

1 **Verification of water-use efficiency estimates via carbon isotope discrimination in potato under**
2 **varying nutrient statuses and CO₂ conditions**

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12 **Abstract**

13 Elevated CO₂ (eCO₂) has the potential to increase plant biomass while decreasing water demand because
14 of enhanced water-use efficiency (WUE), which interacts with nutritional status. Carbon isotope
15 discrimination ($\Delta^{13}\text{C}$) has been shown to be a valid proxy for estimating WUE; however, its validity is
16 uncertain for plants in an environment where the interaction between CO₂ and nutrition strongly affects
17 WUE. Using a single potato cultivar (Irish Cobbler), we examined its validity through three independent
18 trials with varying levels of P, N, or K (Trial P, N, and K, respectively) in growth chambers at two CO₂
19 concentrations. WUE at the plant level varied with CO₂ conditions and nutrient supply rates. Plant
20 biomass was positively regressed against WUE in Trials P and K and against water use in Trial N. WUE
21 was negatively regressed against $\Delta^{13}\text{C}$ across various nutrient supply rates within each CO₂ environment.
22 However, the relationship between WUE and $\Delta^{13}\text{C}$ was altered with CO₂ enrichment by elevating the
23 intercept along the y-axis (WUE) without affecting the slope, implying the involvement of isotopic
24 discrimination in respiration or photorespiration. These results suggest that $\Delta^{13}\text{C}$ can be used to estimate
25 WUE across varied nutrient statuses, not only at the current CO₂ but also at eCO₂ when the comparisons
26 are made within each CO₂ condition.

27 **Keywords**

28 water-use efficiency, carbon isotope discrimination, elevated CO₂, plant growth, nitrogen, phosphorus,
29 potassium, *Solanum tuberosum* L.

30 **Introduction**

31 Producing more crop biomass and yield per water consumption, i.e., increasing water-use efficiency
32 (WUE) as an increase in dry matter per unit of water transpired, has often been explored. This trend
33 seems to be accelerating due to concerns that recent climate change may enhance drought-prone
34 conditions. Terrestrial plants acquire CO₂ but inevitably lose water mainly through their stomata; thus,
35 there is a close relationship between plant biomass production and the amount of transpired water. Partial
36 stomatal closure can increase WUE because it usually reduces water loss much more than CO₂
37 acquisition, but often results in lower growth due to reduced carbon assimilation. Hence, it has been
38 proposed that breeding crops with higher WUE may reduce yield and drought resistance (Blum 2009,
39 Sinclair 2018, Tanner and Sinclair 1983). Although it should be noted that “drought resistance” has
40 different meanings (e.g., productivity during a season or survival over several days) as Passioura (2002)
41 pointed out, higher WUE could usually be achieved at the expense of biomass production.

42 Elevated CO₂ (eCO₂) environments are likely an important exception where the trade-off between
43 WUE and biomass does not hold. Plants under eCO₂ usually exhibit partial stomatal closure, probably
44 due to less requirement for stomatal opening to acquire CO₂, so that both WUE and biomass could
45 simultaneously increase compared to the current ambient CO₂ (aCO₂) (Igarashi et al. 2021, Yi et al. 2019,
46 2020, Yi and Yano 2021). In potato, despite a 1.5-fold increase in biomass due to CO₂ enrichment, water
47 consumption was not affected, but WUE increased by 1.5-fold (Yi et al. 2019). In wheat, water
48 consumption was smaller under eCO₂ despite a 2.2-fold greater biomass because of more than doubled
49 WUE compared to aCO₂ (Igarashi et al. 2021). In addition, WUE under eCO₂ was more variable than
50 that under aCO₂ according to nutrient supply (Igarashi et al. 2021, Yi et al. 2019, 2020, Yi and Yano
51 2021), although WUE has been regarded as very conservative (Sinclair et al. 1984, Tanner and Sinclair
52 1983). These results imply that under eCO₂ compared to aCO₂: 1) higher WUE may reduce plant water
53 demand, 2) WUE may become more critical for biomass production than transpiration, and 3) the WUE
54 may become more dependent on nutritional status.

55 There are various levels of WUE, including leaves, plants, and fields (Fischer and Turner 1978,
56 Jones 2004). At the leaf level, either instantaneous WUE (carbon assimilation to transpiration) or
57 intrinsic WUE (carbon assimilation to stomatal conductance) is obtained by gas exchange measurements
58 during seconds over several mm² of leaf area (Franks et al. 2015), but it is obvious that they do not

59 necessarily represent the actual WUE of the whole plant (Medrano et al. 2015, Wei et al. 2018). WUE
60 at the individual plant level can provide information on how much transpired water is required to achieve
61 a certain biomass production during a growth period more than several days, which is almost impossible
62 to obtain by instantaneous or intrinsic WUE measured on such a small leaf area for several seconds.
63 Hence, WUE at the individual plant level is more informative than instantaneous or intrinsic WUE for
64 agricultural and ecological purposes (Sinclair et al. 1984). However, it is time-consuming to collect
65 water consumption data to determine the WUE.

66 To overcome this problem, carbon isotope discrimination ($\Delta^{13}\text{C}$), which is the difference in $^{13}\text{C}/^{12}\text{C}$
67 composition between plant C and environmental CO_2 , has frequently been used to estimate WUE.
68 Previous studies have demonstrated negative correlations between $\Delta^{13}\text{C}$ and WUE under aCO_2 in
69 various species, such as barley (Hubick and Farquhar 1989), peanut (Hubick et al. 1986, Wright et al.
70 1994), cowpea (Ismail and Hall 1992), wheat (Arslan et al. 2008), legumes (Berriel et al. 2019),
71 grapevines (Bchir et al. 2016), tomatoes (Martin et al. 1999), and potatoes (Jefferies 1995, Monneveux
72 et al. 2013, Ramírez et al., 2016), in which soil water conditions were the cause of variations in WUE.
73 While these previous studies focused on aCO_2 , it was reported that eCO_2 led to higher WUE without
74 changing $\Delta^{13}\text{C}$ (Beerling and Woodward 1995, Polley et al. 1995), whereas WUE under eCO_2 showed
75 complicated patterns of interaction with nutrition (Igarashi et al. 2021, Yi et al. 2019, 2020, Yi and Yano
76 2021). These results suggest the importance of examining the relationship between WUE and $\Delta^{13}\text{C}$
77 especially during interaction between CO_2 and nutritional status, however we are almost lacking the
78 knowledge in the literature.

79 As described above, the importance of WUE may be enhanced under eCO_2 not only for predicting
80 plant water requirements but also for increasing biomass production (although WUE seems to highly
81 fluctuate with nutrient status under eCO_2). Instead of WUE, which is time-consuming to measure, $\Delta^{13}\text{C}$
82 has been used to estimate WUE, however its validity is still uncertain under eCO_2 , where WUE may
83 vary complexly due to the interaction with nutrient status. Therefore, in this study, we examined the
84 validity of estimating WUE via $\Delta^{13}\text{C}$ measurements using a single potato cultivar under different CO_2
85 conditions and nutrient levels in three independent trials.

