Elevated [CO_2] concentration and nitrogen addition affects responses of foliar phosphorus fractions in invasive species to increased phosphorus supply

Running title: Allocation of foliar P fractions

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Abstract: No studies have explored how the invasive species of *Mikania micranatha* and *Chromolaena odoratan* adjust leaf phosphorus (P) among inorganic P (Pi) and organic P fractions to adapt the low soil P availability, especially under elevated CO_2 concentrations ($[CO_2]$) and nitrogen (N) deposition. Here, we address this by measuring foliar total N and P concentrations as well as functional P fractions (i.e. Pi, metabolic P, lipid P, nucleic acids P, and residual P) of both invasive species and a native species (*Paederia. scandens*) growing under different P supplies, N, and N+P addition under both ambient and elevated $[CO_2]$. Phosphorus addition greatly increased plant biomass and foliar P concentrations but did not significantly affect foliar N concentration and leaf mass per unit leaf area (LMA). In response to P addition, the concentration of metabolic P increased the most, followed by that of nucleic acid P, Pi, and lipid P, in all species by an average of 754%, 82%, 53%, and 38%, respectively. However, elevated $[CO_2]$ and N addition weakened this positive effect on concentrations of foliar P fractions in the invasive species. Our results indicate that elevated $[CO_2]$ and N addition allowed the invasive species to acclimate to a low soil P availability, supporting their successful invasion, through greatly reducing P allocation to non-metabolic foliar P fractions (phospholipids and nucleic acid P) to meet their demand for metabolic P and Pi for photosynthesis, rather than altering LMA.

Keywords: Phosphorus availability gradient, Elevated CO_2, Photosynthetic rates, Foliar P fractions, Invasive plant species

Introduction

Phosphorus (P) is an essential plant nutrient and often present in soil at concentrations that limit net primary productivity (Hidaka and Kitayama, 2013; Mo et al., 2019). In subtropical forests, plant productivity is generally limited by a low availability of soil P, rather than by a low availability of nitrogen (N) due to the long-term weathering of bedrock and the gradual loss of P (Walker and Syers, 1976; Jonard et al., 2015; Mo et al., 2019). While P is often limiting, N is increasingly available in subtropical forests because of atmospheric N deposition, which has increased to ~ 30-50 kg N ha $year^{-1}$ in subtropical forests in China (Mo et al., 2006; Luo et al., 2019). Another factor that may greatly affect plant growth is the atmospheric concentration of CO_2 ($[CO_2]$), which has increased from ~280 μmol mol^{-1} in 1840s to ~ 410 μmol mol^{-1} in 2020 (IPCC, 2013; Luo et al., 2019; https://www.co2.earth/). However, the effects of increases in N deposition and atmospheric $[CO_2]$ on the strategies that plants have evolved to use P efficiently in P-impoverished forests are rarely documented in invasive species (Campbell and Sage, 2006; Lewis et al., 2010; Tissue and Lewis, 2010). Invasive species threaten plant diversity (Dukes and Mooney, 1999), and can potentially alter the function and structure of terrestrial ecosystems (Li et al., 2002; Tang et al., 2007; Song et al., 2009; Sage, 2019). Understanding how the strategies of allocation of foliar P for maintaining plant productivity in invasive species is affected by N deposition and elevated $[CO_2]$ would increase the ability to predict and perhaps control plant invasions in tropical P limited forest ecosystems (Song et al., 2009; Wang et al., 2016).

Regulating P allocation to leaves is a vital strategy in plants to acclimate to soil

conditions (e.g., low soil P availability) (Zhang et al., 2018; Wang et al., 2019) and climate change (e.g., elevated [CO_2] and N deposition) (Tissue and Lewis, 2010). Foliar P can be fractionated into inorganic phosphate (Pi) and organic P fractions (metabolic P, lipid P, nucleic acid P and residuals P) (Hidaka and Kitayama, 2013). Pi represents a significant fraction of leaf P, and is generally stored in the vacuole when a plant is unable to acquire adequate Pi from the soil (Veneklaas et al., 2012). The foliar metabolic P fraction consists mainly of intermediates of carbon metabolism, such as bioactive-molecular compound (e.g., phosphorylated sugars, ADP and ATP). The major organic P fraction, i.e. nucleic acid P, often represents more than 50% of the foliar organic P pool; up to 85 % of nucleic acid P consists of rRNA, which is essential for protein synthesis (Matzek & Vitousek, 2009). Lipid P comprises phospholipids, most of which are components of the plasmalemma and organelle membranes (Veneklaas et al., 2012). Finally, the uncharacterized residual fraction may include phosphorylated proteins, some of which regulate cellular processes (Yan et al., 2019). Despite studies on allocation of leaf P fractions following N or P addition (Mo et al., 2019) and in different soil condition (e.g., soil age; Yan et al., 2019), there is little information concerning the interactive effects of elevated [CO_2], N addition, and low soil P availability on the allocation of P to the five foliar P fractions in invasive species (Song et al., 2009; Tissue and Lewis, 2010; Zhang et al., 2016).

