
147 taken from each leaf and mounted on a glass microscope slide to count the number of
148 stomata under the microscope (SZ61, OLYMPUS Co., Tokyo, Japan). One imprint was
149 taken in each plant. Five observations of each imprint were randomly selected to count
150 the number of stomata; thus, data presented are means of five individual measurements
151 per leaf.

152 **2.4. Harvest and sampling**

153 All plants were harvested 33 days after transplanting. Before harvest, the youngest
154 fully expanded leaf was sampled, between 8:00 and 10:00 in the morning, from each

155 plant for starch quantification. Sampled leaves were immediately frozen in liquid N.
156 After leaf area was analyzed, the samples were dried for starch analysis. At harvest, the
157 remaining leaves, stems, roots, and tubers were separated and dried in an oven at 80 °C
158 to a constant mass for dry weight determination. All samples were then ground to
159 powder for N quantification. Leaf area was analyzed in a flatbed scanner (EPSON
160 EXPRESSION 10000XL, Seiko Epson Co., Japan) using software WinRHIZO Pro
161 LA2400 (Regent Instruments Inc., Canada) before drying.

162 **2.5. Starch quantification**

163 Starch content was determined according to [Ono et al. \(1996\)](#). Samples (3-5 mg each)
164 of micro-ground dried tissue from young leaves were placed in 2-mL microtubes
165 containing 0.75 mL 80 % ethanol and heated at 78.5 °C for 10 min on a heating block.
166 The supernatants were transferred after centrifugation (12,000 ×g, room temperature,
167 10 min) and 0.5 mL 80 % ethanol was added into each tube to heat at 78.5 °C for 10
168 min once again. After centrifugation at 18,000 ×g, at room temperature for 10 min, the
169 supernatants were transferred and residues containing starch were dissolved in 400 µL
170 Milli-Q water, heated at 98 °C for 1 h, and then cooled to room temperature
171 (approximately 27 °C). After adding 400 µL amyloglucosidase (70 units G-
172 Amyloglucosidase/mL 50 mM Na-acetate buffer at pH 4.5), samples were incubated at
173 55 °C for 1 h. After digestion of starch to glucose, samples were centrifuged at 18,000
174 ×g, at room temperature for 10 min, and the supernatants were then assayed for glucose
175 using a Glucose CII test kit (Wako Chemicals, Tokyo, Japan). The assay reagents were
176 mixed into the samples and the reaction was incubated for 10 min at room temperature
177 before measuring their absorption at 505 nm (A_{505}) in a microplate reader (Sunrise
178 Rainbow Thermo, Tecan Japan, Co., Ltd., Japan).

179 **2.6. Nitrogen determinations in plant material**

180 Approximately 1.3-1.7 mg of ground dried samples were encapsulated in 0.15 ml tin

181 foil and processed through an elemental analyzer (Vario EL, Elementar
182 Analysensysteme GmbH, Hanau, Germany).

183 **2.7. Critical nitrogen concentration**

184 Simple regressions were analyzed in Origin 9.0 (<https://www.originlab.com>) to
185 calculate critical nitrogen concentration (critical [N]) on mass and area basis. Critical
186 [N] is defined as the minimum [N] in the crop required to reach 90% of maximum
187 growth (Chisholm et al., 1981; Conroy, 1992). Coefficients of the polynomial equations
188 ($y = a + bx + cx^2$) from regression relations between foliar [N] (x) and total biomass
189 (y) were used to calculate critical [N] as follows,

$$190 \text{ Critical [N]} = \frac{-b + \sqrt{b^2 - (4ac + 9b^2)/10}}{2c}$$

191 **2.8. N uptake, N-uptake efficiency and N-utilization efficiency**

192 N uptake was defined as the total N content in the plant. According to Vos (1997),
193 N-uptake efficiency was calculated as follows,

194 N-uptake efficiency (%) = (N content in N-treated plant – N content in N0 plant) (g)/
195 N supply (g).

196 N-utilization efficiency was calculated following Hirose (2011),

197 N-utilization efficiency (g mg⁻¹) = Total plant biomass (g) / Total plant N content (mg).

198 **2.9. Statistical analysis**

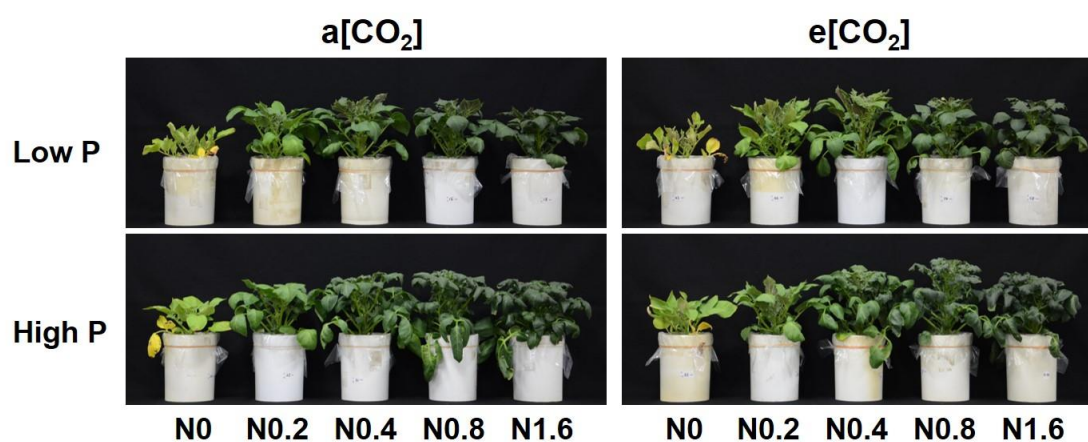
199 The experiment was organized following a factorial design with two CO₂
200 concentrations, two P supply, and five N supply rates with four biological replicates,
201 data are expressed as mean ± standard error (S.E.) for the four biological replicates.
202 Data were analyzed in SPSS 16.0 (SPSS Inc., Chicago, IL., USA) using three-way
203 analysis of variance (ANOVA) at the 0.05 probability level (Supplementary Table S1).
204 Considering the significant interactions between CO₂ concentration and P supply rate
205 from some of the main measured items, a two-way ANOVA was used to analyze the

206 interaction between CO₂ concentration and N supply rate at each P supply rate.

207 3. Results

208 3.1. Plant growth and biomass

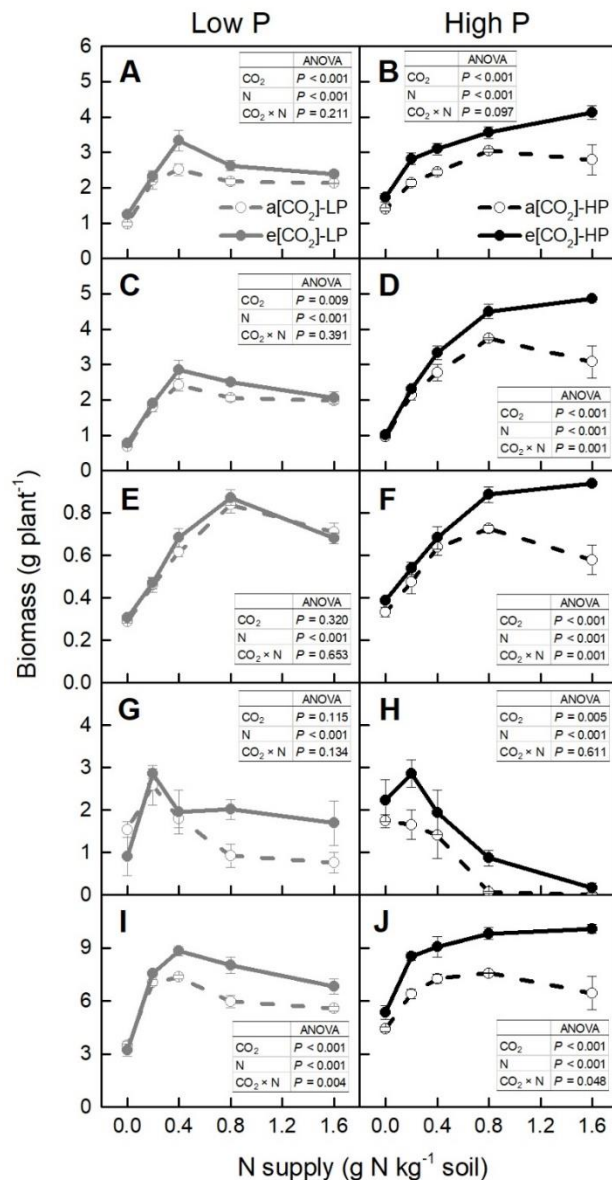
209 Based on the appearance, especially leaf color, plant growth was significantly
210 affected by N supply, and it also appeared to be somewhat affected by both [CO₂] level
211 and P supply (Figure 1). It should be noted that at earlier growth stages, salinity stress
212 was observed under high N supply rates, especially under LP conditions
213 (Supplementary Figure S2).