86 **Materials and methods**

87 **Experimental design and growth conditions**

88 Three independent trials (designated as Trial P, N, and K, summarized in [Table 1](#)) were conducted to
89 study potato plants responding to nitrogen (N), phosphorus (P), and potassium (K) nutrients under
90 different CO₂ conditions in controlled-environment chambers (LPH-410 SPC, Nippon Medical &
91 Chemical Instruments Co., Ltd., Japan). The environmental conditions inside the chambers were set as
92 follows: light intensity, 400 μmol m⁻² s⁻¹; relative humidity, 60%; temperature, 25°C:17°C, light:dark,
93 respectively; and photoperiod, 14 h:10 h, light:dark. The CO₂ concentration was controlled at
94 approximately 400 ppm (aCO₂) and 800 ppm (eCO₂). The plants and CO₂ concentrations were switched
95 weekly between the two chambers to minimize any potential chamber effects. A CO₂ recorder (TR-76Ui,
96 T&D Inc., Japan) was placed inside each chamber to monitor the practical conditions in the chambers
97 every 5 min in Trials N and K, and 10 min in Trial P. The average CO₂ concentrations in the light were
98 approximately 400 and 800 ppm under aCO₂ and eCO₂, respectively, in all three trials ([Table S1](#)). CO₂
99 concentrations under aCO₂ in the dark were approximately 500–560 ppm. This is higher than in-light
100 values, likely due to CO₂ emission from respiration of plants in the chambers. Both temperature and
101 relative humidity were similar under aCO₂ and eCO₂ in each trial ([Table S1](#)).

102 Naturally sprouted potato tubers (*Solanum tuberosum* L., cv. “Irish Cobbler”) were transplanted into
103 1 L pots (diameter, 11.3 cm; depth, 14 cm; one plant per pot) filled with 580 g of dry andosol. Total
104 fertilizer amounts applied in Trial P were 0.8 g N kg⁻¹ soil, 1.6 g K₂O kg⁻¹ soil, and 0, 0.4, 0.8, 1.2, 2.4,
105 3.6 g P kg⁻¹ soil. Total fertilizer amounts applied in Trial N were 0.3 or 3 g P kg⁻¹ soil (designated as low
106 P and high P, respectively), 1.6 g K₂O kg⁻¹ soil, and 0, 0.2, 0.4, 0.8, 1.6 g N kg⁻¹ soil. Total fertilizer
107 amounts applied in Trial K were 0.8 g N kg⁻¹ soil, 0.1 or 1.5 g P kg⁻¹ soil (designated as low P and high
108 P, respectively), and 0, 0.1, 0.2, 0.4, 0.8 g K₂O kg⁻¹ soil.

109 In Trial P, the soil water content was maintained at 60% (w/w) by weighing the pots and
110 supplementary water until 40 days after transplanting. Thereafter, it was maintained at 80% (w/w) until
111 harvest to avoid drought-like conditions caused by rapid daily soil water consumption, which is
112 accompanied by growth. In Trials N and K, the soil water condition was maintained at approximately
113 80% (w/w) during the entire growth period.

114 All three experiments were organized according to a factorial design (two CO₂ concentrations × six
115 P supply rates in Trial P, two CO₂ concentrations × two P supply rates × five N supply rates in Trial N,

116 and two CO₂ concentrations × two P supply rates × five K supply rates in Trial K) with six biological
117 replicates in Trial P and four in Trials N and K. More information about these trials can be found in the
118 literature (Yi et al. 2019, 2020, Yi and Yano 2021).

119 **Measurement of water use**

120 A transparent plastic film was used to cover each pot to prevent water loss through soil evaporation. The
121 pots used had solid bottoms, and thus leaching was not considered. The pots were weighed on the day
122 of planting and before being watered each time until harvest. A decrease in pot weight was regarded as
123 water consumption through transpiration, and the amount of water lost by transpiration was provided to
124 each pot. Considering that plants continue to grow over time, extra water was provided to maintain soil
125 water content at a specific range. The pot weight and amount of water provided to each pot were recorded
126 throughout the growth period. Water use during the growth period was calculated from the cumulative
127 transpiration. WUE was calculated as the total plant biomass/water use according to Jones (2004).

128 **Harvest and sampling**

129 At harvest day (54, 33, and 46 days after transplanting for Trials P, N, and K, respectively), leaves, stems,
130 roots, and tubers were separated and dried in an oven (80°C) for dry weight determination. The powder
131 from all organs was mixed proportionally according to dry weight for carbon isotope analysis in the
132 entire plant, thus avoiding any isotope composition difference in organs resulting from physiological
133 processes within each plant.

134 **Carbon isotope discrimination**

135 The powders of leaves, stems, roots, and tubers were proportionally mixed for carbon isotope
136 discrimination. Samples of approximately 40–100 µg were encapsulated in 0.15 mL tin foil and analyzed
137 using an isotopic ratio mass spectrometer (FLASH 2000, Delta plus, Thermo Fisher Scientific,
138 Worcester, MA, USA). The δ¹³C values were expressed in delta notation relative to the standard (PDB,
139 PeeDee belemnite limestone) as follows:

140

$$141 \quad \delta^{13}\text{C} (\text{‰}) = \left[\left(\frac{{}^{13}\text{C} : {}^{12}\text{C}_{\text{sample}}}{{}^{13}\text{C} : {}^{12}\text{C}_{\text{standard}}} \right) - 1 \right] \times 1,000 \quad \text{Equation [1]}$$

142

143 It is necessary to know the air $\delta^{13}\text{C}$ (δ_a) because δ_a in growth chambers varies and decreases with an
 144 increase in atmospheric CO_2 concentrations (Smith et al. 1976, Stocker et al. 2013). C4 plants (maize in
 145 Trial P and guinea grass in Trials N and K) were grown in the chambers to monitor the air carbon isotope
 146 composition in the growth chambers under different CO_2 conditions. Maize was used in Trial P because
 147 of its general application in estimating δ_a (Marino and McElroy 1991). It was replaced with guinea grass
 148 in Trials N and K because maize grows large and the space is limited in growth chambers. The growth
 149 conditions for C4 species were as follows: one maize plant in 1 L pot filled with 580 g dry andosol in
 150 Trial P; one guinea grass plant in 0.3 L pot filled with 160 g dry andosol in Trials N and K; three
 151 replicates for maize and four replicates for guinea grass. Nutrient supply was as follows: 1.2 g P kg^{-1} dry
 152 soil, 0.8 g N kg^{-1} dry soil, and 1.6 g K_2O kg^{-1} dry soil in Trial P; 3 g P kg^{-1} dry soil, 0.8 g N kg^{-1} dry soil,
 153 and 1.6 g K_2O kg^{-1} dry soil in Trial N; 1 g P kg^{-1} dry soil, 0.8 g N kg^{-1} dry soil, and 0.4 g K_2O kg^{-1} dry
 154 soil in Trial K. C4 plants were harvested on the same day as the potato plants in each trial. δ_a was
 155 calculated using the following equation:

$$\delta_a (\text{‰}) = \Delta_{\text{C4}} (1 + \delta_{\text{C4}} / 1000) + \delta_{\text{C4}} \quad \text{Equation [2]}$$

156
 157
 158 where δ_{C4} is the $\delta^{13}\text{C}$ value of the C4 plants. Δ_{C4} is carbon isotope discrimination in C4 plants, which is
 159 assumed to be 3.3‰ in maize (Marino and McElroy 1991). Although Δ_{C4} of guinea grass has not been
 160 examined in previous studies, it is believed to be like that of maize. This is according to our
 161 supplementary trial where we grew maize and guinea grass under the same conditions, where there was
 162 no significant difference in $\delta^{13}\text{C}$ values in maize and guinea grass (Table S2). This indicates that the
 163 different C4 species in our trials were unlikely to affect our results. $\delta^{13}\text{C}$ in C4 plants was approximately
 164 -15‰ and -20‰ under a CO_2 and e CO_2 , respectively (Table 2). δ_a was calculated to be approximately -
 165 12‰ and -17‰ under a CO_2 and e CO_2 , respectively (Table 2).