Under P deficiency, photosynthesis is generally reduced, due to feedback inhibition resulting for reduced leaf growth (Zhang et al., 2016) or the limitation of orthophosphate (Pi) in the cytosol (Mo et al., 2019). These decreases in photosynthetic activity might increase

85 photosynthetic N-use efficiency (PNUE) and photosynthetic P-use efficiency (PPUE), and
86 also decrease the leaf mass per unit leaf area (LMA; Ghannoum et al., 1999). Plant grow in
87 low soil P availability can reduce their overall need for foliar P by decreasing metabolic P
88 fractions, and buffer direct Pi restriction of photosynthesis (Hadiaka & Kitayama, 2011;
89 Warren, 2011). Moreover, the replacement of phospholipids (lipid P) in membranes by
90 sulfolipids and galactolipids to maintain foliar metabolic P concentration in P-deficiency soil
91 (Lambers et al., 2012; Veneklass et al., 2012). For invasive plants growing in soils with low P
92 availability, however, how they adapt the low soil P availability under elevated [CO_2] and N
93 addition remain unclear.

94 Since their invasion of southern China in the 1980s, *Mikania micranatha* and
95 *Chromolaena odorata* have caused serious damage to secondary forests and crops (Li and
96 Xie, 2002; Song et al., 2009). The rapid spread of both invasive plants has triggered a serious
97 decline in the diversity of native species in terrestrial ecosystems (D'Antonio et al., 2004;
98 Bradley et al., 2010). The photosynthetic rate is faster in invasive species than in co-occurring
99 native species (Baruch and Goldstein, 1999; Deng et al., 2004: Song et al., 2009). Relative to
100 native species, invasive species generally have greater phenotypic plasticity, are more tolerant
101 to environmental change, such as elevated [CO_2], N deposition, or low soil P availability
102 (Alpert et al., 2000; Geng et al., 2006; Feng et al., 2007; Tissue and Lewis, 2010).

103 The objectives of this study were: 1) to determine how the invasive plants *M. micrantha*
104 and *C. odorata* respond to low P availability (in terms of P allocation to leaves and related
105 foliar traits) in order to maintain photosynthetic rates and 2) to determine how those

106 responses are affected by elevated [CO_2], and N deposition. To accomplish these objectives,
107 we conducted an open-top field chamber experiment. We hypothesized that (1) foliar traits
108 (i.e. LMA and N and P concentrations) and the photosynthetic capacity of the invasive
109 species would increase with increasing P-application rate, and that these increases would be
110 greater with elevated [CO_2] than with N addition; (2) the increase in photosynthetic capacity
111 in response to P and N addition under elevated [CO_2] would be more pronounced in invasive
112 species than in a native species; and (3) elevated [CO_2] and N addition would change the
113 pattern of allocation of P to foliar P fractions for photosynthesis and thereby allow the
114 invasive plants to maintain plant growth in a soil with low P availability.

115 **Materials and methods**

116 **Site description**

117 The open-top field chamber experiment was conducted at South China Botanical Garden
118 (23°08´N, 113°17´E), located in Guangzhou Province, China. The region has a subtropical
119 monsoon climate (Zhang et al., 2016; Luo et al., 2019) with a mean annual precipitation of
120 1750 mm, a mean annual temperature of 21.5 °C, and a mean relative air humidity of 77%
121 (Zhang et al., 2016).

122 **Experimental design**

123 The experiment included two widespread invasive species, i.e., *M. micranatha* and *C.*
124 *odorata*. For comparison, the experiment also included a native species that has a similar

125 morphologies as the invasive species, i.e. *Paederia scandens*. *M. micrantha*, *C. odorata* and *P.*
126 *scandens* were collected in South China Botanical Garden.

