


1 **Nitrogen niche partitioning between tropical legumes and grasses conditionally weakens**
2 **under elevated CO₂**

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4 Amber C. Churchill^{1,2,3*}, Haiyang Zhang^{1,4}, Gil Won Kim^{1,5}, Karen L. M. Catunda¹, Ian C.
5 Anderson¹, Forest Isbell², Ben Moore¹, Elise Pendall¹, Jonathan M. Plett¹, Jeff R. Powell¹,
6 Sally A. Power¹

7

8 * Corresponding author: achurchill@binghamton.edu; 

9

10 ¹Hawkesbury Institute for the Environment, Western Sydney University, Hawkesbury
11 Campus, Ground Floor, Building R2, Locked Bag 1797, Penrith 2751, NSW Australia

12

13 ²Department of Ecology, Evolution and Behavior, University of Minnesota, 140 Gortner
14 Laboratory, 1479 Gortner Ave., St. Paul, MN 55108, USA

15

16 ³Environmental Studies Program, Binghamton University, State University of New York,
17 4400 Vestal Parkway E, Binghamton, NY 13902, USA

18

19 ⁴College of Life Sciences, Hebei University, Baoding, China

20

21 ⁵Institute of Agriculture and Life Sciences, Gyeongsang National University, 501 Jinju
22 Daero, Jinju 52828, South Korea

23 **Abstract**

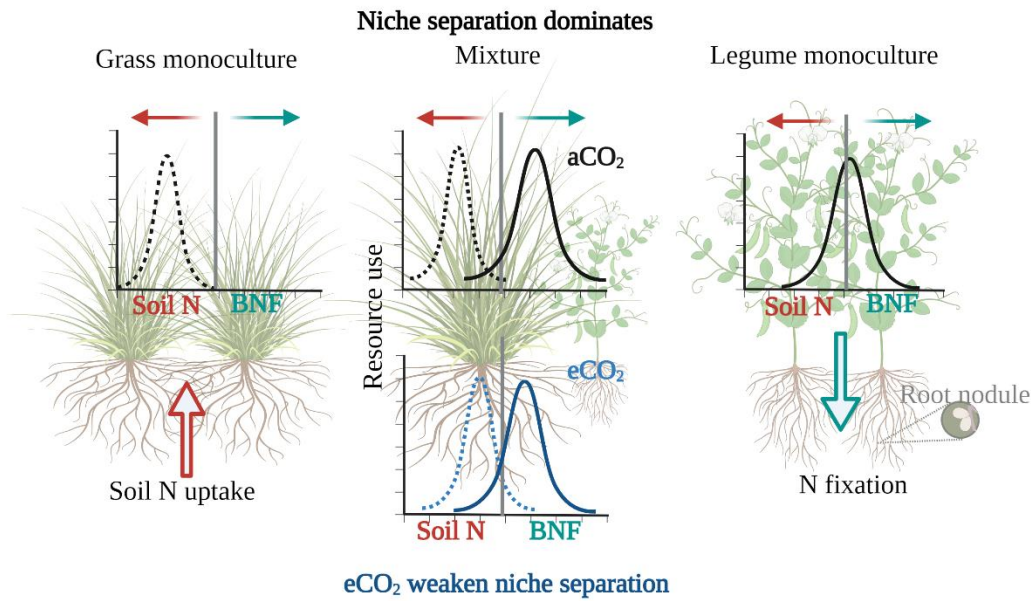
- 24 1. Plant community biodiversity can be maintained, at least partially, by shifts in species
25 interactions between facilitation and competition for resources as environmental
26 conditions change. These interactions also drive ecosystem functioning, including
27 productivity, and can promote over-yielding- an ecosystem service prioritized in agro-
28 ecosystems, such as pastures, that occurs when multiple species together are more
29 productive than the component species alone. Importantly, species interactions that can
30 result in over-yielding may shift in response to rising CO₂ concentrations and changes in
31 resource availability, and the consequences these shifts have on production is uncertain
32 especially in the context of tropical mixed-species grasslands.
- 33 2. We examined the relative performance of two species pairs of tropical pasture grasses
34 and legumes growing in monoculture and mixtures in a glasshouse experiment
35 manipulating CO₂. We investigated how over-yielding can arise from nitrogen (N) niche
36 partitioning and biotic facilitation using stable isotopes to differentiate soil N from
37 biological N fixation (BNF) within N acquisition into aboveground biomass for these
38 two-species mixtures.
- 39 3. We found that N niche partitioning in species-level use of soil N vs. BNF drove species
40 interactions in mixtures. Importantly partitioning and overyielding were generally
41 reduced under elevated CO₂. However, this finding was mixture-dependent based on
42 biomass of dominant species in mixtures and the strength of selection effects for the
43 dominant species.
- 44 4. This study demonstrates that rising atmospheric CO₂ may alter niche partitioning
45 between co-occurring species, with negative implications for the over-yielding benefits
46 predicted for legume-grass mixtures in working landscapes with tropical species.
47 Furthermore, these changes in inter-species interactions may have consequences for
48 grassland composition that are not yet considered in larger-scale projections for impacts
49 of climate change and species distributions.

50

51 **Key Words** (5-8 in alphabetical order):

52 Biological nitrogen fixation, complementarity effect, elevated carbon dioxide, natural
53 abundance ¹⁵N, nitrogen niche partitioning, tropical pastures

54



Graphical abstract (Image by H. Zhang): Among our tropical pasture species we found that grasses (dotted lines) grown in monoculture rely fully on soil nitrogen (N), while legumes (solid lines) grown in monoculture relied approximately equally on soil N and biological nitrogen fixation (BNF) to meet N requirements. When grown with tropical grasses, however, legumes shifted to rely more strongly on BNF, indicative of niche partitioning and decreased competition for soil nutrients with grasses. This separation of niche space was weakened under elevated CO₂ conditions, ultimately reducing legume production.

56 **Introduction**

57 Interactions between plant species within communities and ecosystems vary over time and as
58 environmental conditions change, thereby driving shifts between competition and facilitation
59 for resources (Adler et al., 2012; Arfin Khan et al., 2014). Niche partitioning is a mechanism
60 allowing the co-existence of numerous species that may overlap in fundamental niche space
61 but differ in realized niche space to reduce impacts from competition (Jumpponen et al.,
62 2002; McKane et al., 2002; Zuppinger-Dingley et al., 2014). Additionally, changes in niche
63 space associated with interspecific interactions are a common feature of net biodiversity
64 effects whereby interactions among community members result in greater ecosystem
65 functioning, relative to the sum of individual contributions (Barry et al., 2019; Loreau et al.,
66 2008; Loreau & Hector, 2001); when applied to productivity this concept is often referred to
67 as over-yielding (Vandermeer, 1981). Quantifying mechanisms underpinning interspecific
68 interactions, such as niche partitioning, is fundamental to understanding relationships
69 between biodiversity and ecosystem function.

70

71 Nitrogen (N) is a primary often-limiting nutrient that plays an important role in structuring
72 community interactions and ecosystem function alongside phosphorus (P) and other mineral
73 nutrients (Fay et al., 2015; Vitousek & Howarth, 1991). Plants that host N-fixing bacteria (i.e.
74 N-fixers), such as legumes, are often considered to have an advantage under low soil N
75 conditions as the mutualism can provide an alternative route for accessing N. This biological
76 N fixation (BNF), therefore, can promote biodiversity and ecosystem function, via N niche
77 partitioning allowing different community members to rely on alternative sources of N or
78 facilitation where N resources from BNF are shared among community members (Barry et
79 al., 2019; Elias & Agrawal, 2021; Lee et al., 2003; Mulder et al., 2002; Perring et al., 2010).
80 These processes contribute to the Net Biodiversity Effect through complementarity effects,
81 where more diverse communities contribute to ecosystem functions greater than the weighted
82 average of constituent monoculture species, and selection effects where species with greater
83 than the average among-species-yields dominate in mixture.

84

85 In communities containing legumes, BNF is often assumed to result in N facilitation and an
86 increase in the overall pool of ecosystem N available for neighbouring plants (Lee et al.,
87 2003) that promote and subsequently increase production among component species (Barry et
88 al., 2019; Fornara & Tilman, 2008; von Felton et al., 2009). There are two main pathways

89 enabling this outcome: 1) direct acquisition of BNF resources from legumes by non-legumes
90 (facilitation; i.e. grasses) and 2) via niche partitioning where grasses compete less with
91 legumes for shared soil nitrogen, given that legumes have access to a non-soil pool of N
92 (Barry *et al.*, 2019). There are a number of biotic feedback-based mechanisms enabling the
93 sharing of BNF derived N to non-legume community members, including decomposition of
94 legume leaf litter (Makkonen *et al.*, 2012; Wei *et al.*, 2019), or roots (Fustec *et al.*, 2009;
95 McLaren & Turkington, 2010) that lead to higher soil N. Additionally, some literature has
96 highlighted the potential importance of root exudation of high N-containing compounds that
97 impact N (or carbon: C) availability in the rhizosphere (Henneron *et al.*, 2020; Paynel *et al.*,
98 2001), or transfer through the activity of soil fauna (Gilarte *et al.*, 2020), thereby promoting
99 increased N availability for surrounding community members. Alternatively, niche
100 partitioning can support greater overall ecosystem N availability as legumes rely on BNF for
101 N resources, leaving the soil N pool available to neighbouring community members
102 (Ashworth *et al.*, 2018). These pathways offer numerous strategies that ultimately promote
103 increased community productivity in the presence of legumes representing a potential for
104 over-yielding.