167 Carbon isotope discrimination ($\Delta^{13}\text{C}$) in individual potato plants was calculated using δ_p and δ_a using
 168 the following equation (Farquhar et al. 1982),

$$\Delta^{13}\text{C} = (\delta_a - \delta_p) / (1 + \delta_p / 1000) \quad \text{Equation [3]}$$

172 where δ_a is the $\delta^{13}\text{C}$ value of air in the growth chamber and δ_p is the $\delta^{13}\text{C}$ value of the potato plant.

173 **Statistical analysis**

174 The experiments were organized in a factorial design. Simple regressions were analyzed using
175 standardized major axis tests and routes (SMATR) (Warton et al. 2006). All figures were created using
176 Origin 9.0 (<https://www.originlab.com>).

177 **Results**

178 **WUE and carbon isotope discrimination**

179 The WUE at the plant level was calculated using dry matter production and water consumption during
180 the growth period in this study. As shown in Fig. 1, WUE increased with $e\text{CO}_2$ in all three trials. The
181 increased WUE due to $e\text{CO}_2$ was due to an increase in biomass or a reduction in water use. Additionally,
182 WUE was quite different for different nutrient supplies. P and K supply increased WUE under both CO_2
183 conditions, however N supply showed the opposite effect during the examined growth period. The
184 increase in WUE by $e\text{CO}_2$ along with K supply was dependent on P supply, although the effect of P
185 supply on WUE in Trial N was not as evident. In addition to WUE, a wide range of $\Delta^{13}\text{C}$ variations were
186 observed. Compared to the trends of WUE along with nutrient supply, $\Delta^{13}\text{C}$ showed a completely
187 opposite trend. It decreased with P and K supply but increased with N supply (Fig. 1). $\Delta^{13}\text{C}$ was higher
188 under $e\text{CO}_2$ than under $a\text{CO}_2$ for each K supply rate in Trial K; however, it was not always similar to
189 that in Trials P and N and varied depending on the P and N supply rates (Fig. 1).

190 **Relationship between biomass and water use or WUE**

191 There were different relationships between biomass and water use or WUE in the three trials. Biomass
192 was determined by water use (Trial N), WUE (Trial K), or both (Trial P) (Fig. 2 and Table S3). In Trial
193 N, biomass increased with increasing water use. Linear regression analysis showed that water use could
194 explain over 90% of the variation in biomass. Moreover, the enhancement of biomass under $e\text{CO}_2$ was
195 larger than that under $a\text{CO}_2$ for the same water consumption, resulting in a higher WUE under $e\text{CO}_2$
196 (Fig. 2). In Trial K, biomass was determined by WUE rather than water use under high P; under high P,
197 94% and 96% variations in biomass under $a\text{CO}_2$ and $e\text{CO}_2$, respectively, could be explained by WUE.

2198 Thus, the biomass was enhanced by the K supply, even with little water use. However, under low P, no
2199 clear relationship between biomass and water use or WUE was observed in Trial K. Both water use and
2200 WUE affected biomass in Trial P. More than 93% of the variation in biomass could be explained by
2201 water use or WUE from the linear regression analysis in Trial P.

2202 **Relationship between WUE and carbon isotope discrimination**

2203 A negative relationship between WUE and $\Delta^{13}\text{C}$ was observed in all three trials (Table 3). To further
2204 compare whether the relationship was affected by CO_2 and nutrient status, post-hoc multiple
2205 comparisons of slopes of linear relationships among groups in all three trials were analyzed. The results
2206 suggest that the slopes of the plots in trials under a CO_2 and e CO_2 conditions in the same trial were not
2207 significantly different (Table 4). Furthermore, a common slope was observed in Trials P and K, which
2208 was slightly different from that in Trial N (Table 4). Comparisons of lines with common slopes in each
2209 trial showed clear shifts in the elevation of the intercept between a CO_2 and e CO_2 in all three trials (Table
2210 5). Significant shifts along the common slope were observed only in Trial N ($P = 0.004$). Because there
2211 was a common slope in Trials P and K, and no significant shifts in the elevation of the intercept between
2212 Trial P and Trial K under either CO_2 condition ($P = 0.077$ and $P = 0.222$ under a CO_2 and e CO_2 ,
2213 respectively) (Table S4), we combined data from Trials P and K together in Fig. 3. From Fig. 3, it is
2214 clear that the intercept of the relationship between WUE and $\Delta^{13}\text{C}$ increased remarkably with CO_2
2215 enrichment. The slope of the relationship in Trial N was slightly different from that in Trials P and K.

2216 **Discussion**

2217 It was evident that there were good regressions between $\Delta^{13}\text{C}$ and WUE at the whole-plant level across
2218 various nutritional statuses (Table 3), although the validity of $\Delta^{13}\text{C}$ to estimate WUE would be restricted
2219 within each CO_2 condition (Fig. 3). This is because the intercept of the regression, but not the slope, was
2220 significantly different between a CO_2 and e CO_2 (Tables 3 and 5). Although $\Delta^{13}\text{C}$ has been used to
2221 estimate WUE under a CO_2 (Bchir et al. 2016, Berriel et al. 2019, Hubick et al. 1986, Ismail and Hall 1992,
2222 Liu et al. 2020, Martin et al. 1999, Wright et al. 1994), it has been reported that e CO_2 led to higher WUE
2223 without changing $\Delta^{13}\text{C}$ (Beerling and Woodward 1995, Polley et al. 1995). Our results provide
2224 experimental evidence that CO_2 enrichment increases the intercept in the regression of WUE against
2225 $\Delta^{13}\text{C}$ (Fig. 3), in which WUE was highly variable according to nutrient supply (Fig. 1).

226 The underlying mechanisms that explain theoretical association between $\Delta^{13}\text{C}$ and WUE in C3
227 plants has been described by Hubick and Farquhar (1989) in the following formula,

228

$$229 \text{ WUE} = (1 - \phi_c)Ca(b - d - \Delta^{13}\text{C})/[1.6v(1 + \phi_w)(b - a)] \quad \text{Equation [4]}$$

230

231 where a is the fractionation occurring due to diffusion in air (4.4×10^{-3}); b is the net fractionation caused
232 by RuP2 and PEP carboxylation (27×10^{-3}); Ca is CO_2 concentration in the air; ϕ_c is the proportion of
233 carbon fixed but lost during respiration; ϕ_w is uncontrolled water loss, such as nighttime stomatal
234 transpiration and water loss by non-photosynthetic plant parts; and v is the water vapor pressure
235 difference between the intercellular spaces and the atmosphere. Equation [4] can be simplified as
236 following; $\text{WUE} = X(b - d - \Delta^{13}\text{C})$, where $X = (1 - \phi_c)Ca/[1.6v(1 + \phi_w)(b - a)]$, i.e., the intercept is
237 $X*(b-d)$ and the slope is $X*(-\Delta^{13}\text{C})$.