127 Seedlings were initially grown under suitable soil water and light conditions in a nursery.
128 Seedlings of similar size (about 100 mm tall) were then transplanted into pots (one seedling
129 per pot) that were 280-mm tall and 320-mm in diameter and contained 20 kg of soil. The soil
130 had been collected at 0-400 mm depth from a primary broadleaf forest in South China
131 Botanical Garden; the soil was mixed before it was transferred to the pots. The soil chemical
132 properties (means ± SE) before treatments were: pH= 5.0±0.05; organic C = 16.1±0.6 mg g^{-1};
133 total N = 1.9±0.04 mg g^{-1}; total P = 0.35±0.02 mg g^{-1}; NH_4-N = 30±3.1 mg kg^{-1}; and NO_3-N
134 = 8.1±0.2 mg kg^{-1}. Each species was represented by 120 pots.

135 The experiment used 12 open-top chambers. Six of the chambers were “new”, i.e., they
136 were constructed in January 2016, and were cylindrical, 3.5 m in height, and 5.0 m in
137 diameter. The other six open-top chambers were “old”, i.e., they were constructed in 2012,
138 and were cylindrical, 3.5 m in height, and 3.4 m in diameter. Details on the construction of
139 chambers and on the method of supplying CO_2 were described previously (Zhang et al.,
140 2016).

141 On 18 June 2016, uniform and healthy seedlings of each species were selected and
142 assigned to each chamber; each chamber contained 18 pots (for the narrow chambers) or 24
143 pots (for the wide chambers) of each species (Fig. S1). On 2 July 2016, two old and four new
144 open-top chambers were exposed to elevated [CO_2] (700±50 μmol mol^{-1}), and the other six
145 chambers were exposed to ambient [CO_2] (400±50 μmol mol^{-1}) (Fig. S1). We detected no

146 significant difference in the light, temperature, or moisture conditions between the old and
147 new chambers. For each species in each wider chamber, four pots were not treated with P or
148 N and were used as controls; four pots were treated with N (6.25 g N m^{-2} yr^{-1}); four pots each
149 were treated with P fertilizer at rates of 0.75 (1/2P), 1.5 (1P), or 3.0 (2P) g P m^{-2} yr^{-1}; and four
150 pots were treated with both N and P (6.25 g of N m^{-2} yr^{-1} + 1.5 g of P $m^{-2}yr^{-1}$). In total, there
151 were 12 conditions: three levels of P addition, one level of N addition, one level of N+P
152 addition, the control, and two levels of CO_2 for each of the previous six conditions. For each
153 species in each new chamber, the same treatments were applied to three rather than to four
154 pots per species. Chemically pure NH_4NO_3 was used as the N source, and chemically pure
155 $NaH_2PO_4 \cdot 2H_2O$ was used as the P source (Guangzhou Chemical Reagent Factory, Guangzhou,
156 China). The solutions of N, P, or NP were sprayed on the soil surface of each pot.

157 **Measurement of foliar gas exchange**

158 Healthy sun-exposed mature leaves were chosen for foliar gas exchange measurement
159 from 9: 00 am to 12: 00 am during 11 days in October 2016. For each gas exchange
160 coefficient, at least six individuals for each combination of species and treatment were
161 measured. Following the order of photosynthetic photon flux density (PPFD) 1200, 1000, 800,
162 500, 300, 200, 120, 50, 20, 0 μmol m^{-2} s^{-1}, photosynthetic light-response curves were made.
163 When the measurements were conducted, the vapor pressure deficit was set at 2.0±0.5 kPa,
164 and leaf temperature at 30±1 ^{o}C. The nonrectangular hyperbola model of Thornley (1976)
165 was used to calculate the maximum light-saturated photosynthetic rate.

166 **Measurement of foliar structural traits**

167 After leaf gas exchange was measured, the leaf area of each projected leaf was
168 determined using a leaf area meter (LI-3100C; LI-COR Biosciences, Nebraska, USA); these
169 leaves were then collected and oven-dried at 65 □ to a constant weight for calculating the leaf
170 mass per area (LMA). The remaining mature leaves on the sampled branch of each plant were
171 freeze-dried and ground (after main veins and petioles were removed) to determine their
172 foliar N and P concentrations, and the concentrations of foliar P fractions. Foliar N and P
173 concentrations were measured by a colorimetric assay after sulfuric acid (H_2SO_4) digestion
174 (Sommers et al., 1970; Carter and Gregorich, 2007; Liu et al., 1996).

175 PPUE and PNUE were calculated as the ratio of the maximum photosynthetic rates per
176 unit P or N. Photosynthetic capacity was expressed on a leaf area and dry mass basis.
177 Finally，the plants were harvested, and divided into roots, stems and leaves, then dried and
178 weighed to calculate the biomass.