214 **Figure 1** Appearance of potato plants at harvest (33 days after transplanting).

215 With e[CO₂], increased biomass of leaves, stems, roots, and tubers occurred at each
216 N supply rate under HP, whereas e[CO₂] increased biomass only in leaves ($P < 0.001$)
217 and stems ($P = 0.009$) under LP where clear increases were observed only at some
218 certain N supply rates (N0.4 and N0.8) (Figure 2). Despite the higher tuber biomass
219 under e[CO₂] comparing with a[CO₂] at high N supply (N0.8 and N1.6) under LP,
220 effects of e[CO₂] on tuber biomass was not significant ($P = 0.115$) (Figure 2G). Biomass
221 of leaves, stems, roots, and total plant increased with increases in N supply to a certain
222 range and then decreased, especially under LP (Figure 2). Tuber biomass decreased
223 with the increases in N supply (Figure 2G and 2H). Furthermore, the maximum plant
224 growth under different P supply rates were obtained at different N supply rates (Figure

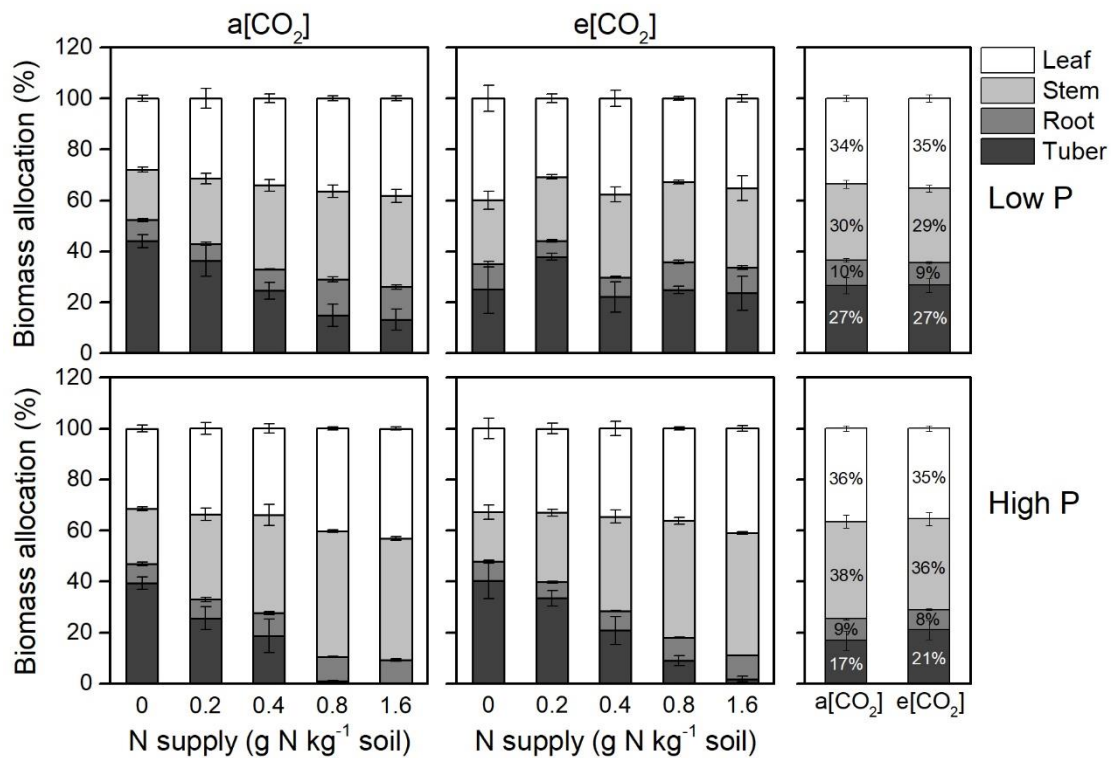
225 2I and 2J). Maximum biomass was achieved at N0.4 in LP under both [CO₂] conditions,
 226 whereas it was achieved at N0.8 and N1.6 under a[CO₂] and e[CO₂], respectively, in
 227 HP.



228 **Figure 2** Biomass of several organs of potato plants grown under a[CO₂] (395 ± 4 ppm)
 229 and e[CO₂] (802 ± 3 ppm) with different N supply rates (0, 0.2, 0.4, 0.8, and 1.6 g N
 230 kg⁻¹ soil) at two P supply rates (0.3 and 3 g P kg⁻¹ soil). Data in each plot are means ±
 231 S.E. (n = 4 biological replicates for each treatment). Statistical comparisons (two-way
 232 ANOVA) between CO₂ concentrations and N supply rates as well as their interaction
 233 (CO₂ × N) are presented. (A) (B) Leaf biomass; (C) (D) stem biomass; (E) (F) root
 234 biomass; (G) (H) tuber biomass; and (I) (J) total plant biomass.

235 **3.2. Biomass partitioning**

236 Under LP, $e[\text{CO}_2]$ did not alter tuber proportion, however, under HP there were
 237 increased biomass allocation to the tuber (Figure 3). To the contrary, high N supply
 238 decreased biomass allocation to tuber under both $[\text{CO}_2]$ and P supply rates (Figure 3).
 239 Compared with LP, HP decreased tuber proportion under both $[\text{CO}_2]$ conditions,
 240 especially at $a[\text{CO}_2]$ (Figure 3).



241

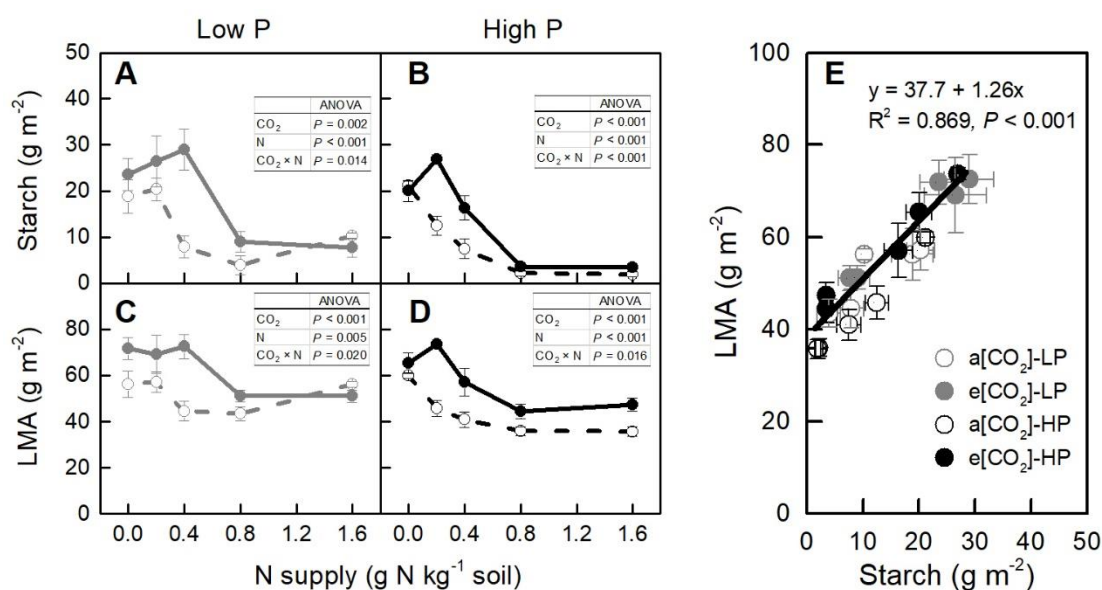
242 **Figure 3** Biomass partitioning in organs of potato plants grown under $a[\text{CO}_2]$ (395 ± 4
 243 ppm) and $e[\text{CO}_2]$ (802 ± 3 ppm) with different N supply rates (0, 0.2, 0.4, 0.8, and 1.6
 244 g N kg⁻¹ soil) at two P supply rates (0.3 and 3 g P kg⁻¹ soil). Data in each plot are means
 245 \pm S.E. (n = 4 biological replicates for each treatment).

246 **3.3. Starch and leaf mass per area**

247 There was a general tendency that starch concentration in the youngest expanded leaf
 248 had the highest peak and decreased along with increases of N supply excepting N1.6,
 249 but the changing patterns were different by $[\text{CO}_2]$ conditions as the significant
 250 interaction effects between them were detected under both P supply rates (Figure 4A

251 and 4B). The highest peak of the starch concentration appeared at higher N supply level
 252 under e[CO₂] than a[CO₂] at both P supply, and the highest peak was observed at lower
 253 N supply level under HP than LP within each [CO₂].