238 As the slope was not affected by CO_2 enrichment in the observed results (Fig. 3), it can be assumed
239 that X was similar between the two CO_2 conditions. Under this assumption, a change in the intercept is
240 caused by $(b - d)$. Because b was proposed to be a constant of 27‰ by Farquhar et al. (1982), d was
241 most likely affected by CO_2 enrichment. The parameter d consists of the sum of the effects of
242 discrimination of CO_2 dissolution, liquid-phase diffusion, and possible discrimination during respiration
243 and photorespiration. Igamberdiev et al. (2004) reported that photorespiration affects C_i/C_a
244 (intercellular CO_2 concentration/ CO_2 concentration in the air) and carbon isotope fractionation by
245 affecting stomata and discrimination of ^{13}C in the glycine decarboxylase reaction. Fractionation for both
246 respiration and photorespiration has been reported to vary among species and environmental conditions
247 (Ghashghaie et al. 2003). This study suggests that variation in d may be involved when the CO_2
248 conditions are different.

249 $\Delta^{13}\text{C}$ has been shown to be a proxy for WUE in many previous studies (Bchir et al. 2016, Berriel et
250 al. 2019, Hubick et al. 1986, Ismail and Hall 1992, Liu et al. 2020, Martin et al. 1999, Wright et al. 1994).
251 Consistent with these studies, WUE was negatively related to $\Delta^{13}\text{C}$ in all three trials in the current study;
252 however, the relationships varied among the three trials (Fig. 3, Tables 3 and 4). Although there were
253 similar slopes in Trials P and K, the slope in Trial N was slightly different, which might be related to the
254 developmental stages. Comparing the tuber biomass proportion in these three trials (mean value

255 approximately 36% in Trial P, 23% in Trial N, and 55% in Trial K, data not shown), it was speculated
256 that plants in Trial N were at a relatively earlier growth stage, when the positive effects of N supply on
257 WUE were not observed (Fig. 1). Therefore, the biomass in Trial N was related to water use rather than
258 WUE (Fig. 2). Zhao et al. (2004) reported that the relationship between $\Delta^{13}\text{C}$ in leaf and total plant WUE
259 in upland rice was markedly different between growth stages, positive relationships from seedling to
260 tillering, negative relationships from tillering to flowering, and undistinguishable relationships from
261 flowering to maturity. In contrast, the relationship was unchanged in tomato plants under various stages
262 (Martin et al., 1999).

263 Our results demonstrated that increases in biomass under eCO₂ would not necessarily require
264 increases in water use during the growth periods, because the highest biomass within each trial was
265 observed under eCO₂ without an increase in water use compared to aCO₂ (Fig. 2). Moreover, a linear
266 regression of biomass was apparent against WUE in either Trial P or Trial K, especially under eCO₂ (Fig.
267 2). The results suggest the significance of WUE not only for predicting water requirements, but also for
268 increasing biomass production under eCO₂. Tanner and Sinclair (1983) and Sinclair et al. (1984)
269 proposed that exploitable variation in plant WUE may be small, although they do not seem to have
270 considered eCO₂. In contrast, our results showed a wide variation in WUE ranging from 3 to 10 g
271 biomass per kg water for a single genotype grown under different CO₂ and nutrient status conditions.
272 $\Delta^{13}\text{C}$ would be useful for estimating such variable WUE unless the comparisons were made across
273 different CO₂ conditions and growth stages.

274 **Conclusions**

275 This study aimed to examine the validity of estimating WUE via $\Delta^{13}\text{C}$ measurements using a single
276 potato genotype under different CO₂ conditions and nutrient levels in three independent trials. As a result,
277 we obtained a highly variable WUE by more than 3-fold and the corresponding $\Delta^{13}\text{C}$ measurements.
278 WUE was negatively regressed against $\Delta^{13}\text{C}$ across various plant nutrient statuses within each CO₂
279 environment. However, the relationship was altered by CO₂ enrichment through elevation of intercept
280 along the y-axis (WUE) without affecting the slope, which may imply the involvement of possible
281 discrimination during respiration and photorespiration. Despite the CO₂ effects, the relationship between
282 WUE and $\Delta^{13}\text{C}$ was stable under various nutrient conditions, and the slopes were slightly different
283 among the different trials, presumably due to different developmental stages. We conclude that $\Delta^{13}\text{C}$

284 allows us to estimate WUE across varied nutrient statuses within the same CO₂ conditions, but not across
285 different CO₂ conditions.

286 **Author Contributions**

287 KY and YY designed the experiments. YY performed the experiments and collected the data for the
288 analysis. YY prepared the manuscript. KY revised the manuscript. All authors approved the final
289 manuscript.

290 **Acknowledgements**

291 This work was supported by the Japan Society for the Promotion of Science (Grant numbers 21H02328
292 and 16H05055). We thank Motohiko Kondo (Graduate School of Bioagricultural Sciences, Nagoya
293 University, Japan) for his valuable comments and encouragement for this work. Yan Yi appreciates the
294 Chinese Scholarship Council (CSC) and the Ministry of Education, Culture, Sports, Science and
295 Technology Japan (MEXT) for supporting her study at the Graduate School of Bioagricultural Sciences,
296 Nagoya University, Japan.

297 **Data availability statement**

298 The data that supports the findings of this study are available in the supplementary material of this article.

299 **Supporting Information**

300 **Table S1** Actual CO₂ concentrations, temperature, and relative humidity during the light and dark time
301 in the growth chambers.

302 **Table S2** Test for carbon isotope composition of maize and guinea grass to CO₂ conditions.

303 **Table S3** Linear regression analysis of the relationship between total plant biomass and water use or
304 water-use efficiency (WUE) in potato plants growing under aCO₂ and eCO₂, respectively in each trial.

305 **Table S4** Comparison of lines with common slope between Trials P and K under different CO₂
306 conditions.