179 **Measurement of leaf P fractions**

180 Foliar P is generally divided into inorganic P (Pi) and organic P (metabolic P, lipid P,
181 nucleic acid P, and residual P). Organic P fractions were sequentially extracted (Hidaka and
182 Kitayama, 2013) following methods of Kedrowski (1983) and Close & Beadle (2004). Foliar
183 Pi was extracted by the acetic-acid extraction method (Yan et al., 2019), and determined
184 using a molybdenum blue-based method (Ames, 1966). The four fractions of organic P were

185 determined in the following steps. First, a 0.5-g subsample of a freeze-dried and ground foliar
186 sample was homogenized with 15 ml of 12: 6: 1 CMF (chloroform, methanol, and formic
187 acid, v/v/v) in a 50-ml centrifuge tube (first tube). The liquid was extracted twice with a total
188 of 19 ml of 1: 2: 0.8 CMW (chloroform, methanol, water, v/v/v), and added with 9.5 ml of
189 chloroform-washed water. The final solvent was 1:1: 0.9 CMW (v/v/v), which caused the
190 extract to separate into a sugar-and nutrient-rich upper layer and a lipid-rich organic bottom
191 layer. The upper layer in the second tube was transferred to a new tube (the third tube), and
192 the bottom layer was used to determine lipid P.

193 A 5-ml volume of 85% methanol (v/v) was added to the material in the third tube, which
194 was then placed in a vacuum dryer for 48 h to remove dissolved chloroform and methanol.
195 The aqueous layer was refrigerated (4 ^{o}C) for 1 hr, and 5 % trichloroacetic acid (TCA)
196 solution was made through adding 1 ml 100 % (w/v) TCA. A 10-ml volume of cold 5 % (w/v)
197 TCA was then added to the tube. After 1 hr, the material in the tube was shaken for 1 hr and
198 then centrifuged at 3000 g for 10 min. The supernatant was prepared for the determination of
199 the sum of Pi and metabolic P. We subtracted Pi from the sum to obtain the metabolic P.

200 Finally, the remaining residue after extraction of the cold TCA was mixed with 35 ml
201 2.5 % TCA (w/v), and extracted for 1 hr at 95 ^{o}C in a hot water bath. Aliquots were
202 centrifuged at 3000 g for 10 min, and taken for analysis of nucleic acid P. The residue
203 remaining from the hot TCA final extraction was the residual P fraction. The determination
204 method of all foliar P fractions was similar to that of foliar total P, and the quantity of the
205 fractions were expressed on a dry mass basis.

206 **Data analyses**

207 The foliar N to total P ratio or the foliar N to P fraction ratios were calculated based on
208 mass. The effects of species, N addition, P addition, elevated [CO_2] and their interactions on
209 foliar P fractions and foliar traits were assessed by multi-way ANOVA with species, N and P
210 addition, and elevated [CO_2] as fixed factors. One-way analyses of variance (ANOVAs) were
211 used to compare the effects of treatments on LMA, PPUE, PNUE, N: P ratios, and the
212 concentrations of N, P, and P fractions. Relationship between plant biomass and concentration
213 of foliar P fractions, total P was conducted with Regression linear analysis. Means were
214 compared with LSD test at significance level of 0.05. SPSS 19.0 (SPSS. Inc., Chicago, IL.,
215 USA) was used for statistical analyses, and figures were drawn with Origin 2015 (Origin Lab.
216 Inc., Massachusetts, USA).

217 **Results**

218 **Photosynthetic capacity (A_{area} and A_{mass}) and leaf mass per area (LMA), and plant**
219 **biomass**

220 Elevated [CO_2], N addition, species, and their interactions significantly affected
221 photosynthetic capacity (A_{area}), but P addition did not significantly affect photosynthetic
222 capacity (A_{area} and A_{mass}) in invasive and native species (Table 1). Elevated [CO_2], N addition,
223 species, and P addition significantly affected plant biomass, but their interactions did not
224 significantly affect plant biomass (Table 1). 2P addition significantly increased plant biomass

of *M. micrantha*, *C. odorata*, *and P. scandens* by 8, 43, and 28%, respectively (Fig. 1). Elevated [CO_2] significantly increased A_{area} and A_{mass} in the invasive species (*M. micrantha* and *C. odorata*) by 17-101% and 5.5- 97%, respectively (Fig. 2d, e, g, h); however, elevated [CO_2] did not significantly affect A_{area} or A_{mass} in the native species (*P. scandens*) (Fig. 2f, i). N addition also significantly increased A_{area} and A_{mass} in *M. micrantha* and *C. odorata*, by 35-38% and 2.8-41%, respectively, under elevated [CO_2] (Fig. 2d, e, g, h), but did not significantly affect A_{area} or A_{mass} in *P. scandens*. Elevated [CO_2], N addition, and P addition did not significantly affect LMA in *M. micrantha* or *C. odorata*, while 2P addition increased LMA significantly more than 1P addition in *P. scandens* (Table 1 and Fig. 2a, b, c).