254 Consistent with starch concentration, leaf mass per area (LMA) of the youngest
 255 expanded leaf was higher under e[CO₂] and low N supply compared to those under
 256 a[CO₂] and high N supply (Figure 4C and 4D). A clear correlation between starch and
 257 LMA was observed. (Figure 4E).



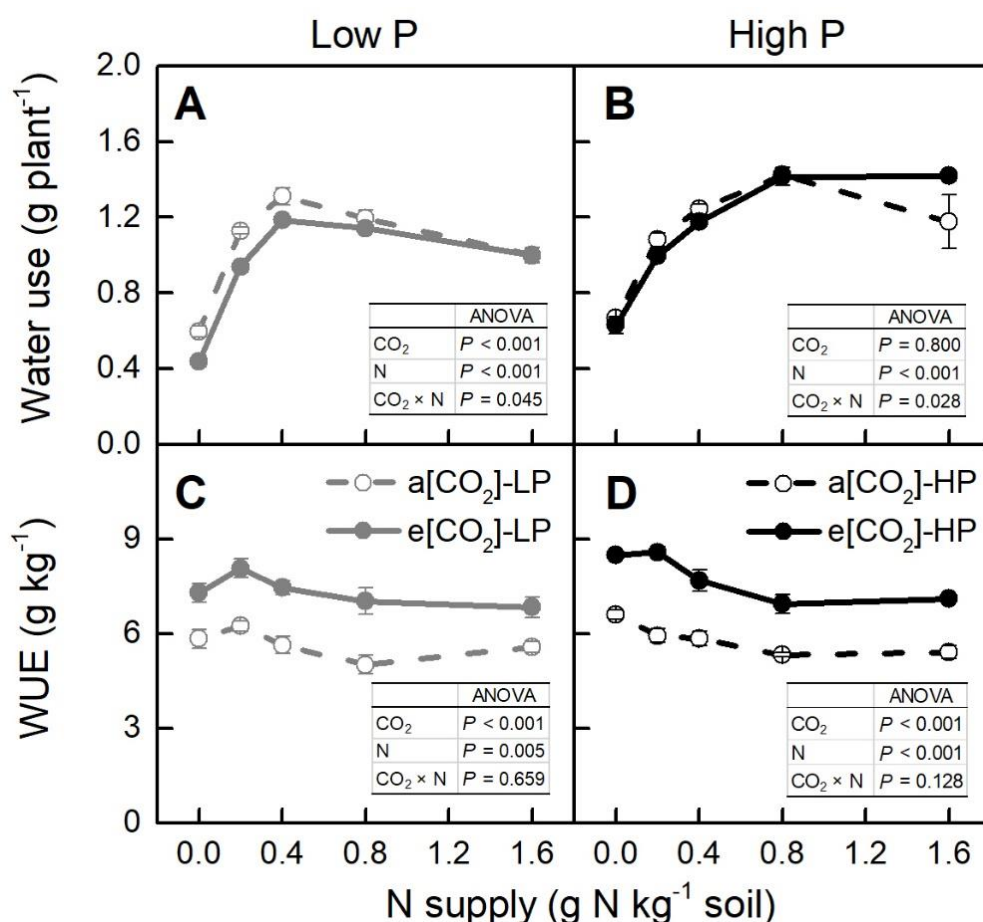
258 **Figure 4 (A) (B)** Starch concentration and **(C) (D)** leaf mass per area (LMA) of the
 259 youngest fully expanded leaf in potato plants grown under a[CO₂] (395 ± 4 ppm) and
 260 e[CO₂] (802 ± 3 ppm) with different N supply rates (0, 0.2, 0.4, 0.8, and 1.6 g N kg⁻¹
 261 soil) at two P supply rates (0.3 and 3 g P kg⁻¹ soil). Data in each plot are means ± S.E.
 262 (n = 4 biological replicates for each treatment). Statistical comparisons (two-way
 263 ANOVA) between CO₂ concentrations and N supply rates as well as their interaction
 264 (CO₂ × N) are presented. **(E)** Relation between starch concentration and LMA of the
 265 youngest fully expanded leaf. Regression is as follows: $y = 37.7 + 1.26x$, $R^2 = 0.869$,
 266 $P < 0.001$.

267 3.4. Water use and water-use efficiency (WUE)

268 We monitored time-course changes in cumulative transpiration as water use in potato

269 plants (Supplementary Figure S3). The interaction effect between [CO₂] and N supply
 270 on water use was significant under both LP ($P = 0.045$) and HP ($P = 0.028$), indicating
 271 the change of water use with N supply was different by [CO₂] conditions (Figure 5A
 272 and 5B). Specifically, water use was lower in e[CO₂] than a[CO₂] at lower N supply
 273 rates, whereas at higher N supply rates, water use was similar (N1.6 at LP and N0.8 at
 274 HP) otherwise became higher (N 1.6 at HP) in e[CO₂]. High N supply increased water
 275 use but from N0.4 under LP, water use gradually decreased (Figure 5A and 5B).

276 A significant increase in WUE by e[CO₂] at each N supply rate was observed under
 277 both P supply rates (Figure 5C and 5D). However, WUE decreased with increases in N
 278 supply until N0.8 and then keep unchanging under both [CO₂] conditions with HP
 279 supply rate, or slightly increasing under a[CO₂] with LP supply rate (Figure 5C and 5D).



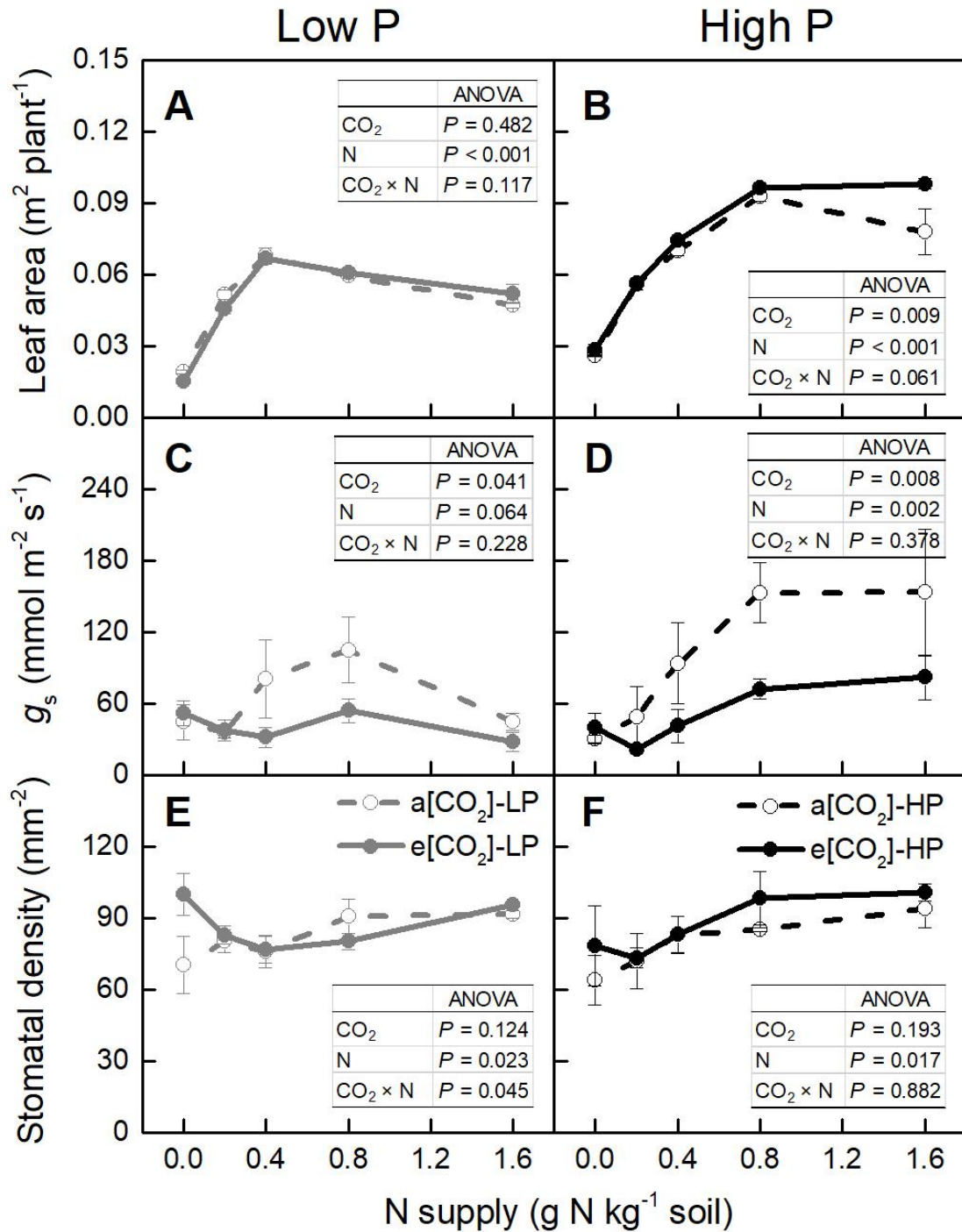
280 **Figure 5 (A) (B)** Water use and **(C) (D)** water-use efficiency (WUE) of potato plants
 281 grown under a[CO₂] (395 ± 4 ppm) and e[CO₂] (802 ± 3 ppm) with different N supply
 282 rates (0, 0.2, 0.4, 0.8, and 1.6 g N kg⁻¹ soil) at two P supply rates (0.3 and 3 g P kg⁻¹