105

106 Atmospheric CO₂ concentrations have increased by 50% since the industrial revolution
107 (IPCC, 2022). There are many ecosystem consequences associated with this change,
108 including an increase in plant productivity (de Graaff *et al.*, 2006; Gill *et al.*, 2002), altered
109 plant community composition and diversity (Reich, 2009; Zelikova *et al.*, 2014), and
110 modified plant interactions within a community that impact ecosystem scale processes
111 (Blumenthal *et al.*, 2013; Gonçalves de Oliveira *et al.*, 2021; Maschler *et al.*, 2022). However,
112 some of these patterns may only occur when water is limiting, nutrients are abundant (Reich
113 *et al.*, 2014; Rogers *et al.*, 2009; Soussana & Lüscher, 2007), or when plant diversity is high
114 (Reich *et al.*, 2001; Shaw *et al.*, 2002). Under well-watered conditions, eCO₂ generally
115 benefits the C₃ photosynthetic pathway over the C₄ pathway (used by warm season or tropical
116 grasses) due to differences in carbon capture (Ainsworth & Long, 2005; Reich *et al.*, 2014,
117 but see Reich *et al.*, 2018). Furthermore, eCO₂ is reported to increase belowground biomass,
118 especially for fine roots (Nie *et al.*, 2013), which may increase access to soil nutrients (de
119 Graaff *et al.*, 2006; Dieleman *et al.*, 2012; Reich *et al.*, 2001).

120

121 Many studies have found that eCO₂ fertilization effects are larger for N fixers than grasses
122 due to greater C resource availability to support the energetically demanding process of N-
123 fixation (Liang et al., 2016). However, N-fixer responses in natural ecosystems have been
124 varied, as potential benefits from increased photosynthesis can become constrained by other
125 limiting nutrients such as phosphorus (Coventry et al., 1985; Hungate et al., 2004; Rogers et
126 al., 2009; Soussana & Lüscher, 2007), or reduced due to temperature or drought conditions
127 (Rogers et al., 2009). Even so, most studies find decreased foliar N concentrations for non-N-
128 fixers under eCO₂ with subsequent increases in C:N ratios in plant tissues (Ainsworth &
129 Long, 2005; Augustine et al., 2018; Gojon et al., 2022; Rogers et al., 2009). However, within
130 mixed grass-legume grassland communities, leaf N and C:N ratios are less likely to be
131 impacted by eCO₂ for the legume component of the sward (Rogers et al., 2009; Winkler &
132 Herbst, 2004). These findings suggest that grassland responses to eCO₂ may depend on the
133 presence of legumes (Jones & Donnelly, 2004). Importantly, the perceived ‘benefit’ of eCO₂
134 for tropical and subtropical ecosystems is less well documented, as most studies have focused
135 on temperate species, with C₃ photosynthetic pathways (Naudts et al., 2013). The potential
136 impacts of eCO₂ on nutrient dynamics in C₄ grasses, including those in mixed C₃-C₄ systems
137 is of particular interest, due to the increasing abundance of warm season grasses in many
138 areas of the world (McKeon et al., 2009). Given the diverse predicted outcomes for
139 ecosystems as eCO₂ concentrations increase (Cannell & Thornley, 1998; de Graaff et al.,
140 2006; Dijkstra et al., 2010), there is a need for improved understanding of the impacts of
141 rising CO₂ in managed and natural grasslands. These include ongoing shifts in grassland
142 composition, driven by species differences in response to climate change (McKeon et al.,
143 2009; Munson & Long, 2017; Sherry et al., 2007; C. Wang et al., 2013; Xie et al., 2022;
144 Zelikova et al., 2014). Increased understanding of the processes that underlie plant-plant
145 interactions between tropical grasses and legumes will therefore increase the accuracy of
146 predictions for how grassland species composition and productivity will be impacted as
147 atmospheric CO₂ concentrations continue to rise and human-induced shifts in ecosystem
148 biodiversity are realized.

149
150 To address this area of research, we examined potential mechanisms by which eCO₂ might
151 benefit warm season (C₄) grasses grown in mixed swards with tropical legumes. Specifically,
152 we evaluated the potential for increased production and nutrient availability within two-
153 species swards relative to individual species, driven by biologically fixed N (from rhizobia)

154 supported by legumes and accessed by grasses. We hypothesized that 1) grasses grown with
155 legumes benefit in terms of both nutrition and productivity, relative to those growing in
156 monoculture, due to access to BNF from legume rhizobia; 2) tropical legumes grown under
157 eCO₂ will increase production as a consequence of increased carbon-use-efficiency; 3)
158 increased C availability under eCO₂ will increase legume N fixation and both legume shoot N
159 and soil N content; and 4) increased available N due to BNF will promote overyielding and a
160 net biodiversity effect in two-species mixtures driven by a combination of complementarity
161 and selection effects that are enhanced by eCO₂.

162

163 **Materials and Methods**

164 *Experimental design*

165 This experiment was conducted in a glasshouse at Western Sydney University's Hawkesbury
166 Campus, Richmond, New South Wales (33°61'S, 150°74'E), Australia. We used six separate
167 glasshouse chambers, with three randomly assigned to ambient conditions (410 - 430 ppm;
168 aCO₂) and three an elevated CO₂ regime (630 - 650 ppm; eCO₂). The ambient treatment
169 matches contemporary atmospheric concentration, while the elevated regime was consistent
170 with the upper-end of predicted end-of-century increase in global atmospheric [CO₂]
171 (Szulejko et al., 2017). We maintained a consistent temperature regime across all chambers at
172 a daily average of 27°C (**Fig S1A**). This included a temperature ramp to mimic diurnal
173 patterns (00-06 hours: 22°C, 06-09 hrs: 27°C, 09-18 hrs: 32°C, 18-21 hrs: 27°C, 21-00 hrs:
174 22°C) for spring to summer temperatures for areas currently cultivating tropical pasture in
175 northern NSW and southern Queensland (BOM, 2020). Additionally, all chambers were
176 controlled at 50-60% relative humidity (**Fig S1B**) with natural lighting between 11 hours
177 (August) and 14 hours (December) per day.

178

179 The soil used in this experiment was collected adjacent to the Pastures and Climate Extremes
180 field experimental facility (Churchill et al., 2022). Soil was sieved to 5 mm before being air
181 dried and mixed with quartz sand (soil:sand 7:3 v/v). Each pot (3.7L, 150 mm diameter, 240
182 mm height) was filled with 3.9 kg of the soil-sand mixture before planting (Zhang et al.,
183 2021). The final soil mixture was ~ 84% sand, with an average pH of 5.6, plant available N at
184 55 mg kg⁻¹, plant available (bray) P at 39 mg kg⁻¹ and soil organic carbon at 1.1%. All pots
185 were maintained under well-watered conditions using an automated irrigation system such

186 that soil water capacity was brought to 100% every two days (Zhang et al., 2021). In order to
187 promote legume nodulation, no nutrients were added.

188

189 Two tropical grasses (C₄) and two tropical legumes (C₃) were selected, based on current
190 widespread use or consideration for future use by key pasture industries within Australia
191 (**Table 1**). All species are commonly grown in multi-species improved pastures in southern
192 Queensland and northern New South Wales, Australia, as well as among other sub-tropical
193 grasslands world-wide (Thomas & Sumberg, 1995; Bell *et al.*, 2012; Miegoue *et al.*, 2016;
194 Melesse *et al.*, 2017; DPI, 2018). Specific species-pairs were the legume *Macroptilium*
195 *bracteatum* (Nees. & Marti.) growing with the grass *Chloris gayana* (Kunth), and the legume
196 *Desmodium intortum* (Mill) Urb with grass *Panicum maximum* var. *trichoglume* (Robyns).
197 Specific species pairing within pots was randomly assigned at project initiation, as all species
198 are typically grown in mixtures. We included non-factorial pairings between grasses and
199 legumes to maximize species included in the study, while balancing time and space
200 constraints in the greenhouse. All seeds were supplied by Heritage Seeds and contained
201 AgriCote advanced seed coating technology; legume seed came pre-inoculated with
202 appropriate rhizobia (Barenbrug Co., Heritage Seeds, New South Wales, Australia). Seeds
203 were planted directly into seedling trays (~5 seeds per cell) and thinned to a single individual
204 over time. After three weeks, two individuals for the monoculture or one individual from
205 each species for the mixture were then transplanted into the pots. The experimental design
206 therefore included the two CO₂ treatments (aCO₂ and eCO₂), six types of plant combinations
207 in pots (grass₁, legume₁, mixed₁, grass₂, legume₂, mixed₂), and twelve replicates per plant
208 combination with four of these in each of three chamber replicates per CO₂ treatment). This
209 included a total of 288 pots distributed among six climatically isolated greenhouse chambers.

210

211 *Aboveground sampling*

212 After transplanting the seedlings, all plants were grown for 11 weeks before a final harvest,
213 just as individuals were reaching the flowering phenophase. All aboveground standing plant
214 material (shoot; leaves and stems/tillers together) was cut at soil level and for mixed species
215 pots, separated by species, then weighed. To accommodate differing methodologies for
216 nutritional analysis of harvested biomass (Catunda et al., 2022), the fresh aboveground
217 biomass was frozen at -18°C. Within one month all samples were then microwaved at
218 medium power for 90 seconds to halt enzymic degradation of plant tissue compounds

219 (Landh usser et al., 2018), and oven dried at 70 C for 48 hours then weighed. All dried plant
220 material was homogenized, ground to pass through a 2-mm screen in a laboratory cyclone
221 mill (Foss Cyclotec Mill, Denmark) then ball-milled to a fine powder (Retsch  MM400;
222 Hann, Germany) for nitrogen (N) and isotopic (i.e. $\delta^{15}\text{N}$) measurements, which were
223 determined using an elemental analyzer interfaced to a continuous flow isotope ratio mass
224 spectrometer at the UC Davis Stable Isotope Facility (Davis, CA, USA).