PPUE and PNUE

The interaction of species, elevated [CO_2], N addition, and P addition significantly affected PNUE and PPUE (Table 1). On average, elevated [CO_2] increased PNUE and PPUE by 62% and 51%, respectively, in *C. odorata*, and by 79% and 41%, respectively, in *P. scandens* (Fig. 3b, c, e, f). Elevated [CO_2] significantly increased PPUE in *M. micrantha* (by 73%), but only when combined with 2P addition (Fig. 3d). N addition did not significantly affect PNUE in the invasive species, but increased PPUE in *M. micrantha* (Fig. 3a, b, d, e). Under elevated [CO_2], 1/2P, 1P and 2P addition significantly increased PNUE by 24%, 55% and 57%, respectively, in *C. odorata*, and 2P addition significantly increased PNUE by 34% in *P. scandens* (Fig. 3a, b, c). However, PPUE significantly decreased in all species as the quantity of P added was increased further. 2P addition significantly decreased PPUE by 25%

in *C. odorata* and by 32% in *P. scandens* under elevated [CO_2] (Fig. 3d, e, f).

Foliar P and N

Species and its interaction with elevated [CO_2], N addition, and P addition significantly affected foliar P and N concentrations (Table 1). N addition significantly increased the foliar N concentration, but slightly decreased the foliar P concentration (Fig. 4). Under elevated [CO_2], 2P addition significantly decreased N concentrations by 15% in *C. odorata* and by 13% in *P. scandens* (Fig. 4a, b, c). Foliar P concentrations significantly increased with increases in the amount of P added in *M. micrantha* and *C. odorata*, and the increases were greater for the invasive species than for *P. scandens*; this effect of added P was slightly reduced by elevated [CO_2]. 2P addition significantly increased foliar P concentrations by 50 and 96% in *M. micrantha* and *C. odorata*, respectively, and by 58.3% in *P. scandens* (Fig. 4d, e, f).

Foliar P fractions and N:P ratios

Overall, both species and P addition significantly affected foliar P fractions, i.e. Pi, metabolic P, nucleic P, lipid P and residual P (Table 1). N addition slightly decreased the concentrations of all foliar P fractions in all species (Fig. 5). In *M. micrantha* and *C. odorata*, Pi (Fig. 5a, b), metabolic P (Fig. 5d, e), nucleic acid P (Fig. 5g, h) and lipid P (Fig. 5j, k) were significantly increased by an average of 53, 754, 38, and 82%, respectively, by 2P addition, but residue P was not significantly increased by P addition (Fig. 5m, n). In *P. scandens*, foliar

264 P fractions significantly increased with the amount of P added from 1/2P to 1P, but tended to
265 decrease with 2P addition (Fig. 5f, i, l, o). In response to P addition, metabolic P increased the
266 most, followed by nucleic acid P, Pi, and lipid P in all species, while residual P did not
267 respond in a consistent pattern to P addition. Elevated [CO_2] slightly weakened the enhancing
268 effect of P addition on foliar P fractions in all species (Fig. 5). The concentration of foliar Pi,
269 metabolic P, nucleic P, lipid P and residual P are all significantly and positively correlated
270 with plant biomass, suggested that plant biomass increased with the increase of the
271 concentration of foliar P fractions (Fig. 6).

272 As the amount of P added was increased, the foliar N: P ratios, foliar N: phosphate ratios,
273 foliar N: metabolic P ratios and foliar N: lipid P ratios decreased more in *M. micrantha* and *C.*
274 *odorata* than in *P. scandens* under ambient [CO_2] (Table 2). Under ambient [CO_2], however,
275 the foliar N: residue P ratio did not significantly differ among 1/2P, 1P, and 2P treatments in
276 any species, except between 1/2P and 1P in *C. odorata* (Table 2). Elevated [CO_2]
277 significantly decreased the foliar N: phosphate ratio, foliar N: lipid P ratio, and foliar N:
278 residue P ratio in *C. odorata* (Table 2). In the invasive species, the concentrations of foliar P
279 fractions (phosphate, metabolic P, nucleic P, lipid P and residual P) were positively correlated
280 with A_{mass}, N concentration, and P concentration, but negatively with LMA, PPUE, and N: P
281 ratios (Table 3).