283 soil). Data in each plot are means \pm S.E. ($n = 4$ biological replicates for each treatment).
284 Statistical comparisons (two-way ANOVA) between CO₂ concentrations and N supply
285 rates as well as their interaction (CO₂ \times N) are presented.

286 **3.5. Leaf area, stomatal conductance (g_s), and stomatal density**

287 The most important factors affecting water use in potato plants, leaf area, stomatal
288 conductance (g_s), as well as stomatal density were examined. Leaf area was not affected
289 by e[CO₂] under LP (Figure 6A). Two-way ANOVA results suggest that e[CO₂]
290 decreased g_s but did not change stomatal density (Figure 6C and 6E), which indicates
291 the decreased water use by e[CO₂] under LP was attributed to decreased g_s . Under HP,
292 e[CO₂] slightly increased leaf area (Figure 6B) without increase in water use (Figure
293 5B), which was also related to g_s as a clear decrease in g_s under e[CO₂] was observed
294 (Figure 6D) while stomatal density was not changed (Figure 6F).

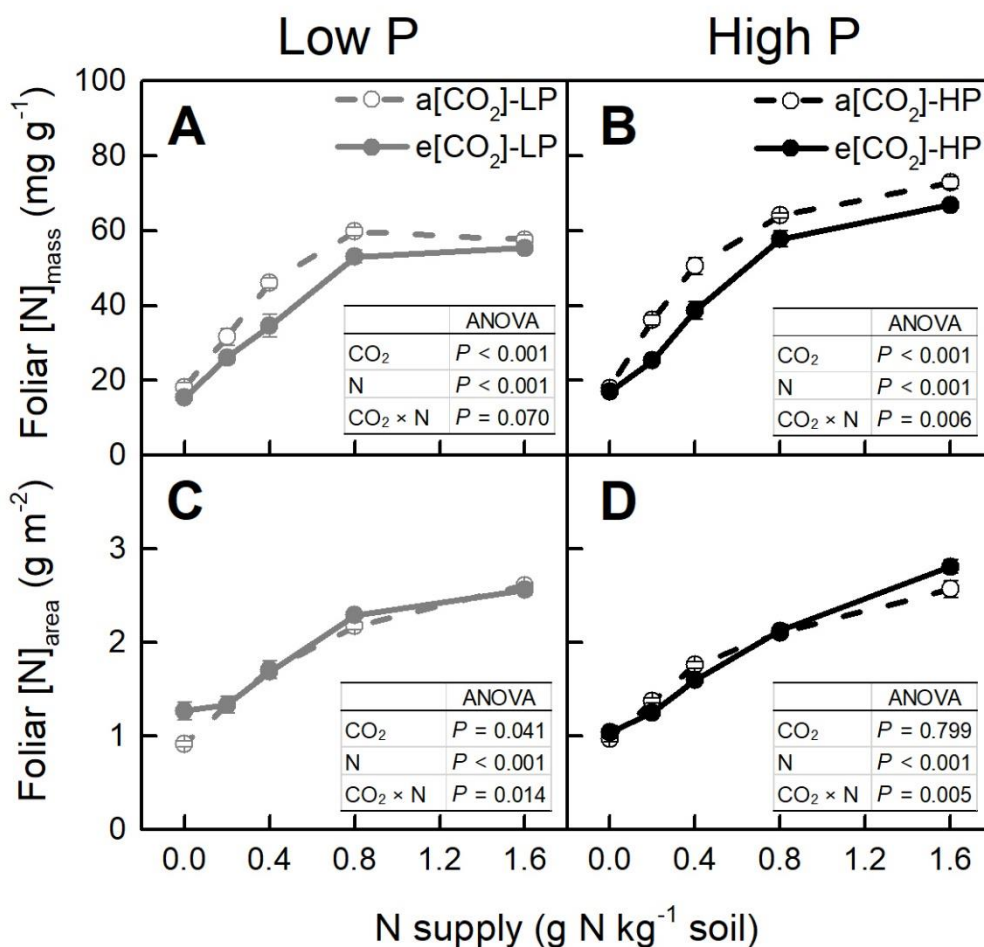
295 Changes in leaf area and water use with an increase in N supply were similar (Figure
296 5A, 5B, 6A and 6B). We found a clear positive correlation between leaf area and water
297 use (Supplementary Figure S4). Additionally, g_s was little affected by N supply under
298 LP ($P = 0.064$), but increased with the increases in N supply under HP ($P = 0.002$)
299 (Figure 6D). High N supply increased stomatal density under HP (Figure 6F), but the
300 effects of N supply became complicated under LP (Figure 6E).



301 **Figure 6** (A) (B) Leaf area, (C) (D) stomatal conductance (g_s), and (E) (F) stomatal
 302 density of potato plants grown under a[CO₂] (395 ± 4 ppm) and e[CO₂] (802 ± 3 ppm)
 303 with different N supply rates (0, 0.2, 0.4, 0.8, and 1.6 g N kg⁻¹ soil) at two P supply
 304 rates (0.3 and 3 g P kg⁻¹ soil). Data in each plot are means ± S.E. (n = 4 biological
 305 replicates for each treatment). Statistical comparisons (two-way ANOVA) between CO₂
 306 concentrations and N supply rates as well as their interaction (CO₂ × N) are presented.

307 **3.6. Foliar N concentration**

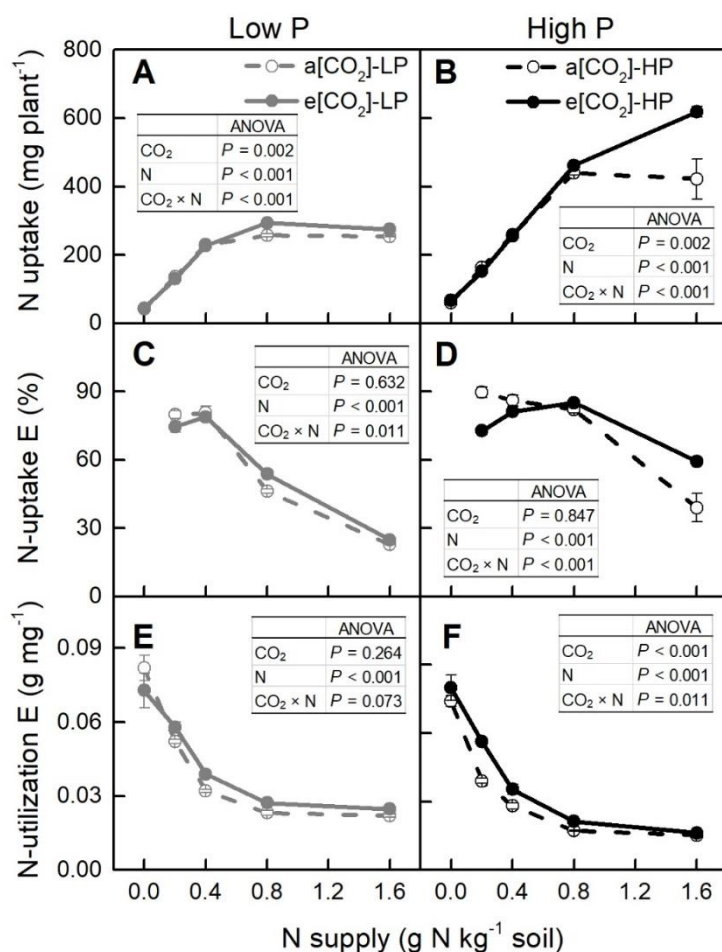
308 As LMA markedly changed (Figure 4C and 4D), foliar N concentration was
 309 calculated based on mass and area. When N concentration was calculated based on mass,
 310 [CO₂] effects were clearly observed under both P supply rates ($P < 0.001$) (Figure 7A
 311 and 7B). However, the effects of [CO₂] were reduced under LP ($P = 0.041$) and
 312 disappeared under HP ($P = 0.799$) when N concentration was calculated based on area
 313 (Figure 7C and 7D).