225

226 *Belowground biomass*

227 After aboveground material was removed, belowground biomass was determined. All pot
228 soils were carefully sieved using 2 mm sieves to retain root fragments and roots were oven
229 dried to constant mass. We assessed legume nodule number and weight for a subset of fresh
230 root material in each legume- or mixed species pot by randomly subsetting roots and nodules
231 from different locations within the root system (~25g fresh root or ~ 80% of total root mass).
232 From this subset, we separated the nodules from the roots and weighed the fresh biomass for
233 each component separately. Fresh nodules were incubated with acetylene for 1 hour and
234 ethylene evolution tested as a proxy to estimate the average instantaneous rate of nitrogenase
235 activity of the bacteria within the nodules using acetylene reduction assays (ARA; Plett *et al.*,
236 2016). After ARA measurement, root nodules were oven dried and weighed as above. Final
237 estimates of biological nitrogen fixation (BNF) activity were calculated as the rate of
238 ethylene production per gram of incubated nodule dry biomass.

239

240 *Soil nutrients*

241 Nutrient availability was assessed using ion exchange resins (IONAC NM-60 H^+/OH^- Form;
242 J.T. Baker) for nitrate (NO_3^-), ammonia (NH_4^+) and phosphate (PO_4^{3-}). We enclosed 5 g of
243 mixed bed resin in a 3 cm x 5 cm nylon bag (42 μm mesh) and placed one bag in each pot in
244 the upper 10 cm of soil. Resin bags were collected during the biomass harvest, briefly rinsed
245 with deionized water to remove attached soil and root particles before immediate storage at -
246 70  C prior to extraction with 0.5 M HCl solution through a Whatman No. 1 paper (Carrillo
247 et al., 2012; Dijkstra et al., 2010). Extractable NO_3^- , NH_4^+ and PO_4^{3-} concentrations in the
248 resin bag were determined on a SEAL AQ2 Discrete Analyser (SEAL Analytical Inc., USA).

249

250 *Quantifying contributions of biological nitrogen fixation*

251 The contribution of BNF to plant total N was calculated as described in Chalk *et al.* (2016)
252 and Bell *et al.* (2017), where lower $\delta^{15}\text{N}$ is indicative of a higher N-fixation amount. The
253 minimum observed values for $\delta^{15}\text{N}$ for both legume species in this experiment (*B*;
254 *Macroptilium*: -2.7 ‰ under eCO₂ in a mixed pot; *Desmodium*: -2.0 ‰ under aCO₂ in a
255 mixed pot) were used to represent when the maximum possible amount of N is derived from
256 BNF in our experimental conditions. Note that while this could overestimate the absolute
257 estimate of BNF, the general patterns among different treatments should remain unaffected
258 (Chalk *et al.*, 2016). Mean $\delta^{15}\text{N}$ values for companion grass species in monoculture at each
259 treatment level of CO₂ were used as baselines for soil derived N (*Ref*; *Chloris*: 3.9 ‰, 3.7‰;
260 *Panicum*: 4.9 ‰, 4.4 ‰; aCO₂, eCO₂ respectively), and we then calculated the relative
261 contribution of BNF to plant aboveground N following Equation 1:

262
$$(Equation\ 1)\ BNF\ contribution\ to\ plant\ N = 100 * \frac{(Ref - Sample_{\delta^{15}N})}{Ref - B}$$

263 Calculations of the percentage of shoot N derived from BNF were performed for each species
264 within a pot, based on species specific shoot $\delta^{15}\text{N}$. The contribution of soil N to shoot N was
265 then calculated as the difference between 100% and BNF values for each species within a
266 pot. Species level shoot N content derived from either BNF or soil was then calculated by
267 multiplying total shoot N content by the relative percentage for both sources.

268

269 *Calculations and statistical analyses*

270 Effects of CO₂ treatment on aboveground plant responses measured on pot level totals
271 (aboveground shoot biomass, total shoot N content, soil derived shoot N content,
272 belowground biomass and resin extractable soil nutrients) were analysed using linear mixed
273 effects models. For these analyses, ‘Species Pair’, ‘CO₂’ and ‘Pot Type’ treatments were
274 included as fixed effects with all two-way and three-way interactions. For analysing shoot %
275 N we conducted a linear mixed effect model with ‘Species Pair’, ‘CO₂’ and ‘Plant Type’.
276 ‘Chamber’ was also included as a random effect in all models to account for non-
277 independence among replicates within each chamber due to potential among-chamber
278 environmental variation. Treatments of CO₂ included two levels (aCO₂ and eCO₂), ‘Species
279 Pair’ included ‘*Macroptilium* & *Chloris*’ and ‘*Desmodium* & *Panicum*’, the ‘Pot Type’
280 category included ‘Grass’, ‘Legume’, and ‘Mixed’ pots, and the ‘Plant Type’ category
281 included ‘Grass’, ‘Legume’, grass grown with legume: ‘L-Grass’, and legume grown with
282 grass: ‘G-Legume’. Nodule data were also analysed using linear mixed effects models, such

283 that ‘CO₂’, ‘Species Pair’, and ‘Pot Type’ were included as interactive fixed effects and
284 ‘Chamber’ as a random effect. For these analyses ‘Pot Type’ only included ‘Legume’ and
285 ‘Mixed’ pots.

286

287 Community over-yielding was determined as the increased productivity of a mixed pasture
288 over the discrete contributions of the constituent species grown in monoculture (Isbell et al.,
289 2018). We calculated over-yielding for each species pair based on mixture total shoot
290 biomass (and total N content in aboveground plant material) and the associated shoot biomass
291 of the constituent monoculture grass and legume species standardized by the number of
292 individuals per pot in each chamber, following Equation 2:

293

$$(Equation\ 2)\ Overyield\ effect\ of\ mixtures = \frac{\overline{Mixed}}{\frac{\overline{Grass}}{2} + \frac{\overline{Legume}}{2}} - 1$$

294 Effects of eCO₂ on over-yielding were examined by calculating over-yield estimates based
295 on average monoculture aboveground biomass by species and average mixed pots for the
296 component species pairs in each chamber (n = 6; 3 for each CO₂ level). To examine
297 differences in over-yield effect between CO₂ treatments and species pair swards, we then ran
298 a linear mixed effects model with over-yield effect as the response, ‘CO₂’ level and ‘Species
299 Pair’ as the fixed effects and ‘Chamber’ as a random effect. Statistical significance of the
300 effect size for over-yielding was determined by calculating mean treatment values and 95%
301 confidence intervals (CI) across all replicates (n = 12 for each plant type and CO₂ level) and
302 over-yielding was deemed significant if CIs did not overlap with 0. For these results, CIs that
303 did not overlap with 0 indicate either a gain in shoot biomass in mixed pots relative to
304 monoculture pots (positive values) or a loss in shoot biomass in mixed pots relative to
305 monoculture pots (negative values).

306

307 The net biodiversity effect, and its constituents- the complementarity effect and selection
308 effect- were calculated following Loreau & Hector (2001) based on averages for monoculture
309 and mixed pots of each species pair in each chamber and analysed statistically as described
310 for the over-yield metric above. Net biodiversity effect was therefore defined as the
311 difference between the observed yield of a mixture and the expected yield in the absence of
312 complementarity or selection (Loreau & Hector, 2001). NBE is considered the net balance of
313 complementarity effects and selection effects. Complementarity effects are positive when

314 mixture yields are higher than expected based on the weighted average of constituent
315 monoculture species, while selection effects are positive when species with greater than the
316 average among-species-yields dominate in mixture.

317

318 All analyses were conducted in R version 4.1.1 (R Core Team, 2020) using the package lme4
319 (Bates et al., 2015) with statistical contrasts based on Kenward-Roger degrees of freedom
320 calculated using the ‘Anova’ function from the ‘car’ package (Fox & Weisberg, 2019).

321 Pairwise comparisons to determine treatment differences in plant responses for all models
322 were conducted using the R package emmeans (Length, 2020) and using the Tukey method
323 for *P*-value adjustment. All data used in these analyses are publicly available on Dryad
324 (Churchill et al., 2024).

325

326 **Results**

327 *Shoot biomass and N content*

328 Shoot biomass differed strongly between our species pairs, with greater productivity in pots
329 with the *Macroptilium* & *Chloris* monocultures and mixture, compared with *Desmodium* &
330 *Panicum* (**Fig 1; Table 2**). Elevated CO₂ (eCO₂) had limited effects on shoot biomass, with
331 increased productivity only observed for *Macroptilium* monoculture pots (**Fig 1a**). There
332 were no significant productivity differences among pot types (grass, legume, mixed) for the
333 *Macroptilium* & *Chloris* pairing, while *Desmodium* in monoculture produced less shoot
334 biomass than *Panicum* or the mixed pot type (**Fig 1b**). Despite little change in pot-level
335 biomass among monocultures and mixed pots, the contribution of grasses and legumes to
336 mixed pot total biomass was heavily weighted toward grass production in both species-pairs,
337 with *Chloris* contributing 65% and 61% of total pot biomass under aCO₂ and eCO₂ and
338 *Panicum* 90% and 95%, respectively. Relative to growth of individual plants in monoculture
339 pots, this change represented a 38%/52% gain in biomass under aCO₂ and eCO₂ by *Chloris*
340 and 20%/28% reduction in growth by *Macroptilium* (**Fig 1a**). This trend was stronger for the
341 *Desmodium* & *Panicum* species pair with 96%/87% gain for *Panicum* and 65%/86% loss for
342 *Desmodium* between monoculture and mixed pots (**Fig 1b**).