307 **References**

- 308 Arslan A, Zapata F, Kumarasinghe KS (2008) Carbon isotope discrimination as indicator of water-use
309 efficiency of spring wheat as affected by salinity and gypsum addition. *Commun Soil Sci Plant*
310 *Anal* 30: 2681-2693
- 311 Bchir A, Escalona JM, Gallé A, Hernández-Montes E, Tortosa I, Braham M, Medrano H (2016) Carbon
312 isotope discrimination ($\delta^{13}\text{C}$) as an indicator of vine water status and water use efficiency
313 (WUE): looking for the most representative sample and sampling time. *Agric Water Manage*
314 167: 11-20
- 315 Beerling DJ, Woodward FI (1995) Leaf stable carbon isotope composition records increased water-use
316 efficiency of C3 plants in response to atmospheric CO₂ enrichment. *Funct Ecol* 9: 394-401
- 317 Berriel V, Perdomo C, Monza J (2019) Carbon isotope discrimination and water-use efficiency in
318 Crotalaria cover crops under moderate water deficit. *J Soil Sci Plant Nutr* 20: 537-545
- 319 Blum A (2009) Effective use of water (EUW) and not water-use efficiency (WUE) is the target of crop
320 yield improvement under drought stress. *Field Crops Res* 112: 119-123
- 321 Drake BG, González-Meler MA, Long SP (1997) More efficient plants: a consequence of rising
322 atmospheric CO₂? *Annu Rev Plant Biol* 48: 609-639
- 323 Farquhar GD, O'Leary MH, Berry JA (1982) On the relationship between carbon isotope discrimination
324 and the intercellular carbon dioxide concentration in leaves. *Funct Plant Biol* 9: 121-137
- 325 Fischer R, Turner NC (1978) Plant productivity in the arid and semiarid zones. *Annu Rev Plant Physiol*
326 29: 277-317
- 327 Franks PJ, W. Doheny-Adams T, Britton-Harper ZJ, Gray JE (2015) Increasing water-use efficiency
328 directly through genetic manipulation of stomatal density. *New Phytol* 207: 188-195
- 329 Ghashghaie J, Badeck FW, Lanigan G, Nogués S, Tcherkez G, Deléens E, Cornic G, Griffiths H (2003)
330 Carbon isotope fractionation during dark respiration and photorespiration in C3 plants.
331 *Phytochem Rev* 2: 145-161
- 332 Hubick K, Farquhar G (1989) Carbon isotope discrimination and the ratio of carbon gained to water lost
333 in barley cultivars. *Plant Cell Environ* 12: 795-804
- 334 Hubick K, Farquhar G, Shorter R (1986) Correlation between water-use efficiency and carbon isotope
335 discrimination in diverse peanut (*Arachis*) germplasm. *Funct Plant Biol* 13: 803-816

336 Igamberdiev AU, Mikkelsen TN, Ambus P, Bauwe H, Lea PJ, Gardeström P (2004) Photorespiration
337 contributes to stomatal regulation and carbon isotope fractionation: a study with barley, potato
338 and *Arabidopsis* plants deficient in glycine decarboxylase. *Photosynth Res* 81: 139-152
339 Igarashi M, Yi Y, Yano K (2021) Revisiting why plants become N deficient under elevated CO₂:
340 Importance to meet N demand regardless of the fed-form. *Front Plant Sci*: 2377
341 Ismail AM, Hall A (1992) Correlation between water-use efficiency and carbon isotope discrimination
342 in diverse cowpea genotypes and isogenic lines. *Crop Sci* 32: 7-12
343 Jefferies RA (1995) Physiological determinants of genotypic differences in carbon isotope
344 discrimination in potato grown in well-watered conditions. *Ann Appl Biol* 127: 585-592
345 Jones H (2004) What is water use efficiency. In: Bacon MA (ed) *Water Use Efficiency in Plant Biology*.
346 Blackwell Publishing Ltd, Oxford, pp 27-41
347 Liu Z, Ma Fy, Hu Tx, Zhao Kg, Gao Tp, Zhao Hx, Ning Ty (2020) Using stable isotopes to quantify
348 water uptake from different soil layers and water use efficiency of wheat under long-term tillage
349 and straw return practices. *Agric Water Manage* 229: 105933
350 Marino BD, McElroy MB (1991) Isotopic composition of atmospheric CO₂ inferred from carbon in C4
351 plant cellulose. *Nat* 349: 127-131
352 Martin B, Tauer CG, Lin RK (1999) Carbon isotope discrimination as a tool to improve water-use
353 efficiency in tomato. *Crop Sci* 39: 1775-1783
354 Medrano H, Tomás M, Martorell S, Flexas J, Hernández E, Rosselló J, Pou A, Escalona J-M, Bota J
355 (2015) From leaf to whole-plant water use efficiency (WUE) in complex canopies: limitations
356 of leaf WUE as a selection target. *The Crop J* 3: 220-228
357 Monneveux P, Ramírez DA, Pino MT (2013) Drought tolerance in potato (*S. tuberosum* L.): Can we
358 learn from drought tolerance research in cereals? *Plant Sci* 205: 76-86
359 Passioura JB (2002) Environmental biology and crop improvement. *Funct Plant Biol* 29: 537-546
360 Polley H, Johnson H, Mayeux H (1995) Nitrogen and water requirements of C3 plants grown at glacial
361 to present carbon dioxide concentrations. *Funct Ecol* 9: 86-96
362 Ramírez DA, Yactayo W, Rens LR, Rolando JL, Palacios S, De Mendiburu F, Mares V, Barreda C,
363 Loayza H, Monneveux P (2016) Defining biological thresholds associated to plant water status
364 for monitoring water restriction effects: Stomatal conductance and photosynthesis recovery as

365 key indicators in potato. *Agric Water Manage* 177: 369-378

366 Sinclair TR (2018) Effective water use required for improving crop growth rather than transpiration
367 efficiency. *Front Plant Sci* 9: 1442

368 Sinclair TR, Tanner C, Bennett J (1984) Water-use efficiency in crop production. *Biosci* 34: 36-40

369 Smith BN, Oliver J, Millan CM (1976) Influence of carbon source, oxygen concentration, light intensity,
370 and temperature on $^{13}\text{C}/^{12}\text{C}$ ratios in plant tissues. *Bot Gaz* 137: 99-104

371 Stocker et al (2013) Technical summary. In: Stocker TF, Qin D, Plattner GK, Tignor M, Allen SK,
372 Boschung J, Nauels A, Xia Y, Bex V, Midgley PM (eds) *Climate Change 2013: The Physical*
373 *Science Basis: Contribution of Working Group I to the Fifth Assessment Report of the*
374 *Intergovernmental Panel on Climate Change*. Cambridge University Press, Cambridge and New
375 York, pp 33-115

376 Tanner C, Sinclair T (1983) Efficient water use in crop production: research or re-research? In Taylor HM,
377 Jordan WR, Sinclair TR (eds) *Limitations to Efficient Water Use in Crop Production*. American
378 Society of Agronomy, Crop Science Society of America, Soil Science Society of America, pp
379 1-27

380 Warton DI, Wright IJ, Falster DS, Westoby M (2006) Bivariate line-fitting methods for allometry. *Biol*
381 *Rev* 81: 259-291

382 Wei Z, Du T, Li X, Fang L, Liu F (2018) Interactive effects of CO₂ concentration elevation and nitrogen
383 fertilization on water and nitrogen use efficiency of tomato grown under reduced irrigation
384 regimes. *Agric Water Manage* 202: 174-182

385 Wright G, Rao RN, Farquhar G (1994) Water-use efficiency and carbon isotope discrimination in peanut
386 under water deficit conditions. *Crop Sci* 34: 92-97

387 Yi Y, Sugiura D, Yano K (2019) Quantifying phosphorus and water demand to attain maximum growth
388 of *Solanum tuberosum* in a CO₂-enriched environment. *Front Plant Sci* 10, 1417

389 Yi Y, Sugiura D, Yano K (2020) Nitrogen and water demands for maximum growth of *Solanum*
390 *tuberosum* under doubled CO₂: interaction with phosphorus based on the demands. *Environ Exp*
391 *Bot* 176: 104089

392 Yi Y, Yano K (2021) Plant growth and water economy of *Solanum tuberosum* in response to doubled
393 CO₂: Interaction between potassium and phosphorus. *J Agron Crop Sci* 207: 901-912

394 Zhao B, Kondo M, Maeda M, Ozaki Y, Zhang J (2004) Water-use efficiency and carbon isotope
395 discrimination in two cultivars of upland rice during different developmental stages under three
396 water regimes. *Plant Soil* 261: 61-75

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Table 1 Summary of design and growth periods for the three trials.