314 **Figure 7** Foliar N concentration on mass basis ([N]_{mass}) (A) (B), and area basis ([N]_{area})
 315 (C) (D) of potato plants grown under a[CO₂] (395 ± 4 ppm) and e[CO₂] (802 ± 3 ppm)
 316 with different N supply rates (0, 0.2, 0.4, 0.8, and 1.6 g N kg⁻¹ soil) at two P supply
 317 rates (0.3 and 3 g P kg⁻¹ soil). Data in each plot are means ± S.E. (n = 4 biological
 318 replicates for each treatment). Statistical comparisons (two-way ANOVA) between CO₂
 319 concentrations and N supply rates as well as their interaction (CO₂ × N) are presented.

320 3.7. N uptake, N-uptake efficiency and N-utilization efficiency

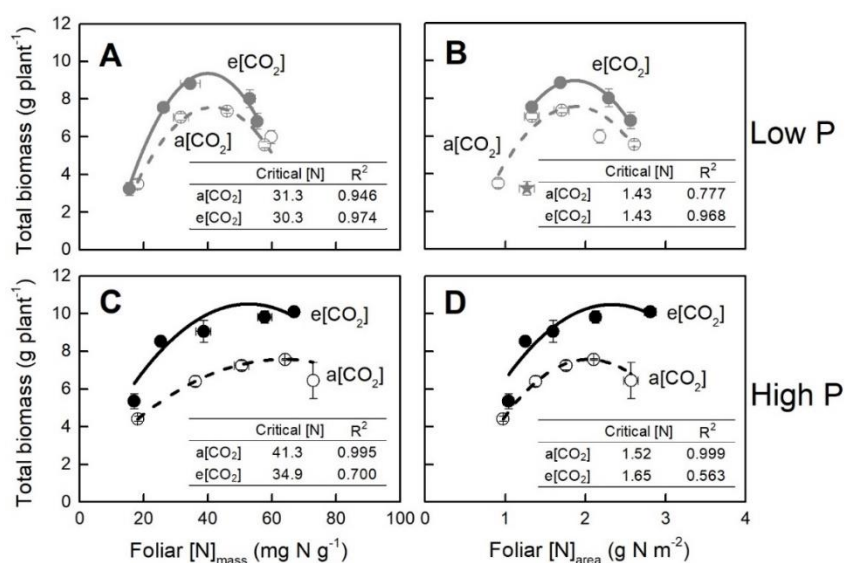
321 N uptake was increased by e[CO₂] when N supply was high particularly at N0.8
 322 under LP and N 1.6 under HP, as supported by the strong interaction effect (P < 0.001)
 323 between [CO₂] and N supply under both P supply rates (Figure 8A and 8B). The effect
 324 of [CO₂] on N-uptake efficiency was also dependent on N supply rate due to the
 325 significant interaction effect between them under both P supply rates; e[CO₂] decreased
 326 the N-uptake efficiency with low N supply, whereas that increased the efficiency with
 327 high N supply (Figure 8C and 8D). The trend was more remarkable under HP
 328 comparing to LP. N-utilization efficiency was increased by e[CO₂] under HP (Figure
 329 8F), but it was not affected by e[CO₂] under LP (Figure 8E). As for the effects of N
 330 supply, N uptake increased with an increase in N supply, especially under HP (Figure
 331 8A and 8B). On the contrary, N-uptake efficiency and N-utilization efficiency
 332 decreased with an increase in N supply under both P supply rates (Figure 8).



333 **Figure 8 (A) (B)** N-uptake, **(C) (D)** N-uptake efficiency (N-uptake E), and **(E) (F)** N-
 334 utilization efficiency (N-utilization E) of potato plants grown under a[CO₂] (395 ± 4
 335 ppm) and e[CO₂] (802 ± 3 ppm) with different N supply rates (0, 0.2, 0.4, 0.8, and 1.6
 336 g N kg⁻¹ soil) at two P supply rates (0.3 and 3 g P kg⁻¹ soil). Data in each plot are means
 337 ± S.E. (n = 4 biological replicates for each treatment). Statistical comparisons (two-way
 338 ANOVA) between CO₂ concentrations and N supply rates as well as their interaction
 339 (CO₂ × N) are presented. N-uptake efficiency (%) = (N content in N-treated plant – N
 340 content in N0 plant) (g) / N supply (g). N-utilization efficiency (g mg⁻¹) = Total plant
 341 biomass (g) / Total plant N content (mg).

342 3.8. Critical N concentration

343 To evaluate N demand in potato plant growth, we analyzed critical N concentration
 344 (critical [N]), the minimum [N] required to achieve 90% maximum growth. We
 345 calculated critical [N] in two ways, that is foliar [N] per dry weight ([N]_{mass}) and foliar
 346 [N] per area ([N]_{area}). The two different calculation methods produced inconsistent
 347 results. Under LP condition, critical [N]_{mass} and [N]_{area} under both [CO₂] showed similar
 348 values, 30 mg N g⁻¹ and 1.43 g N m⁻², respectively (Figure 9A and 9B). Under HP,
 349 however, critical [N]_{mass} decreased under e[CO₂] from 41.3 to 34.9 mg N g⁻¹ (Figure
 350 9C). On the contrary, critical [N]_{area} increased under e[CO₂] from 1.52 g N m⁻² to 1.65
 351 g N m⁻² (Figure 9D).



352 **Figure 9** Relations between total biomass with foliar N concentration on mass basis
353 ($[N]_{\text{mass}}$) under low P (LP) (A) and high P (HP) (C). Relationships between total
354 biomass with foliar N concentration on area basis ($[N]_{\text{area}}$) under LP (B) and HP (D).
355 Critical [N] is defined as the minimum concentration of N required by the crop to reach
356 90% of maximum growth. Critical [N] and R^2 values for regressions are presented. Data
357 in each plot are means \pm S.E. ($n = 4$ biological replicates for each treatment).
358 Regressions are as follows: (A) a[CO₂]: $y = -4.873 + 0.599x - 0.007x^2$, $R^2 =$
359 0.946 ; e[CO₂]: $y = -6.556 + 0.797x - 0.010x^2$, $R^2 = 0.974$. (B) a[CO₂]: $y =$
360 $-6.252 + 14.771x - 3.945x^2$, $R^2 = 0.777$; e[CO₂]: $y = -7.453 + 17.554x -$
361 $4.698x^2$, $R^2 = 0.968$. (C) a[CO₂]: $y = 1.394 + 0.194x - 0.0015x^2$, $R^2 = 0.995$;
362 e[CO₂]: $y = 1.298 + 0.350x - 0.0033x^2$, $R^2 = 0.700$. (D) a[CO₂]: $y = -3.979 +$
363 $11.322x - 2.772x^2$, $R^2 = 0.999$; e[CO₂]: $y = -1.703 + 10.460x - 2.244x^2$, $R^2 =$
364 0.563 . The fitting line of e[CO₂] in (B) was fitted with four plots excluding the plot
365 (star) shown in the figure, because fitting is unavailable when that plot is included.

366 4. Discussion

367 4.1. Which is suitable to assess critical [N], mass basis or area basis?

368 Generally, plants growing under e[CO₂] are expected to require a larger N supply to
369 take full advantage of the CO₂-fertilization effect (Ainsworth and Long, 2005), because
370 reduction in leaf N concentration caused by the dilution effect under e[CO₂] has
371 significant effect on leaf photosynthesis and carbohydrate metabolic process (Li et al.,
372 2016; Sanz-Sáez et al., 2010). In this study, considering the significant difference in
373 LMA under different [CO₂] conditions (Figure 4C and 4D), critical [N], as an index to
374 evaluate N nutrition demand in plants, was assessed in two ways; based on mass ($[N]_{\text{mass}}$)
375 (Figure 9A and 9C) and on area ($[N]_{\text{area}}$) (Figure 9B and 9D). Similar to previous reports
376 in cotton and wheat (Rogers et al., 1993), critical $[N]_{\text{mass}}$ was also decreased by e[CO₂]
377 under HP in potato plants in this study (Figure 9C). However, critical $[N]_{\text{area}}$, which had
378 not yet been examined in previous studies increased with e[CO₂] under HP (Figure 9D).