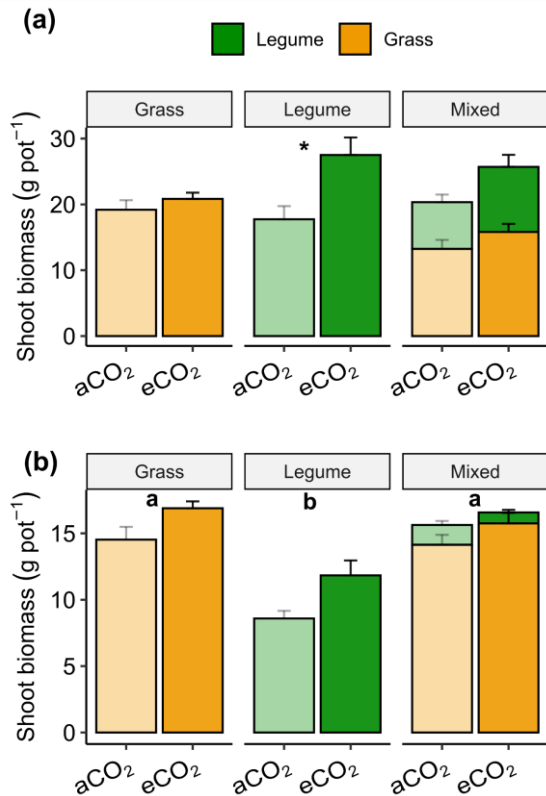


Figure 1. Shoot biomass of grass and legume grown as monoculture or mixtures for two species pairs under ambient (aCO₂) and elevated CO₂ (eCO₂) comprising (a) *Macroptilium* & *Chloris* and (b) *Desmodium* & *Panicum*. Bars indicate mean values, plus standard error. Significant differences ($p < 0.05$) between CO₂ treatments are indicated by ‘*’, and significant differences among pot types (grass, legume, mixed) are indicated by differing letter designations.

343

344

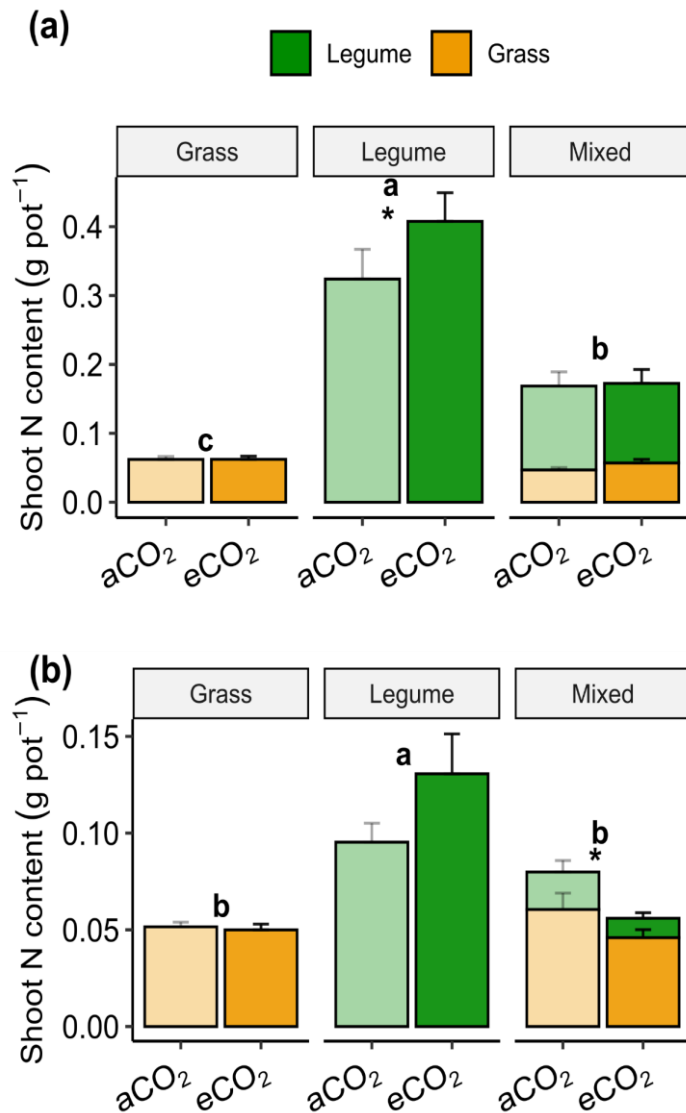


Figure 2. Shoot nitrogen (N) content (%N multiplied by shoot biomass) partitioned between grasses and legumes grown as monocultures or mixtures under ambient (aCO₂) and elevated CO₂ (eCO₂), for (a) *Macrotidium & Chloris* and (b) *Desmodium & Panicum*. Bars indicate mean values, plus standard error. Notations follow Fig. 1.

345 We found that legumes had a higher shoot % N than grasses, and this resulted in greater total
 346 shoot N content for the legumes in both species pairs (**Fig S2; Table S1**). Additionally, the
 347 *Macrotidium & Chloris* species pair generally had greater total shoot N content than
 348 *Desmodium & Panicum*, driven by the differences in aboveground biomass rather than shoot
 349 N concentrations. *Macrotidium* exhibited increased shoot N content under eCO₂ in
 350 monoculture, however there was no change in N content in the mixed pots that included this
 351 species (**Fig 2a**). In contrast, *Desmodium* N content was not significantly affected by eCO₂ in
 352 monoculture, however pot total N content in the mixtures with *Panicum* was reduced under

353 eCO₂ (**Fig 2b**). Legume contributions to shoot N content in mixed pots also differed between
354 the two species pairs, with *Macroptilium* contributing 72%/67% under aCO₂ and eCO₂ and
355 *Desmodium* contributing 24%/18% of the total. Importantly, in both species pairs the shoot N
356 content per individual plant in grasses grown with legumes increased (*Chloris*: 51%/83%,
357 *Panicum*: 135%/84%; aCO₂/eCO₂, respectively). At the same time, the corresponding shoot
358 N content per individual in the mixed pot legumes declined compared with monoculture pots
359 (*Macroptilium*: -25%/-43%, *Desmodium*: -59%/-85%). For the *Desmodium* & *Panicum* pair
360 this change was large enough that the *Panicum* grown with *Desmodium* contained
361 comparable shoot N content to the *Desmodium* grown in monoculture. Differences in shoot N
362 content among individual species between monoculture and mixed pots (based on
363 ‘PlantType’) are shown in **Table S1** and **Figure S3**.

364

365 *N uptake source partitioning: soil N uptake*

366 Despite variability in shoot N contents, the portion of pot total shoot N derived from soil
367 nutrients (**Fig 3**) remained similar among pot types within each species pair (**Table 2**). There
368 were no effects of eCO₂ on soil-derived shoot N for either species pair, however the fraction
369 of soil-derived N in aboveground plant material in mixed pots was not equally divided
370 between grasses and legumes. Instead, grasses dominated soil nutrient uptake, accounting for
371 74%/83% for aCO₂ and eCO₂ pots in the *Macroptilium* & *Chloris* species pair and 94%/95%
372 in *Desmodium* & *Panicum*. Raw values for $\delta^{15}\text{N}$ for each pot type and CO₂ treatment are
373 included in **Table S2**. Resin extractable soil nutrients indicated that eCO₂ did not impact
374 availability (**Fig S4**) of NO₃⁻, NH₄⁺ or PO₄⁻³ ($p > 0.05$). We did, however, find differences in
375 the soil availability of NO₃⁻ and NH₄⁺ among pot types within the *Macroptilium* & *Chloris*
376 species pair. *Chloris* monoculture pots had less NO₃⁻ than in mixed pots (**Fig S4a**), and
377 *Macroptilium* monoculture pots had less NH₄⁺ than mixed pots (**Fig S4c**).

378

379 The ability of plants to take up soil nutrients is contingent on belowground traits, and we
380 found that there were key differences in the root biomass associated with eCO₂
381 (*Macroptilium* & *Chloris* **Fig S5a**; **Table S3**) and among pot types (legume/grass/mixed,
382 *Desmodium* & *Panicum*; **Fig S5b**). In general, there was increased root biomass in pots under
383 eCO₂, driven by significant increases in *Macroptilium* roots in monoculture and for mixed
384 roots in the *Macroptilium* & *Chloris* pots (**Fig S5a**). In the *Desmodium* & *Panicum* species

385 pair there was no impact of eCO₂, however *Panicum* had the greatest root biomass, followed
386 by the mixed pots and then *Desmodium* in monoculture (**Fig S5b**). Despite these changes in
387 belowground biomass, there were no shifts in the root mass fraction between species pairs,
388 among pot types or associated with eCO₂ (**Fig S5c & d; Table S3**).

389

390 *N uptake source partitioning: biological nitrogen fixation*

391 We found that a large fraction of the total shoot N was derived from BNF in legume
392 monoculture and mixed species pots, although this differed between legume species and
393 between pot types (**Fig 4; Table 3**). *Macroptilium* derived more shoot N via BNF than
394 *Desmodium* (*Macroptilium*: 77/82% of total shoot N in aCO₂ vs. eCO₂, *Desmodium*: 32/39%;
395 **Table 3**) in monoculture. Pot level shoot N relied more strongly on BNF in mixtures, with
396 *Macroptilium* & *Chloris* pots increasing reliance on BNF to 84/93% under aCO₂/eCO₂ and
397 *Desmodium* & *Panicum* shoot N increasing to 72/48% (**Fig S3b & d; Table S1**). Importantly,
398 although both legumes derived less shoot N from BNF in mixture than monoculture (**Fig 4a**
399 & c), BNF contributed a greater percentage of the total shoot N in contrast to soil N (**Fig 4d**)
400 for *Desmodium* especially under aCO₂. Across both species pairs there were low levels of
401 BNF-derived N uptake by grasses (*Chloris*: 3/1%, *Panicum*: 6/1%). However, by mass, the
402 BNF derived N accounted for 29% of the shoot N in *Panicum* in mixtures under aCO₂, but
403 only 2% under eCO₂ (**Fig 4b & d**).