	Growth period (days)	CO ₂ conditions	N supply (g N kg ⁻¹ dry soil)	P supply (g P kg ⁻¹ dry soil)	K supply (g K ₂ O kg ⁻¹ dry soil)
Trial P	54	aCO ₂ , eCO ₂	0.8	0, 0.4, 0.8, 1.2, 2.4, 3.6	1.6
Trial N	33	aCO ₂ , eCO ₂	0, 0.2, 0.4, 0.8, 1.6	0.3, 3	1.6
Trial K	46	aCO ₂ , eCO ₂	0.8	0.1, 1.5	0, 0.1, 0.2, 0.4, 0.8

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400 Table 2 $\delta^{13}\text{C}$ values of the C4 plants and their corresponding $\delta^{13}\text{C}$ values of air (δ_a) in the growth
401 chambers. Data are expressed as mean \pm standard error (S.E.). n = 3 for maize; n = 4 for guinea grass.

	C4 species	$\delta^{13}\text{C}$ in C4 plants (‰)		δ_a (‰)	
		aCO ₂	eCO ₂	aCO ₂	eCO ₂
Trial P	Maize	-14.71 \pm 0.04	-19.53 \pm 0.17	-11.46	-16.29
Trial N	Guinea grass	-15.60 \pm 0.19	-21.02 \pm 0.05	-12.35	-17.80
Trial K	Guinea grass	-15.34 \pm 0.16	-19.87 \pm 0.06	-12.09	-16.63

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404 Table 3 Linear regression analysis of the relationship between water-use efficiency (WUE) and carbon
405 isotope discrimination ($\Delta^{13}\text{C}$) in potato plants growing under aCO₂ and eCO₂, respectively in each trial.
406 Parameters of the linear regression of WUE and $\Delta^{13}\text{C}$ are presented.

Group	n	R ²	P-value	Slope	Intercept	
Trial P	aCO ₂	6	0.668	0.047	-2.05	41.0
	eCO ₂	6	0.816	0.014	-1.62	36.7
Trial N	aCO ₂	10	0.745	0.001	-0.49	14.2
	eCO ₂	10	0.579	0.011	-0.46	15.7
Trial K	aCO ₂	10	0.542	0.015	-1.00	23.3
	eCO ₂	10	0.721	0.002	-1.00	25.9

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409 Table 4 Post-hoc multiple comparisons of slopes of linear relationships between WUE and $\Delta^{13}\text{C}$ among
410 groups in all three trials.

Group	Trial P		Trial N		Trial K	
	aCO ₂	eCO ₂	aCO ₂	eCO ₂	aCO ₂	eCO ₂
Trial P	aCO ₂	1				
	eCO ₂	0.528	1			
Trial N	aCO ₂	0.004	0.004	1		
	eCO ₂	0.006	0.001	0.820	1	
Trial K	aCO ₂	0.082	0.157	0.030	0.031	1
	eCO ₂	0.097	0.121	0.008	0.009	0.988

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414 Table 5 Comparisons of lines with common slope between aCO₂ and eCO₂ in each trial.

	Trial P	Trial N	Trial K
Test for common slope between aCO ₂ and eCO ₂			
<i>P</i> -value	0.529	0.814	0.991
Testing for shifts in elevation between aCO ₂ and eCO ₂ using WALD statistic			
<i>P</i> -value	< 0.001	< 0.001	< 0.001
Testing for shifts along the common slope between aCO ₂ and eCO ₂ using WALD statistic			
<i>P</i> -value	0.364	0.004	0.509

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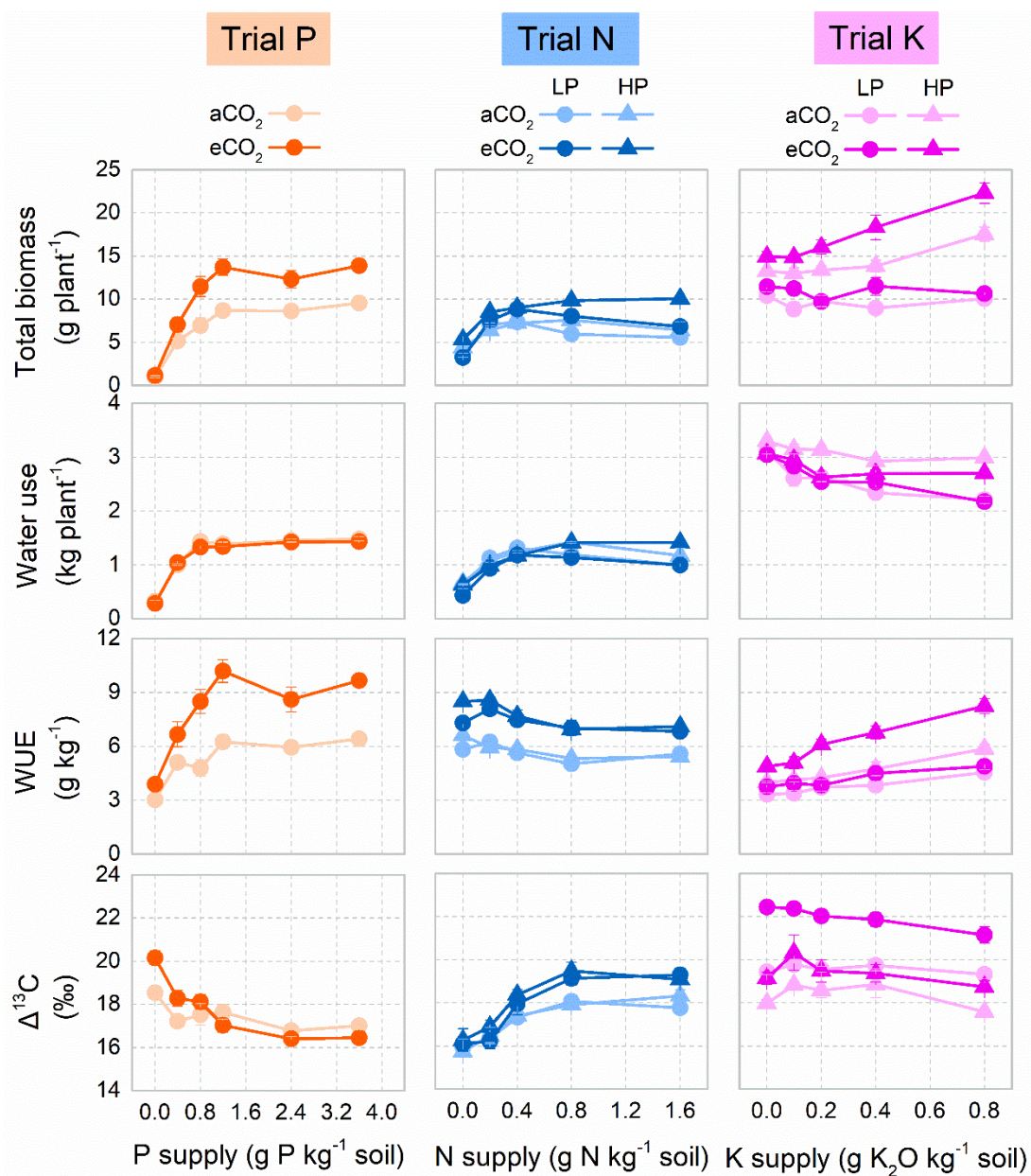
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417 **Figure legends**