379 The discrepancy may be due to more carbohydrates (e.g., starch) accumulating under
380 e[CO₂] conditions (Figure 4B), which finally resulted in a higher LMA (Figure 4D).
381 Both critical [N]_{mass} and critical [N]_{area} were similar between a[CO₂] and e[CO₂]
382 regardless of different LMA under LP, implying an involvement of P nutritional status
383 into the above discrepancy; however, the mechanism is unclear. Considering the light-
384 capturing function of leaves in addition to such changes in LMA, it would be suitable
385 to adopt per unit area ([N]_{area}) rather than mass basis ([N]_{mass}) as the critical [N] at leaf
386 level, particularly when LMA is altered. Therefore, we have evaluated if N requirement
387 was affected by [CO₂] and P nutrition on the basis of critical [N]_{area} in addition to the
388 minimum N supply for the maximum growth.

389 **4.2. Is N requirement for the maximum growth affected by [CO₂] with interaction** 390 **of P nutrition?**

391 Foliar critical [N]_{area} was around 1.43 g N m⁻² under both [CO₂] conditions under LP
392 (Figure 9B), but it increased from 1.52 to 1.65 g N m⁻² under e[CO₂] under HP (Figure
393 9D). Consistent with critical [N]_{area}, the minimum N supply for the maximum plant
394 growth was N0.4 under both [CO₂] conditions under LP (Figure 2I), whereas it
395 increased under e[CO₂] from N0.8 to N1.6 under HP (Figure 2J), especially in leaf
396 biomass (Figure 2B). These results reveal that N requirement for the maximum growth
397 increased under e[CO₂] unless P was deficient for the plant as mentioned below.

398 In our previous study, foliar critical P concentration ([P]) in potato plants was around
399 at 110 mg P m⁻² (Yi et al., 2019). From Supplementary Figure S5, foliar [P] under the
400 LP condition was below this value except for N0 under e[CO₂] probably owing to the
401 most severe N-limiting condition; however, foliar [P] under HP was above the value.
402 The result clearly indicates P was sufficient for HP plants but not for LP ones,
403 supporting that our hypothesis that LP and HP treatments provided remarkable contrast
404 that allows comparison between them. As for N status, previous studies showed that
405 foliar [N] in potato plants range from 20 to 90 mg N g⁻¹ (Vos and van der Putten, 1998).

406 In our study, foliar [N] ranged from 15 to 73 mg N g⁻¹, which covers a large range of N
407 levels from N-deficient to N-sufficient. Based on these results, we have confirmed that
408 the plants examined here had a wide range of N nutrition together with a high contrast
409 of P nutrition.

410 As expected, e[CO₂] increased foliar N requirement for the maximum growth
411 biomass only at HP (Figure 9D). Contrary to that, LP decreased the requirement
412 compared to HP for each [CO₂] level (Figure 9), suggesting the strong growth limitation
413 by P decreased N demand. Taub and Wang (2008) suggested two hypotheses to explain
414 why [N]_{mass} in plant tissue decreases under e[CO₂]; 1) dilution of N by increased C, 2)
415 decrease in the specific uptake rates (per unit mass or length of root). As for the latter,
416 they pointed out two factors; decreased N demand by shoots and decreased N supply
417 from soil to root that is induced with transpiration-driven mass flow. In our results, N
418 demand was higher in e[CO₂] unless P was deficient. Furthermore, e[CO₂] did not
419 always suppress N uptake (Figure 9B and 9D) considering the similar leaf area
420 excepting N1.6 at HP (Figure 6B) as well as plant transpiration (Figure 5B). These
421 results would not likely support that e[CO₂] decreased either N demand by shoots or N
422 supply from soil to root.

423 There was significant interaction between [CO₂] and N supply on total plant biomass
424 ($P = 0.004$ and 0.048 under LP and HP, respectively) (Figure 2I and 2J), indicating that
425 additional N supply is required to sustain the positive effect of e[CO₂] perhaps via
426 utilizing additional carbohydrates for the development of new sink organs (Uprety and
427 Mahalaxmi, 2000). The N-utilization efficiency is a long-term indicator of availability
428 of N utilization for C acquisition in plants (Wang et al., 2010). Grown under lower N
429 condition would exacerbate the shortage of leaf N relative to C in plant (Stitt and Krapp,
430 1999), thus resulting in increased N-utilization efficiency (Figure 8E and 8F). Similarly,
431 higher N-utilization efficiency under e[CO₂] also indicates relative N shortage
432 comparing with that under a[CO₂], thus more N supply is required in a CO₂ enrichment
433 condition.

434 The maximum total plant biomass was increased at $e[\text{CO}_2]$ by 1.2- and 1.4-fold under
435 LP and HP, respectively (Figure 9), which confirmed the CO_2 -fertilization effect in
436 potato plants. However, enhancement of the maximum total plant biomass by $e[\text{CO}_2]$
437 was lower than our previous study (1.5-fold) (Yi et al., 2019). As Ainsworth et al. (2002)
438 and Dong et al. (2017) pointed out, plant growth response to $e[\text{CO}_2]$ is related to the
439 growth stage. Thus, the difference in the increments of maximum plant biomass
440 between our previous study (Yi et al., 2019) and this study might be related to different
441 growth stages. It is widely known that potato growth would be suppressed by nutrient
442 deficiency, including N-deficiency (Mokrani et al., 2018; Vos, 1997; Vos and van der
443 Putten, 1998) and P-deficiency (Yi et al., 2019). The increment of biomass by $e[\text{CO}_2]$
444 was found to depend on both N and P supply in the current study (Figure 2I and 2J).
445 Additionally, starch was increased under both low N and low P supply as well as $e[\text{CO}_2]$
446 (Figure 4A and 4B). This indicates photosynthesis may be inhibited by both nutrient
447 deficiencies and $e[\text{CO}_2]$, because over-accumulation of starch in leaves may damage
448 internal organization of chloroplasts (Pritchard et al., 1997; Yelle et al., 1989) or hinder
449 CO_2 diffusion in the chloroplasts (Jauregui et al., 2018; Makino and Mae, 1999; Sawada
450 et al., 2001).

451 **4.3. Is water requirement for the maximum growth affected by $[\text{CO}_2]$ with** 452 **interaction of P nutrition?**

453 Water use for the maximum plant biomass was unchanged by $e[\text{CO}_2]$ under HP (N0.8
454 at $a[\text{CO}_2]$ and N1.6 at $e[\text{CO}_2]$) (Figure 5B) but decreased under LP (N0.4 at the both
455 $[\text{CO}_2]$) (Figure 5A). Based on these results, it is likely that the water requirement for
456 the maximum growth does not increase under the doubled CO_2 condition. This could
457 be explained by the decreased g_s for $e[\text{CO}_2]$ under both P supply rates (N0.4 vs. N0.4 in
458 Figure 6C and N0.8 vs. N1.6 in Figure 6D) besides the similar leaf area (Figure 6A and
459 6B) at the corresponding N treatment achieving the maximum biomass.

460 Consistent with previous studies (Ainsworth and Rogers, 2007; Polley, 2002), $e[\text{CO}_2]$

461 increased WUE significantly under each treatment in this study (Figure 5C and 5D).
462 Moreover, both $e[\text{CO}_2]$ and HP increased WUE independently from N supply
463 (Supplementary Table S1). Stomatal conductance under $e[\text{CO}_2]$ was lower than that
464 under $a[\text{CO}_2]$ at each N supply rate, especially under HP (Figure 6C and 6D). However,
465 stomatal density was unaffected by $e[\text{CO}_2]$ (Figure 6E and 6F). In line with the
466 statement by Finnan et al. (2005), decreases in g_s on long-term exposure to $e[\text{CO}_2]$ could
467 be expected to improve WUE in potato plants.

468 As for the effects of P supply, WUE slightly increased under HP than LP at each N
469 supply rate, except for a distinct increase at the N0 supply rate (Figure 5A and 5B). Like
470 the effects of $e[\text{CO}_2]$ on WUE, HP was also reported to increase WUE by decreasing g_s
471 in N-sufficient potato plants, despite no effect on stomatal density (Yi et al., 2019).
472 However, g_s was higher under HP compared to LP in this study (Figure 6C and 6D).
473 Though the cause of the discrepancy of g_s in the two studies is not clear, it can be
474 concluded that HP could increase WUE in potato plants.