404

405 The ability of legumes to support rhizobia in nodules is the main constraint on the potential
406 role of BNF as a source for shoot N, and we found clear differences between species in terms
407 of root nodules (**Fig 5; Table 4**). In parallel with total shoot N content, *Macroptilium* had
408 greater nodule biomass (**Fig 5a**) and number of nodules (**Fig 5b**), compared to *Desmodium*,
409 in monoculture. Additionally, both legumes had a significant reduction in nodule biomass and
410 number of nodules when grown with grass, and there were no impacts of eCO₂ on these
411 measurements. Despite the difference in nodule biomass and number between legume
412 species, *Desmodium* had equivalent levels of instantaneous nodule activity to *Macroptilium*,
413 based on production of ethylene as a proxy for the rate of nitrogen fixation following harvest
414 (**Fig 5c**) and in monoculture there was no impact of eCO₂ for either species. However, for
415 *Desmodium* grown in mixture with *Panicum*, real-time nodule activity significantly increased
416 for individuals grown under eCO₂ (**Fig 5c**).

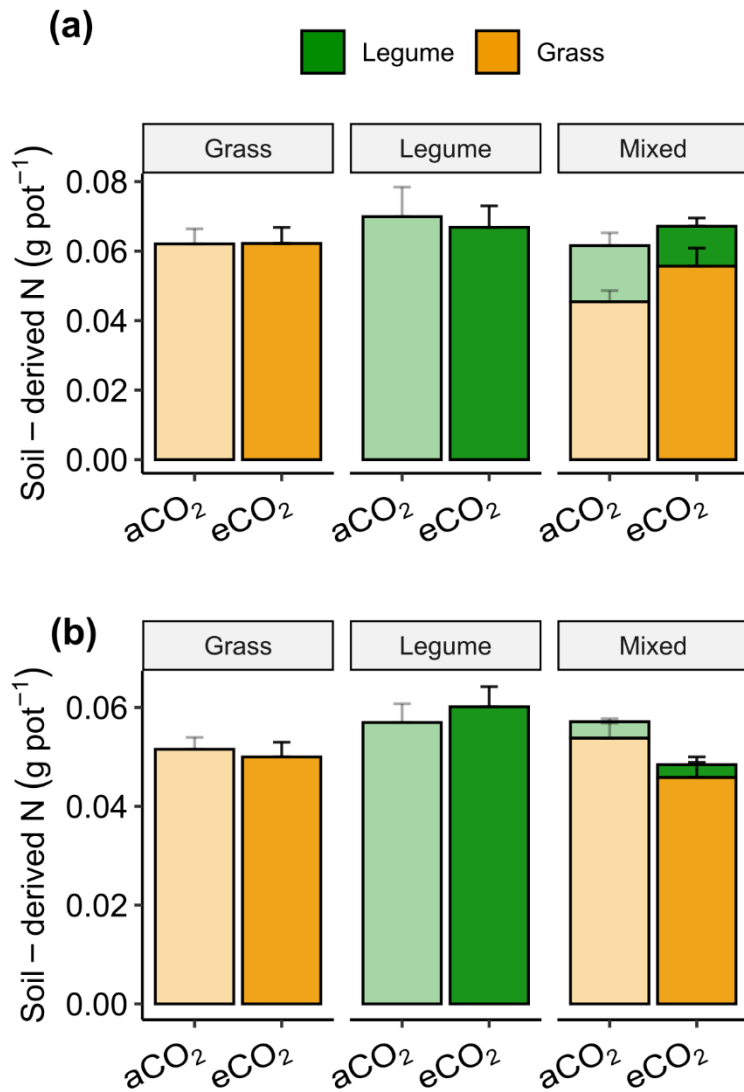


Fig 3. Shoot N content derived from soil for (a) *Macroptilium* & *Chloris* and (b) *Desmodium* & *Panicum* under ambient (aCO_2) and elevated (eCO_2) concentrations of CO_2 . Values shown are pot-level means + 1 SE. Note no significant differences with CO_2 treatment or among pot types were observed.

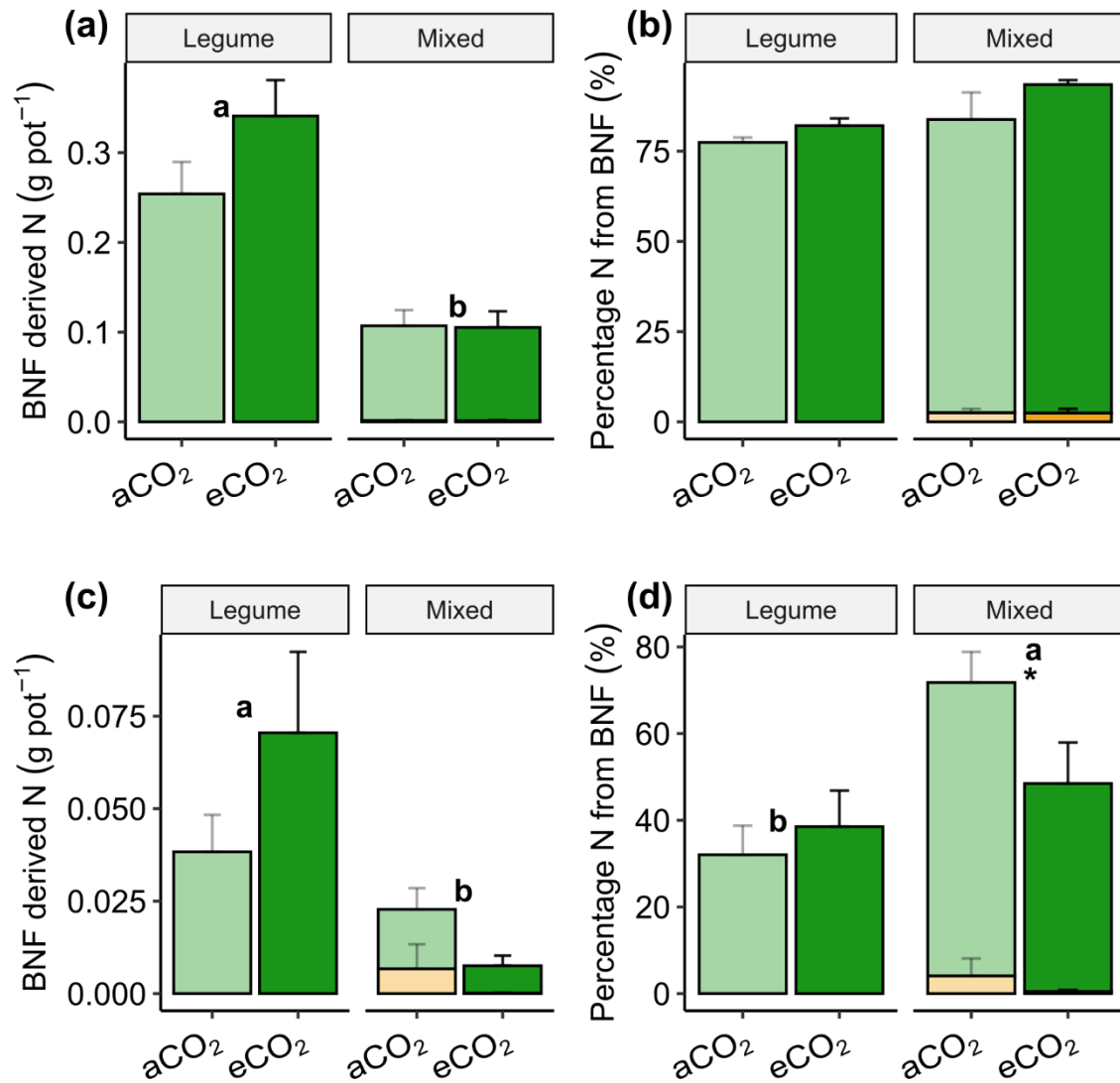


Fig. 4. Shoot N content derived from biological N fixation (BNF; a & c) and the proportion of aboveground N content derived from BNF (b & d) for *Macroptilium & Chloris* (a & b) and *Desmodium & Panicum* (c & d). Values shown are means \pm 1 SE. Colours in panels match Fig 1, with green indicating N in legume shoots, and orange indicating N in grass shoots. Notations from Fig. 1.