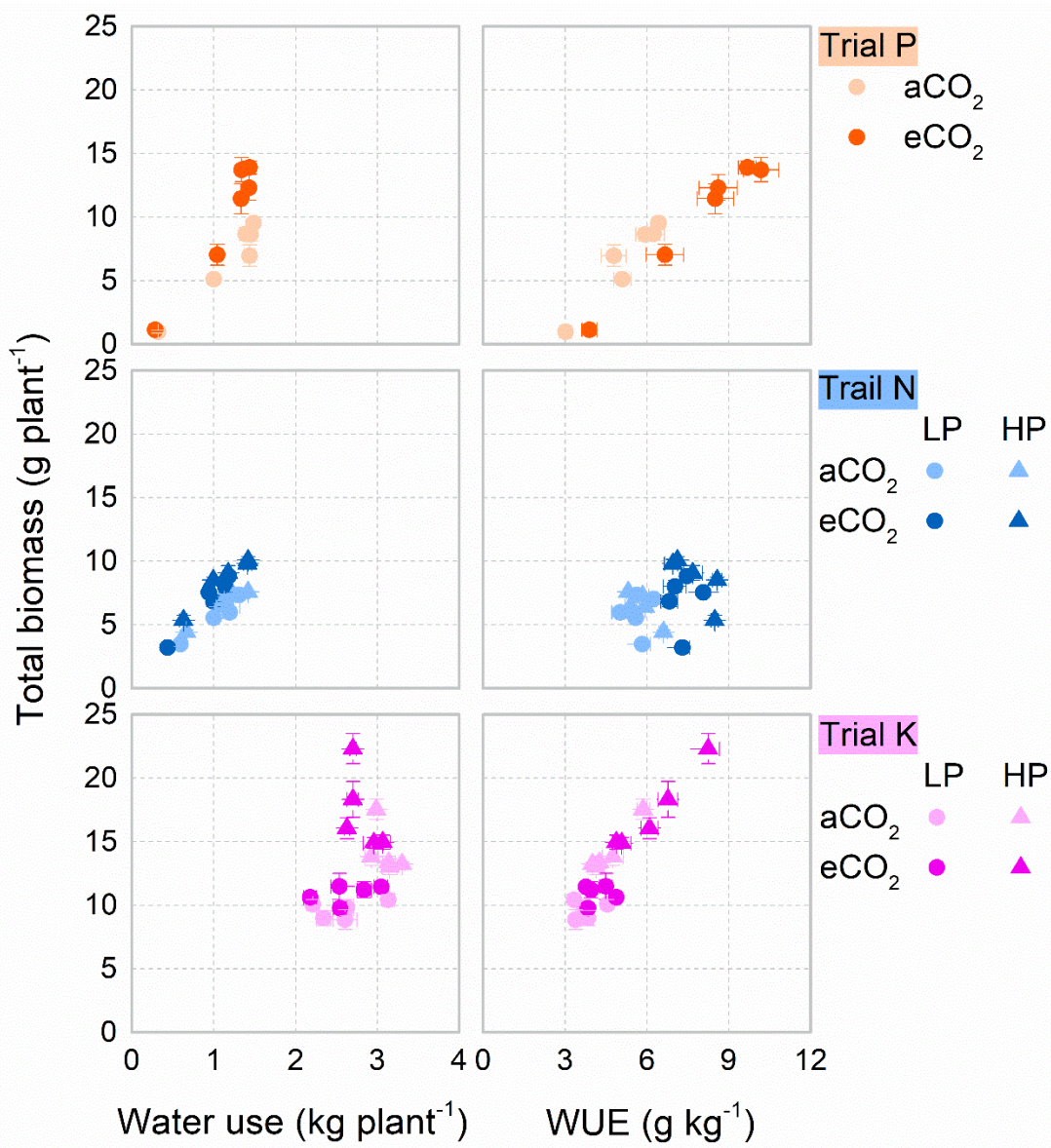
418 **Figure 1** Total biomass, water use, water-use efficiency (WUE, total biomass/water use) and total plant
419 $\Delta^{13}\text{C}$ value of potato plants. For Trial P, potato plants grown under aCO₂ and eCO₂ with different P
420 supply rates (0, 0.4, 0.8, 1.2, 2.4, and 1.6 g P kg⁻¹ soil). For Trial N, potato plants grown under aCO₂ and
421 eCO₂ with different N supply rates (0, 0.2, 0.4, 0.8, and 1.6 g N kg⁻¹ soil) at low P (LP, 0.3 g P kg⁻¹ soil)
422 and high P (HP, 3 g P kg⁻¹ soil). For Trial K, potato plants grown under aCO₂ and eCO₂ with different K
423 supply rates (0, 0.1, 0.2, 0.4, and 0.8 g K₂O kg⁻¹ soil) at low P (LP, 0.1 g P kg⁻¹ soil) and high P (HP, 1.5
424 g P kg⁻¹ soil). Data in each plot are means \pm S.E. (n = 5 or 6 (in Trial P) or 4 (in Trials N and K) biological
425 replicates for each treatment). Data for biomass, water use and WUE in Trials P, N, and K were reused
426 from [Yi et al. \(2019, 2020\)](#) and [Yi and Yano \(2021\)](#).

427 **Figure 2** Relationship between total plant biomass and water use or water-use efficiency (WUE). Linear
428 regression analysis of the relationship between total plant biomass and water use or WUE are shown in
429 Table S3.

430 **Figure 3** Relationship between water-use efficiency (WUE) and carbon isotope discrimination ($\Delta^{13}\text{C}$)
431 in potato plants growing under aCO₂ and eCO₂, respectively. Data from Trials P and K were combined
432 into one figure because the same slope without any shift in elevation were evaluated under each CO₂
433 condition. Regressions are as follows: in Trial N, aCO₂: $y = -0.49x + 14.15$, $R^2 = 0.745$, $P = 0.001$; eCO₂:
434 $y = -0.46x + 15.74$, $R^2 = 0.579$, $P = 0.011$; in Trials P and K, aCO₂: $y = -1.06x + 24.09$, $R^2 = 0.625$, $P <$
435 0.001 ; eCO₂: $y = -1.09x + 27.59$, $R^2 = 0.823$, $P < 0.001$.