475 Surprisingly, high N supply decreased WUE in potato plants with an increase in g_s
476 as well as stomatal density (Figure 5 and 6), despite numerous literatures demonstrating
477 increased WUE according to N supply (review by Brueck, 2008). Wei et al. (2018)
478 reported that plant WUE (total dry weight/plant water use) was decreased under high N
479 supply in tomato plants even with the higher intrinsic WUE (photosynthesis/ g_s) and
480 instantaneous (photosynthesis/transpiration) compared to low N supply. This
481 discrepancy indicates that intrinsic WUE or instantaneous WUE may not always reflect
482 plant WUE, implying a difficulty to integrate intrinsic or instantaneous WUE measuring
483 several mm^2 on a certain leaf at several minutes to a whole plant growing in several
484 days. In our study, we speculated that the increase in biomass accumulation was smaller
485 than the increase in water use during the growth period examined, thus lower WUE was
486 observed at high N supply rate. This finding would be important for N fertilizer
487 management at different growth stages in potato production.

488 5. Conclusions

489 The current study aimed at quantifying the growth response of potato plants to N
490 supply under different [CO₂] conditions and P supply rates to clarify how much N and
491 water are required for maximum growth under each [CO₂] condition and their
492 dependencies on P supply. After carrying out trials, we found area based foliar critical
493 [N] to be more suitable than mass based foliar critical [N] to evaluate plant N state.
494 Based on this, we concluded that N requirement to obtain the maximum growth during
495 the early growth stage in potato plants under e[CO₂] would be dependent on P supply;
496 HP increased the N requirement, but LP would not affect it, probably owing to the
497 limited growth by P. As for water requirement for the maximum growth, the doubled
498 CO₂ condition would not likely increase it with enhanced WUE.

499 **Supporting Information**

500 Supplementary Table S1 Output of three-way ANOVA for the measurements.
501 Supplementary Figure S1 Actual conditions in growth chambers during growth period.
502 Supplementary Figure S2 Appearance of potato plants at 8 days after transplanting.
503 Supplementary Figure S3 Water use by potato plants over the experimental period.
504 Supplementary Figure S4 Relation between leaf area and water use.
505 Supplementary Figure S5 Foliar P concentration on area basis ([P]_{area}).

506 **Author Contributions**

507 YY designed and performed the experiment and collected data for analysis. KY
508 supervised the experiment. YY and KY prepared the manuscript. DS revised the
509 manuscript. All authors approved the final manuscript.

510 **Declaration of Competing Interest**

511 The authors declare that the study was conducted without any commercial or
512 financial relationships which could be construed as a potential conflict of interest.

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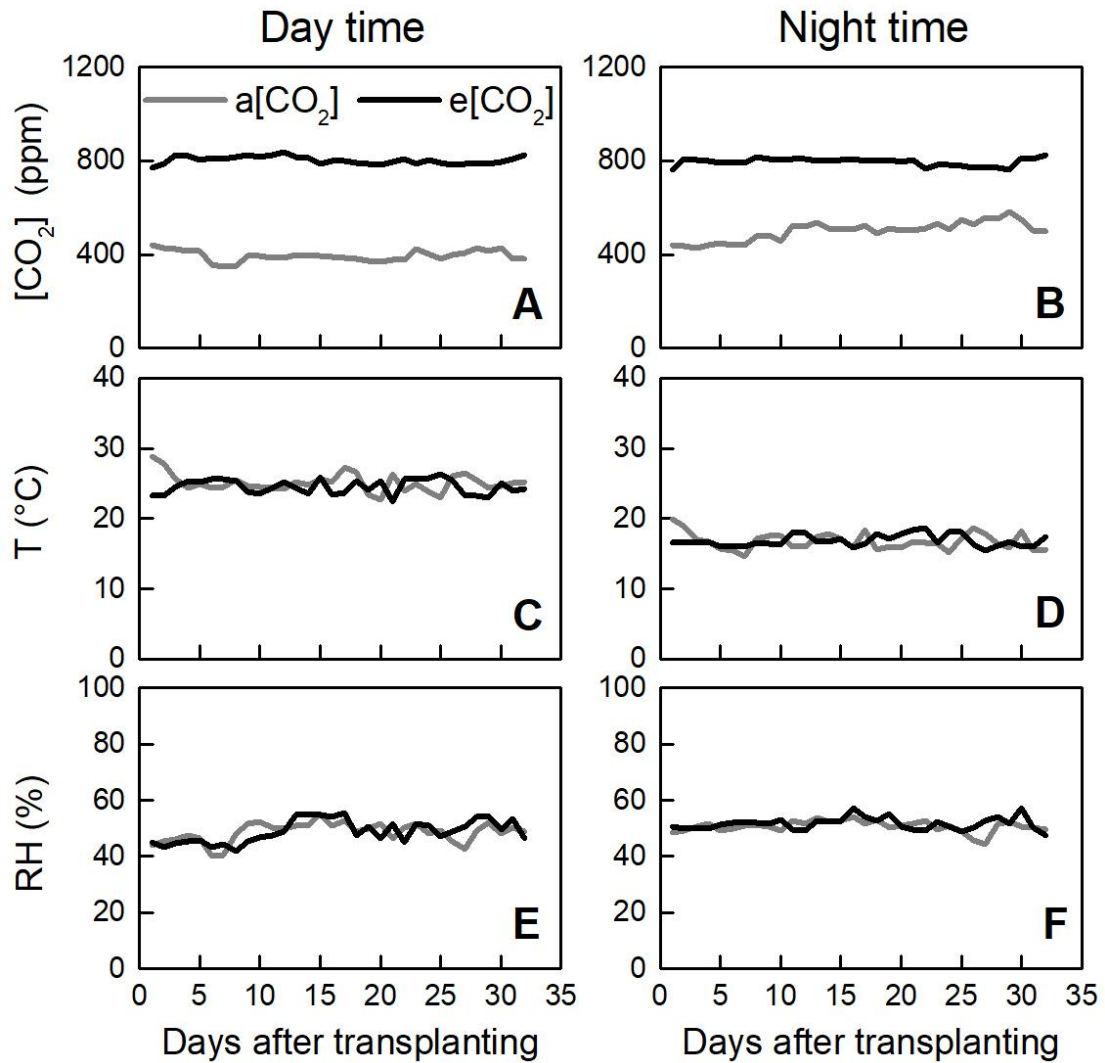
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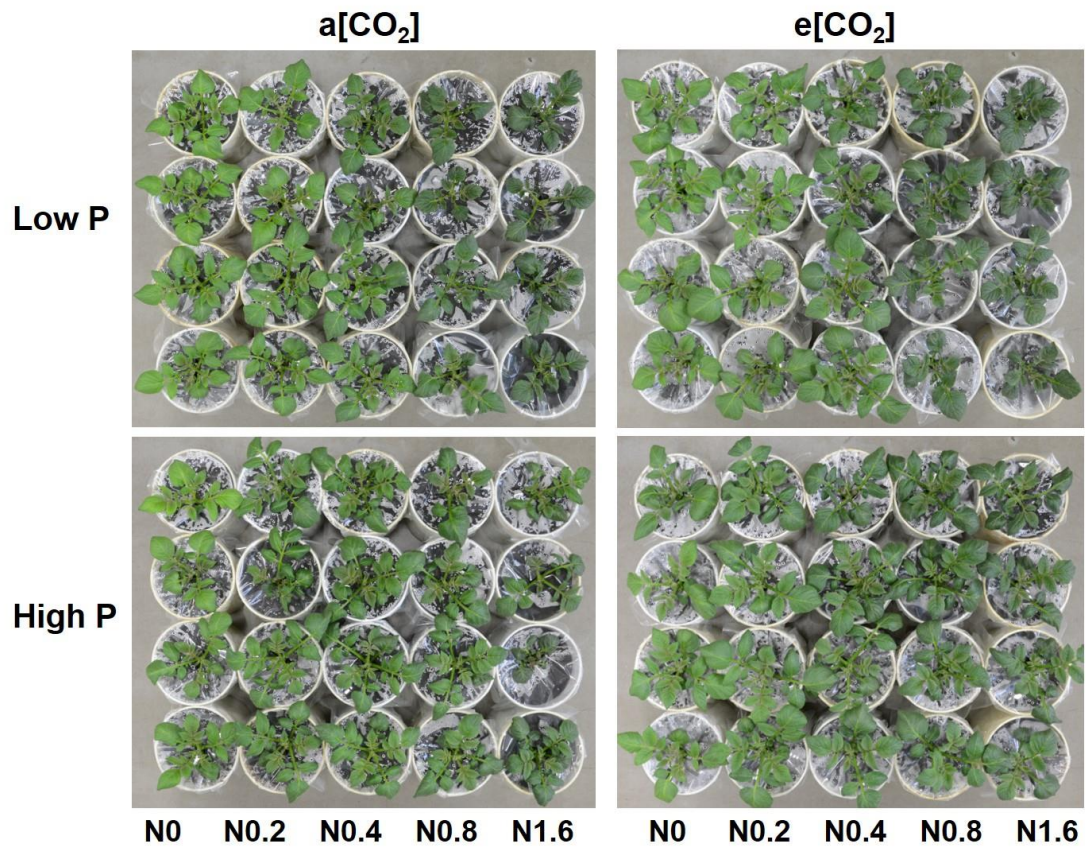
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Supplementary Table S1 Output of three-way ANOVA for the measurements of potato plants grown under a[CO₂] (395 ± 4 ppm) and e[CO₂] (802 ± 3 ppm) with different N supply rates (0, 0.2, 0.4, 0.8, and 1.6 g N kg⁻¹ soil) at two P supply rates (0.3 and 3 g P kg⁻¹ soil).