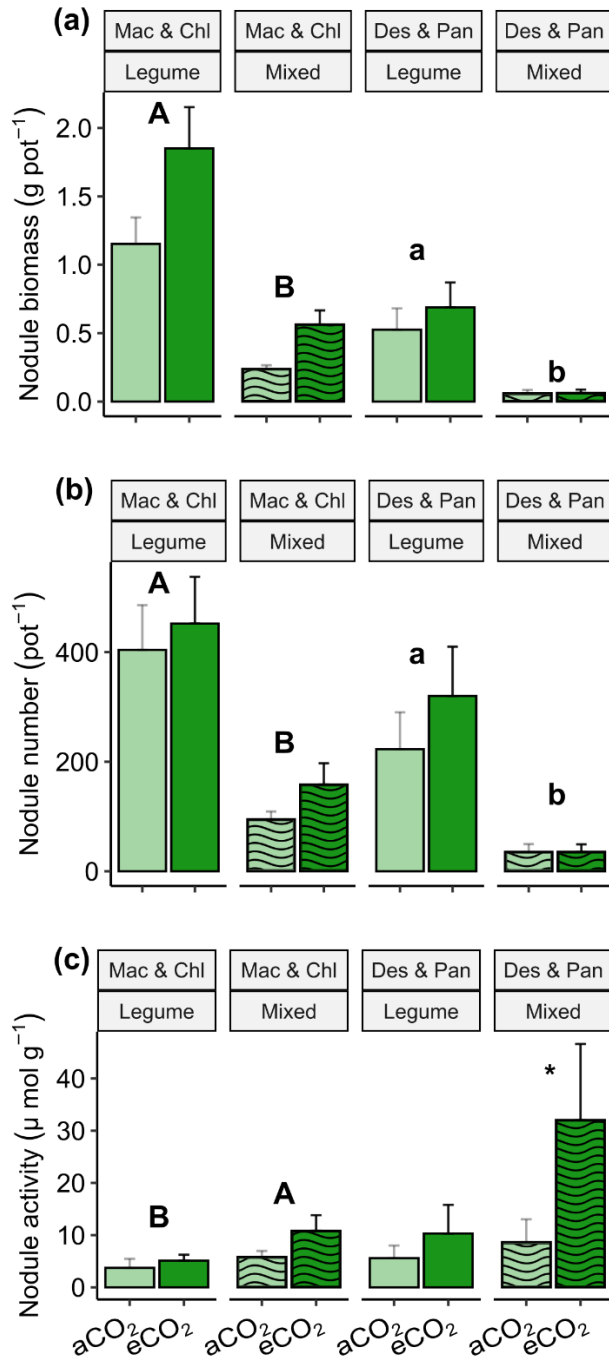


Fig 5. Effects of eCO₂ on metrics of nitrogen fixation capacity relating to root nodules for legumes grown in monoculture (Legume) and in mixtures with grasses (Mixed; *Macroptilium* and *Chloris*: Mac & Chl, *Desmodium* and *Panicum*: Des & Pan) including (a) the total biomass of nodules per pot, (b) the number of nodules per pot, and (c) root nodule activity as measured by the production of ethylene as a proxy for the rate of nitrogen fixation. Values shown are means ± 1 SE. Differences between plant types within a species pair are indicated by letters (Mac & Chl uppercase, Des & Pan lowercase) and significant differences between CO₂ treatments are indicated by ‘*’.

419 *Over-yielding and Complementarity vs. Selection Effects*

420 We found a non-significant positive response of over-yielding for both species' pairs (**Fig.**
421 **6a**). In *Macroptilium* & *Chloris* mixture, over-yielding was mainly determined by the
422 complementarity effect but not the selection effect (**Fig. 6bc**). By contrast, for the
423 *Desmodium* & *Panicum* mixture, the over-yielding was driven more by selection than the
424 complementarity effect (**Fig. 6bc**).

425

426 We also found eCO₂ significantly reduced over-yielding (**Fig. 6a**). In the *Macroptilium* &
427 *Chloris* mixture, complementarity effects shifted from neutral under aCO₂ to significantly
428 positive under eCO₂. Meanwhile, selection effects shifted from neutral under aCO₂ to
429 significantly negative under eCO₂, meaning that the dominant species had less biomass than
430 expected in the mixture (**Fig. 6c**). In the *Desmodium* & *Panicum* mixture, however, the
431 complementarity effect shifted from significantly positive under aCO₂ to neutral under eCO₂,
432 meaning that niche partitioning was weakened. At the same time, the selection effect was
433 unaffected by CO₂ (**Fig. 6c**), although values were significantly positive under both CO₂
434 scenarios, meaning that the dominant species (*Panicum*) consistently had greater biomass in
435 mixtures than proportional production would suggest from monocultures, regardless of CO₂
436 treatment. There were no significant effects of eCO₂ among the two species pairs for either
437 complementarity (CO₂: $F_{1,4} = 0.3$, $p = 0.62$; Species pair: $F_{1,44} = 1.9$, $p = 0.18$; CO₂ x Species
438 pair: $F_{1,44} = 2.1$, $p = 0.15$) or selection effects (CO₂: $F_{1,4} = 1.0$, $p = 0.37$; Species pair: $F_{1,44} =$
439 94.9 , $p < 0.01$; CO₂ x Species pair: $F_{1,44} = 2.6$, $p = 0.11$). Summing these components of the
440 net biodiversity effect supports the general outcomes of biomass over-yielding only in the
441 *Desmodium* & *Panicum* mixture, with reduced over-yielding under eCO₂ (**Fig. 6d**). There
442 were, however, no difference between the species pairs in their response to elevated CO₂
443 (CO₂: $F_{1,4} = 0.7$, $p = 0.44$; Species pair: $F_{1,44} = 3.0$, $p = 0.09$; CO₂ x Species pair: $F_{1,44} = 0.8$, p
444 $= 0.37$).

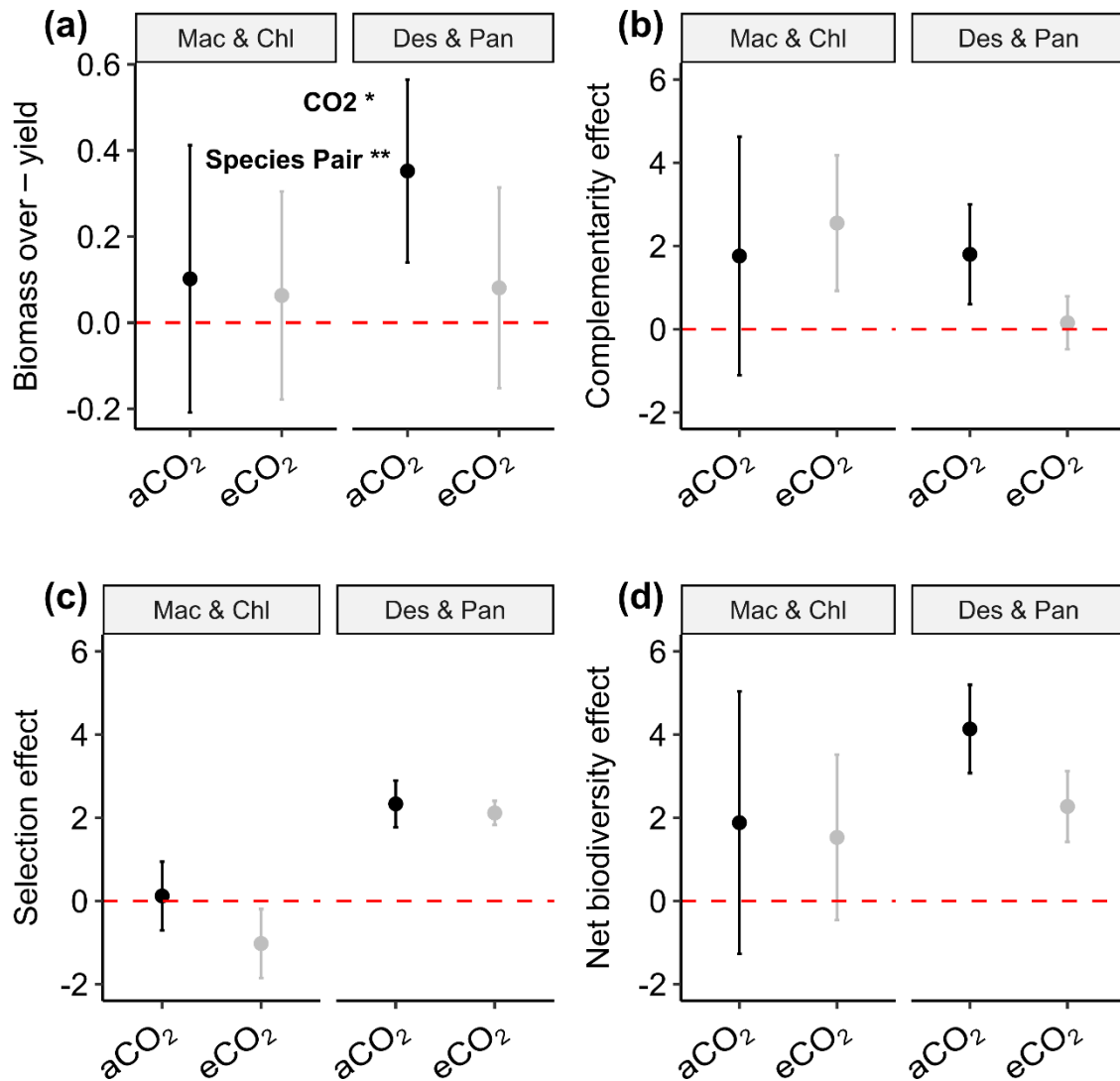


Fig. 6. Over-yield effect size for (a) aboveground productivity for pots grown with mixed grass-legume plant types in comparison with standardized summed totals for grasses and legumes grown in monoculture. Species-pair calculations for examining (b) complementarity effect, (c) selection effect and (d) the net biodiversity effect. Positive values indicate an increase relative to monoculture pots (a) or a net positive effect in interaction type between species (b-d). Non-overlapping bars indicate a significant difference from 0. Points shown are means values with 95% CI for *Macroptilium* & *Chloris* (Mac & Chl) mixtures and *Desmodium* & *Panicum* (Des & Pan) mixtures.