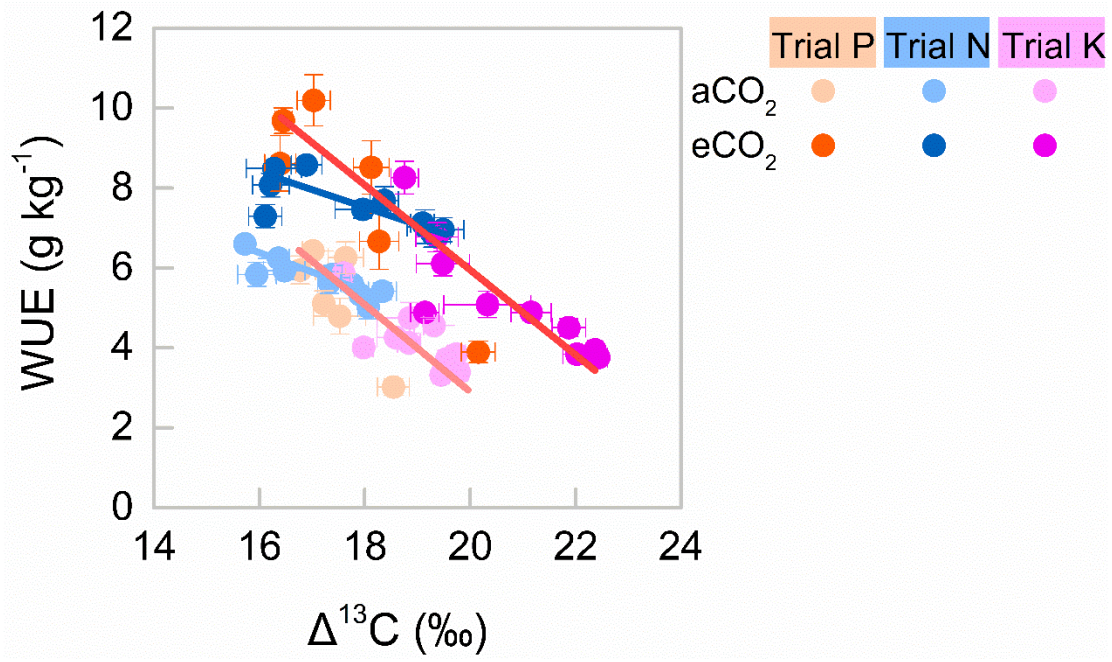


438 **Figure 2**



439

440 **Figure 3**



441

442 **Verification of water-use efficiency estimates via carbon isotope discrimination in potato under**
 443 **varying nutrient statuses and CO₂ conditions**

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449 **Table S1** Actual CO₂ concentrations, temperature, and relative humidity during the light and dark time
 450 in the growth chambers.

		CO ₂ concentration (ppm)		Temperature (°C)		Relative humidity (%)	
		aCO ₂	eCO ₂	aCO ₂	e[CO ₂]	aCO ₂	eCO ₂
Light time	Trial P	439 ± 9	825 ± 17	24.3 ± 0.3	25.6 ± 0.4	36.8 ± 1.2	36.2 ± 1.2
	Trial N	395 ± 4	802 ± 3	25.1 ± 0.2	24.5 ± 0.2	48.6 ± 0.6	48.9 ± 0.7
	Trial K	407 ± 9	793 ± 6	24.6 ± 0.2	24.9 ± 0.3	48.7 ± 0.5	47.9 ± 0.5
Dark time	Trial P	543 ± 14	809 ± 6	17.8 ± 0.3	18.6 ± 0.3	48.5 ± 1.3	48.3 ± 1.2
	Trial N	500 ± 7	795 ± 3	16.7 ± 0.2	16.8 ± 0.1	50.7 ± 0.4	51.7 ± 0.4
	Trial K	562 ± 7	793 ± 7	17.1 ± 0.1	17.6 ± 0.1	52.6 ± 0.3	51.3 ± 0.3

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455 **Table S2** Test for specific response to CO₂ conditions. Maize and guinea grass were grown under the
 456 same chambers with different CO₂ conditions (401 ± 5 and 839 ± 2 ppm under aCO₂ and eCO₂,
 457 respectively).

C4 species	δ ¹³ C in C4 plants (‰)	
	aCO ₂	eCO ₂
Maize	-13.95 ± 0.20	-22.20 ± 0.26
Guinea grass	-14.12 ± 0.39	-22.27 ± 1.06

ANOVA	
Species (S)	0.718
CO ₂ (C)	< 0.001
S × C	0.939

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460 **Table S3** Linear regression analysis of the relationship between total plant biomass and water use or
 461 water-use efficiency (WUE) in potato plants growing under aCO₂ and eCO₂, respectively in each trial.
 462 Parameters of the linear regression of total plant biomass against water use or WUE are presented. Values
 463 in brackets are the results of test for a common slope between aCO₂ and eCO₂ in each trial.

	Group	n	R ²	P-value	Slope		Intercept	
Linear regression analysis of the relationship between water use and biomass								
Trial P	aCO ₂	6	0.936	0.002	6.97	(*)	-1.59	
	eCO ₂	6	0.937	0.001	11.2		-2.91	
Trial N	LP	aCO ₂	5	0.893	0.015	5.57	(ns)	0.06
		eCO ₂	5	0.956	0.004	7.31		0.02
	HP	aCO ₂	5	0.968	0.002	4.38	(ns)	1.52
		eCO ₂	5	0.939	0.007	5.77		2.06
Trial K	LP	aCO ₂	5	0.189	0.464	1.91	-	4.68
		eCO ₂	5	0.225	0.420	2.22		5.07
	HP	aCO ₂	5	0.285	0.354	-12.9	-	54.3
		eCO ₂	5	0.335	0.307	-16.6		64.0
Linear regression analysis of the relationship between water-use efficiency and biomass								
Trial P	aCO ₂	6	0.927	0.002	2.51	(ns)	-6.53	
	eCO ₂	6	0.983	< 0.001	2.15		-7.08	
Trial N	LP	aCO ₂	5	0.004	0.917	3.46	-	-13.7
		eCO ₂	5	0.019	0.824	4.57		-26.6
	HP	aCO ₂	5	0.729	0.066	-2.42	-	20.5
		eCO ₂	5	0.589	0.130	-2.52		28.2
Trial K	LP	aCO ₂	5	0.029	0.784	1.37	-	4.45
		eCO ₂	5	0.004	0.920	1.50		4.62
	HP	aCO ₂	5	0.941	0.006	2.49	(ns)	2.73
		eCO ₂	5	0.957	0.004	2.28		3.13

464 *, $P < 0.05$; ns, not significant.

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 467
 468 **Table S4** Comparison of lines with common slope between Trial P and K under different CO₂ conditions.

	aCO ₂	eCO ₂
Test for common slope between Trail P and K		
P-value	0.107	0.140
Testing for shifts in elevation between Trial P and K using WALD statistic		
P-value	0.077	0.222
Testing for shifts along the common slope between Trial P and K using WALD statistic		
P-value	0.006	0.003

469