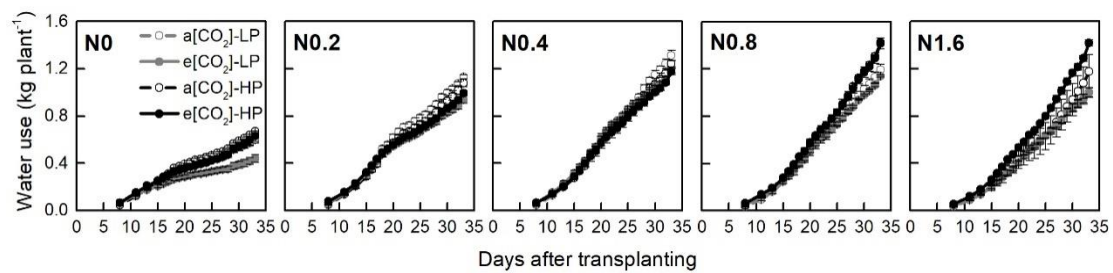
	CO ₂	P	N	CO ₂ × P	CO ₂ × N	P × N	CO ₂ × P × N
Leaf biomass	0.000	0.000	0.000	0.041	0.172	0.000	0.082
Stem biomass	0.000	0.000	0.000	0.005	0.004	0.000	0.003
Root biomass	0.000	0.105	0.000	0.000	0.064	0.142	0.001
Tuber biomass	0.002	0.010	0.000	0.369	0.279	0.002	0.257
Total plant biomass	0.000	0.000	0.000	0.001	0.001	0.000	0.187
Starch concentration	0.000	0.000	0.000	0.405	0.000	0.456	0.044
Leaf mass per area	0.000	0.000	0.000	0.116	0.010	0.761	0.023
Water use	0.016	0.000	0.000	0.005	0.001	0.000	0.547
Water-use efficiency	0.000	0.010	0.000	0.239	0.284	0.055	0.494
Leaf area	0.050	0.000	0.000	0.008	0.005	0.000	0.625
Stomatal conductance	0.001	0.020	0.001	0.224	0.089	0.019	0.878
Stomatal density	0.057	0.713	0.001	0.771	0.376	0.201	0.499
Foliar [N] _{mass}	0.000	0.000	0.000	0.289	0.001	0.000	0.503
Foliar [N] _{area}	0.082	0.291	0.000	0.158	0.002	0.077	0.012
N-uptake	0.000	0.000	0.000	0.015	0.000	0.000	0.000
N-uptake efficiency	0.670	0.000	0.000	0.919	0.000	0.000	0.000
N-utilization efficiency	0.000	0.000	0.000	0.035	0.009	0.267	0.088
Foliar [P] _{area}	0.000	0.000	0.000	0.533	0.000	0.000	0.019



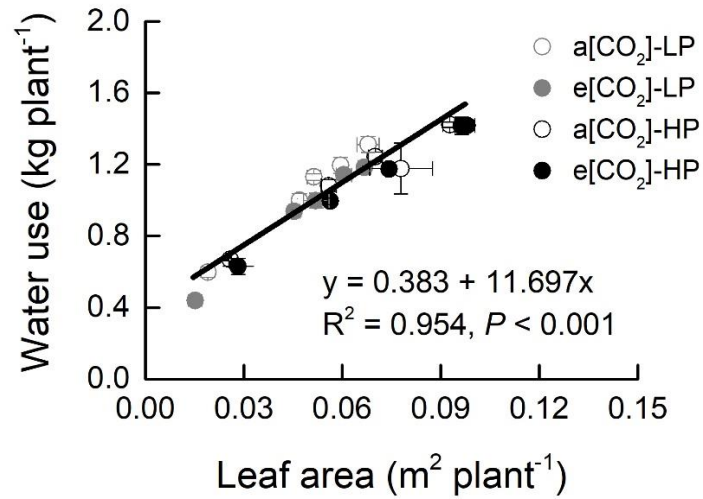
Supplementary Figure S1 Actual conditions in growth chambers during growth period (33 days after transplanting). Actual CO₂ concentrations at day time (**A**) and night time (**B**). Actual temperature (T) at day time (**C**) and night time (**D**). Actual relative humidity (RH) at day time (**E**) and night time (**F**).



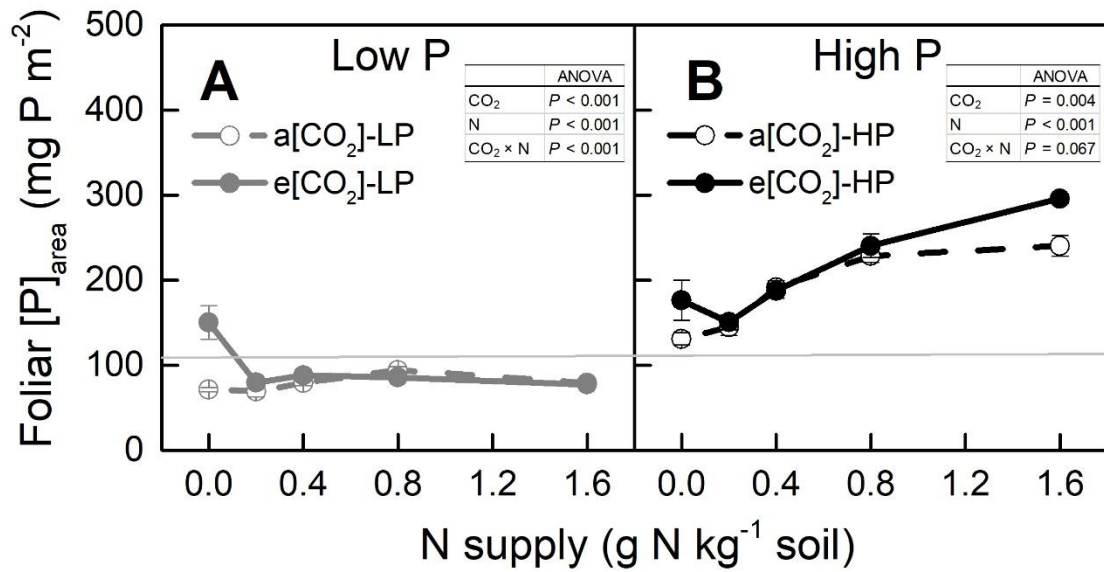
Supplementary Figure S2 Appearance of potato plants at the 8th day after transplanting.



Supplementary Figure S3 Water use by potato plants over the experimental period (33 days after transplanting) under $a[\text{CO}_2]$ (395 ± 4 ppm) and $e[\text{CO}_2]$ (802 ± 3 ppm) with different N supply rates (0, 0.2, 0.4, 0.8, and 1.6 g N kg⁻¹ soil) at two P supply rates (0.3 and 3 g P kg⁻¹ soil). Data in each plot are means \pm S.E. ($n = 4$ biological replicates for each treatment).



Supplementary Figure S4 Relation between leaf area and water use in potato plants grown under a[CO₂] (395 ± 4 ppm) and e[CO₂] (802 ± 3 ppm) with different N supply rates (0, 0.2, 0.4, 0.8, and 1.6 g N kg⁻¹ soil) at two P supply rates (0.3 and 3 g P kg⁻¹ soil). Data in each plot are means ± S.E. (n = 4 biological replicates for each treatment). Regression is as follows: $y = 0.383 + 11.679x$, $R^2 = 0.954$.



Supplementary Figure S5 Foliar P concentration on area basis ($[P]_{\text{area}}$) of potato plants grown under $a[\text{CO}_2]$ (395 ± 4 ppm) and $e[\text{CO}_2]$ (802 ± 3 ppm) with different N supply rates (0, 0.2, 0.4, 0.8, and 1.6 g N kg^{-1} soil) at low P supply (0.3 g P kg^{-1} soil) (A) and high P supply (3 g P kg^{-1} soil) (B). Data in each plot are means \pm S.E. ($n = 4$ biological replicates for each treatment). Statistical comparisons (two-way ANOVA) between CO_2 concentrations and N supply rates as well as their interaction ($\text{CO}_2 \times \text{N}$) are presented.