445

446

447 **Discussion**

448 In mixed grasslands or pastures containing both grasses and legumes, nutrient niche
449 partitioning among community members can promote species coexistence and ecosystem
450 function, particularly by influencing nutrient availability (Craven et al., 2018; da Silveira
451 Pontes et al., 2015; Fornara & Tilman, 2009). While direct-nutrient sharing is often predicted
452 among community members (Pirhofer-Walzl et al., 2012), nutrient, especially N, niche
453 partitioning is a more common finding in ecological and agricultural studies under field
454 conditions (Nyfeler et al., 2011; Pelzer et al., 2014; Zuppinger-Dingley et al., 2014). In these
455 situations, community members access different pools of available resources, over time
456 and/or space, enabling the persistence of many species by reducing competitive interactions
457 (Adler et al., 2012; Zuppinger-Dingley et al., 2014). Our results found that (1) within tropical
458 grass-legume mixtures, N niche partitioning drove plant-plant interactions such that legumes
459 increased reliance on biological nitrogen fixation (BNF) relative to soil N when grown with
460 grasses leading to increased grass growth per tiller. (2) Elevated CO₂ (eCO₂) had varied
461 impact on individual tropical forage species, with benefits in growth for one legume and no
462 direct benefit for grasses, (3) while nodule activity and N content increased for one legume,
463 we found community productivity and N content from tropical legume-grass mixtures were
464 lower under eCO₂ than aCO₂, suggesting that legume-grass pairings may benefit ecosystem
465 function to a lesser extent in the face of rising CO₂; and (4) the species-specific responses
466 associated with over-yielding and responses to eCO₂, demonstrated differences in
467 complementarity and selection effects operating between the two mixed communities and a
468 lessening of net biodiversity effects under eCO₂ associated with reduced niche partitioning
469 between the grass and legume for one species pair.

470

471 *Tropical grass-legume mixtures*

472 Relationships between plant functional group diversity and ecosystem function typically
473 predict and find that increased diversity supports greater ecosystem productivity (Hector et
474 al., 1999; Küchenmeister et al., 2012; Loreau & Hector, 2001; Mason et al., 2017; Tilman et
475 al., 2014). In testing this hypothesis, many studies have included grass-forb-legume or grass-
476 shrub-forb combinations experimentally to examine potential over-yielding benefits (Finn et
477 al., 2013), but fewer ecological studies have included C₄ grasses alongside legumes (Lee et
478 al., 2003; Lilley, Bolger, & Gifford, 2001; Rasmussen et al., 2013). This is despite early
479 results suggesting that the high competitive ability of some C₄ grasses may functionally

480 contribute to over-yielding (Lambers et al., 2004) and the economic importance of such
481 mixtures in many agricultural grasslands around the world (Butler et al., 2013; Duchene et
482 al., 2017). Previous results have shown that, at a global scale, tropical grasses show lower
483 productivity benefits from the addition of legumes, relative to temperate mixed-species
484 systems (Ashworth et al., 2018). Therefore, increased understanding of the mechanisms that
485 may promote over-yielding are of broad interest, especially in tropical systems. Our study
486 found strong evidence for productivity gains by the grass component in tropical grass-legume
487 mixtures (per tiller), accompanied by declines in the legume partner, which translated into
488 minimal changes at the pot or sward level over a short-term glasshouse experiment. This
489 result aligns with earlier tropical mixed pasture trials that concluded that the relative
490 abundance of grasses and legumes has an important impact on the transfer of N and sward
491 productivity benefits of mixtures, compared to monocultures of the component species (Baba
492 et al., 2011).

493

494 Increased productivity in mixed species grasslands, relative to monocultures, is primarily
495 attributed to increased access to resources, including light, water or nutrient availability in
496 ways that promote facilitation. This facilitation can include mechanisms of niche partitioning
497 such that different species access different resource pools in time or space to minimize
498 competition (da Silveira Pontes et al., 2015; Jumpponen et al., 2002; Thilakarathna et al.,
499 2016). Biological or environmental conditions under which different mechanisms promoting
500 nutrient facilitation may dominate are still an area of active research (Brooker et al., 2015),
501 and our study addressed the potential for niche partitioning vs. direct facilitation for N
502 between co-occurring grasses and legumes (Ajayi et al., 2008; Baba et al., 2011; Bell et al.,
503 2017; Miegoue et al., 2016). Despite only limited gains in shoot N for mixed pots, relative to
504 the grass monocultures over our short-term species interactions, this indicated niche
505 partitioning as the main mechanism promoting plant N access between the component
506 species. Under these conditions, the proportion of shoot N derived from BNF increased for
507 legumes even as the total mass of shoot N declined in comparison with monoculture pots, as
508 evidenced by one of our species pairs (*Desmodium-Panicum*). In agricultural-grassland
509 settings mixtures of grasses and legumes are often used to specifically improve forage quality
510 and production in the absence of fertilizer applications (Brooker et al., 2016; Li et al., 2015;
511 Warwick et al., 2016). This practice is predicted to play an important role in grassland

512 management for grazed systems as C₄ grasses become more dominant under changing
513 climate (Butler et al., 2013; McKeon et al., 2009; Still et al., 2019).

514

515 There are a variety of mechanisms that enable N niche complementarity in mixed-species
516 grasslands, and it is worth noting that the duration of species associations may play a role in
517 the potential for direct-facilitation of BNF-derived N between for companion grasses in
518 mixed grasslands (Heichel & Henjum, 1991; Thilakarathna et al., 2016). Indeed, a time lag
519 may be associated with the decomposition of legume leaf and root litter that can provide
520 sources for increased ecosystem N availability on longer time scales (Cannell & Thornley,
521 1998; Kohmann et al., 2019). While there was likely root turnover over the course of our
522 glasshouse experiment, and any senesced leaves were retained in pots, these mechanisms are
523 likely more substantial in field settings especially where soil disturbance may promote
524 incorporation of plant materials directly into soils or where legume residues have sufficient
525 time to undergo decomposition (Brooker et al., 2015; Thilakarathna et al., 2016).

526 Furthermore, under field conditions, legumes often shift to BNF as plants mature (Edmeades
527 & Goh, 1984; Wery et al., 1986) and this can impact the potential facilitation provided to
528 companion species (Bell et al., 2017). In our study all plants were harvested immediately
529 prior to the first flowering phenophase, and consequently observed patterns are from first-
530 year perennial plants in a rapid growth phase when individuals may rely more strongly on
531 available soil N pools. Grasses in mixed pots in our study did show some evidence for
532 incorporation of BNF-derived N into aboveground plant tissue (up to 29% of shoot N for
533 *Panicum* grown with *Desmodium*), thereby providing evidence of direct facilitation under
534 ambient conditions within this species pair. Additionally, this direct contribution is predicted
535 to increase as swards mature (Thilakarathna et al., 2016).

536

537 *Consequences of elevated CO₂*

538 Previous work has shown that eCO₂ can increase grassland and crop productivity (Shaw et
539 al., 2002; Terrer et al., 2021), or have no impact over differing time scales, and can result in
540 shifts in the abundance of component species (Bloor et al., 2010; Carroll et al., 2003; Mueller
541 et al., 2016). Under well-watered conditions, C₄ grasses are not predicted to benefit directly
542 from eCO₂ and our results follow those predictions (Ainsworth & Long, 2005; Soussana &
543 Lüscher, 2007; Wang *et al.*, 2022; but see Reich *et al.*, 2018). On the other hand, we found
544 some limited effects of eCO₂ on legume production, with increased growth in *Macroptilium*

545 likely due to increases in shoot N and root biomass. In contrast, *Desmodium* did not respond
546 to eCO₂, potentially due to a greater reliance on soil N rather than BNF in monoculture, that
547 may have induced stoichiometric constraints in responding to eCO₂. This result is in line with
548 research showing that the strongest predictor for an individual's or species' positive response
549 to eCO₂ in legumes is the ability to form nodules (Cernusak et al., 2011; Parvin et al., 2020).
550 For example, *Macroptilium* responded positively to eCO₂ and also had greater nodule
551 production than *Desmodium*, although the number of nodules was unaffected by eCO₂.
552 Indeed, despite the positive effect of eCO₂ on *Desmodium* nodule activity in mixtures, low
553 nodule numbers and biomass overall contributed to a proportional reduction in legume
554 biomass within the mixed sward.

555

556 At the sward level, despite some gain in productivity for legume biomass and total shoot N in
557 monoculture (*Macroptilium* only), we didn't find an increase in productivity for mixed pots
558 under eCO₂. While this result is in contrast to C₃ grass-legume mixtures for temperate
559 pastures in Australia where eCO₂ has been shown to increase total shoot N yield (Lilley,
560 Bolger, Peoples, et al., 2001), it is consistent with evidence for limited responses to eCO₂ by
561 C₄ grasses and legumes. One explanation may be related to shifts in the nutritional quality of
562 component species under eCO₂, as reductions in quality for aboveground biomass have been
563 reported across ecosystems (Augustine et al., 2018; Bhargava & Mitra, 2021). Our species
564 experienced limited shifts in quality based on shoot N%, including lower N in *Macroptilium*
565 when grown with *Chloris* and in *Panicum* when grown with *Desmodium*. Even so, these
566 patterns align with other studies measuring nutritional shifts in both tropical grasses and
567 legumes under low soil nutrients where legume shoot N% declined in mixture under eCO₂
568 (Edwards et al., 2006).

569

570 While the use of confined soil space to test mechanisms for the source of nutrient use and
571 acquisition between grasses and legumes is typical in glasshouse settings, there are some key
572 factors that are likely to impact the species interactions and dynamics in response to eCO₂
573 that are not captured here that are important for overarching messages in relation to nutrient
574 facilitation. Our study found that eCO₂ conditions differentially altered the shoot N%
575 between legumes in mixture but had no impact on legumes grown in monoculture. Under
576 field settings, the effects of herbivory on legumes that maintain a higher nutritive quality
577 relative to surrounding species may result in a comparative loss in abundance or persistence

578 within the community, ultimately reducing the potential over-yield or net biodiversity effect
579 (Rogers et al., 2009). Additionally, literature reviews on field studies have generally found an
580 increase in root length and biomass associated with eCO₂ in grasslands (Dieleman et al.,
581 2012; Nie et al., 2013), a pattern that was seen in pots containing *Macroptilium* in this study.
582 While our pots were not root-bound by the end of the experiment, other key differences
583 between field and glasshouse conditions may have limited further responses, for example Nie
584 et al. (2013) also found a shift in the depth distribution of roots that is not possible under non-
585 field settings. Such spatial re-distribution of roots with depth can play a major role in
586 complementarity effects between species within mixtures (Oram et al., 2018). Finally, field
587 conditions also typically introduce variation in other resources that modify the interactions
588 between eCO₂ and nutrient-use, in particular water availability. Typically, C₄ grasses have
589 been shown to benefit from eCO₂ under drier soil conditions, further altering the potential
590 interactions between grasses and legumes in mixed swards (Ainsworth & Long, 2005; Reich
591 *et al.*, 2014, but see Reich *et al.*, 2018).

592

593 *Over-yield implications under future CO₂ scenarios*

594 Reliance on different sources of N among species within mixed plant communities can
595 ultimately reduce competition especially under low nutrient availability (Ball et al., 2021;
596 Elias & Agrawal, 2021). The increased N niche partitioning between grasses and legumes
597 grown in mixture found in this study support this conclusion. Furthermore, the species pair
598 that had a substantial shift in N source use between monoculture and mixtures was also
599 associated with a significant over-yielding benefit. This suggests that greater plasticity in
600 niche space may promote species coexistence and enhance ecosystem production.
601 Belowground, the idea of nutrient form (nitrate vs. ammonium) niche plasticity supporting
602 coexistence has been well-established for cold-climate grasslands (Ashton et al., 2010) and
603 aboveground light-use plasticity has been shown to translate into increased production in
604 experimental grasslands (Meilhac et al., 2020).

605

606 Importantly, however, we found that eCO₂ reduced over-yielding for both aboveground
607 biomass and total shoot N in our tropical grass-legume mixtures. While there were no
608 statistical effects of eCO₂ on complementarity and selection effects directly, we found key
609 differences in the relative importance of these effects in contributing to the over-yield/net

610 biodiversity effect, with positive complementarity and selection effects together resulting in
611 increased sward level over-yielding that was greatest under aCO₂.

612

613 **Conclusions**

614 Plant responses to eCO₂ are largely dependent on soil water and nutrient availability.
615 Therefore, shifts in plant-plant interactions including competition (evidenced by niche
616 partitioning) or facilitation among community members may drive diverse ecosystem
617 responses to changes in CO₂ concentrations. Among our tropical pasture species, we found
618 that legumes grown in monoculture relied approximately equally on available soil N pools
619 and BNF for their N requirements. When grown with tropical grasses, however, legumes
620 shifted to rely either equally or more strongly on BNF, the latter indicative of stronger niche
621 partitioning and less successful competition for soil nutrients with grasses. This separation of
622 niche space was weakened under elevated CO₂ conditions, ultimately reducing legume
623 production and minimizing over-yielding benefits. Disentangling contributions of niche
624 partitioning and abiotic facilitation, here quantified using stable isotopes, are key to
625 understanding grass-legume contributions to ecosystem function under future climate
626 conditions.

627

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639

640 **Conflict of Interest**

641 The authors declare no conflicts of interest.

642

643 **Author Contributions**

644 Authors ACC, HZ, and GWK designed the experiment with input from SAP, EP, BM, JRP,
645 JMP, and KLMC. ACC, HZ, GWK, and KLMC performed experiments and conducted
646 glasshouse work. ACC conducted statistical analyses with input from HZ, JRP and FI; ACC
647 and HZ wrote the paper with input from all co-authors on draft iterations.

648

649 **Data Availability**

650 The data that support the findings of this study are openly available in Dryad at

651 <https://doi.org/10.5061/dryad.nk98sf7ww>.

652

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1103

Table 1. Tropical species and associated mixtures

Scientific Name*	Common Name	Variety	Growth form	Paired species
<i>Chloris gayana</i> (Kunth, 1830)	Rhodes grass	Katambora	Grass	Burgundy
<i>Desmodium intortum</i> (Mill. Urb.)	Greenleaf Desmodium	Greenleaf	Legume	Panic
<i>Macroptilium bracteatum</i> (Nees & Mart.)	Burgundy Bean	Presto	Legume	Rhodes
<i>Panicum maximum</i> var. <i>trichoglume</i> (Robyns)	Panic grass	Megamax 059	Grass	Desmodium

**Species are referenced by genera in text*

Table 2) Effects of CO₂ treatment (CO₂; ambient, elevated), species pair (SP; *Macroptilium/Chloris* and *Desmodium/Panicum*) and pot type (PT; grass, legume, mixed-species), for pot level total shoot biomass, total shoot N, total shoot N derived from soil (shoot N- soil).

Response	Factors	F Statistic*	P Value	r ² -M	r ² -C
Shoot biomass [^]	CO ₂	5.23 _{1,4}	0.08	0.40	0.45
	Species pair	49.9 _{1,143}	<0.01		
	Plant type	12.1 _{2,143}	<0.01		
	CO ₂ x SP	1.9 _{1,143}	0.17		
	CO ₂ x PT	2.5 _{2,143}	0.08		
	SP x PT	5.6 _{2,143}	<0.01		
	CO ₂ x SP x PT	1.0 _{2,143}	0.38		
	Shoot N content [^]	CO ₂	0.3 _{1,4}		
Species pair	102.7 _{1,143}	<0.01			
Plant type	79.4 _{2,143}	<0.01			
CO ₂ x SP	1.9 _{1,143}	0.18			
CO ₂ x PT	3.7 _{2,143}	0.03			
SP x PT	14.7 _{2,143}	<0.01			
CO ₂ x SP x PT	0.4 _{2,143}	0.64			
Shoot N-soil	CO ₂	0.1 _{1,4}	0.79	0.14	0.15
	Species pair	17.8 _{1,143}	<0.01		
	Plant type	2.7 _{2,143}	0.07		
	CO ₂ x SP	0.4 _{1,143}	0.54		
	CO ₂ x PT	0.1 _{1,143}	0.92		
	SP x PT	0.04 _{2,143}	0.95		
	CO ₂ x SP x PT	1.3 _{2,143}	0.27		

*Subscripts indicate degrees of freedom, [^]data were ln transformed to meet assumptions of normality. All models included growth chamber as a random effect.

Table 3) Effects of CO₂ treatment (CO₂; ambient, elevated), species pair (SP; *Macroptilium/Chloris* and *Desmodium/Panicum*) and pot type (PT; legume and mixed-species only), for pot level percent of N derived from biologically fixed N (Shoot N- BNF %; legume and mixed plant types only) and total shoot N derived from BNF (Shoot N- BNF content).

Response	Factors	F Statistic*	P Value	r²-M	r²-C
Shoot N- BNF %	CO ₂	0.03 _{1,4}	0.87	0.44	0.45
	Species pair	59.0 _{1,94}	<0.01		
	Plant type	13.8 _{1,94}	<0.01		
	CO ₂ x SP	2.5 _{1,94}	0.12		
	CO ₂ x PT	2.1 _{1,94}	0.15		
	SP x PT	2.8 _{1,94}	0.10		
	CO ₂ x SP x PT	3.3 _{1,94}	0.07		
Shoot N- BNF content [^]	CO ₂	0.1 _{1,4}	0.80	0.59	0.63
	Species pair	132.3 _{1,94}	<0.01		
	Plant type	30.8 _{1,94}	<0.01		
	CO ₂ x SP	0.7 _{1,94}	0.39		
	CO ₂ x PT	3.5 _{1,94}	0.06		
	SP x PT	1.0 _{1,94}	0.32		
	CO ₂ x SP x PT	0.2 _{1,94}	0.62		

*Subscripts indicate degrees of freedom, ^data were ln transformed to meet assumptions of normality. All models included growth chamber as a random effect.

Table 4) Effects of CO₂ treatment (CO₂; ambient, elevated) for pot-level total biomass of root nodules (Nodule biomass), number of root nodules present (Nodule number), and the activity of nodules at the time of harvest (Nodule activity) by species pair (SP; *Macroptilium/Chloris* and *Desmodium/Panicum*) and plant type (PT; legumes in monoculture vs mixed pots only)

Response	Factors	F Statistic*	P Value	r²-M	r²-C
Nodule biomass [^]	CO ₂	4.0 _{1,4}	0.12	0.68	0.69
	Species pair	65.6 _{1,68}	<0.01		
	Plant type	97.2 _{1,68}	<0.01		
	CO ₂ x SP	0.6 _{1,68}	0.43		
	CO ₂ x PT	0.1 _{1,68}	0.80		
	SP x PT	4.6 _{1,68}	0.04		
	CO ₂ x SP x PT	0.3 _{1,68}	0.58		
Nodule number [^]	CO ₂	0.4 _{1,4}	0.55	0.55	0.58
	Species pair	28.8 _{1,68}	<0.01		
	Plant type	72.9 _{1,68}	<0.01		
	CO ₂ x SP	0.02 _{1,68}	0.88		
	CO ₂ x PT	0.1 _{1,68}	0.8		
	SP x PT	4.0 _{1,68}	0.05		
	CO ₂ x SP x PT	0.4 _{1,68}	0.52		
Nodule activity [^]	CO ₂	4.7 _{1,4}	0.10	0.16	0.16
	Species pair	0.7 _{1,68}	0.42		
	Plant type	7.0 _{1,68}	0.01		
	CO ₂ x SP	0.6 _{1,68}	0.44		
	CO ₂ x PT	1.0 _{1,68}	0.33		
	SP x PT	0.01 _{1,68}	0.90		
	CO ₂ x SP x PT	1.9 _{1,68}	0.17		

*Subscripts indicate degrees of freedom, ^data were ln transformed to meet assumptions of normality. All models included growth chamber as a random effect.