282 **Discussion**

283 Phosphorus addition significantly increased the plant biomass of *M. micrantha* and *C.*

odorata, which was consistent with previous studies that P limitation of plant growth occurred in subtropical forest ecosystems (Hidaka and Kitayama, 2013; Hou et al., 2020). Recent studies, however, the invasive species *M. micrantha* and *C. odorata* in a soil with low P availability under ambient conditions of [CO_2] and N deposition still maintain high plant biomass, and rapid invasion in the forest ecosystem (Tang et al., 2007; Song et al., 2009; Zhang et al., 2016). In the present study, we found that changes in allocation of foliar P fractions after P addition which may help explain how invasive species can maintain their photosynthetic capacity and therefore possibly their invasiveness in soils with low P availability under conditions of elevated atmospheric [CO_2] and N deposition.

P addition affected foliar traits and photosynthetic capacity under the combination of elevated [CO_2] and N addition

Although we observed no significant increase of foliar N in response to P addition in any of the studied species, we found a marked increase in response to P addition in foliar P concentration and plant biomass in the two invasive species, and this increase was greater than that in the native species. These results indicated that P addition alleviated the P limitation of plant growth (Buenemann et al., 2011; Novriyanti et al., 2012). However, N and P addition did not significantly affect photosynthesis under ambient [CO_2], including that photosynthesis were not limited by N or P in ambient [CO_2] (Dissanayaka et al., 2018). Elevated [CO_2] did not significantly affect LMA or concentrations of foliar N or P, The most likely explanation for this is that plants that experienced increased A_{area} and A_{mass} under

elevated [CO_2] could not use the newly fixed carbohydrates for new growth (Presoctt et al., 2020). In our study, A_{area} and A_{mass} increased with an increase in P supply under elevated [CO_2] and N addition in the invasive species, consistent with previous studies that elevated [CO_2] and N deposition generally increased photosynthetic capacity (A_{area} and A_{mass}) even in low-P soils (Campbell and Sage, 2006), and consequently increased plant growth (Zhang et al., 2016). P addition can alleviate P limitation for plant growth and can increase photosynthesis under elevated [CO_2], consistent with our first two hypotheses. With increasing soil P availability under elevated [CO_2], the invasive plants showed a remarkable increase in foliar P concentrations, and provided enough metabolic P for intermediates of nucleotide and carbon metabolism for photosynthetic rates (Hidaka and Kitayama, 2013; Mo et al., 2019). We also found that the interaction of N and P addition under elevated [CO_2] increased the photosynthesis to a greater degree in invasive species than in the native species.

Plants in P-deficient soils also change foliar PNUE and PPUE to maintain their growth (Zhang et al., 2016; Mo et al., 2019). In our study, the interaction of P addition and elevated [CO_2] significantly increased PNUE, and N addition did not significantly increased plant biomass of all species, which both indicated that the soil even without N addition contained sufficient N to support increased growth. In our study, PNUE was higher in invasive than in native species despite insignificant differences in foliar N concentration in treatments that differed in quantities of P added under elevated [CO_2], probably due to the higher photosynthetic capacity (A_{area} and A_{mass}) of invasive species than native species. We also found that PPUE in invasive species greatly decreased with increasing rates of P addition, but

increased with N addition and elevated [CO_2], indicating that plants experiencing low levels of P use P more efficiently than plants experiencing adequate levels of P, and that soil P availability affected PPUE more in invasive species than in native species. The results were in agreement with a previous study, which found that PPUE decreased in response to increasing soil P availability, because the accumulation of P in plants was not adequately be used (Hidaka and Kitayama, 2011).

P addition affected foliar P fractions under the combination of elevated [CO_2] and N addition

In our study, the foliar N: total P and N: P fractions ratios were >20, and plant biomass increased with the increasing amount of P application in the invasive species, indicating that P limitation occurred in untreated plants (Güsewell, 2004; Tang et al., 2007; Mo et al., 2019). P addition increased plant biomass by increasing foliar P fractions (Pi, metabolic P, lipid P and nucleic acid P), and decreased foliar N:P ratios, which might promote the photosynthesis and growth of invasive species by increasing the amount of foliar P available for synthesis of rRNA and membrane phospholipids (Reef et al., 2010).

Changes in the foliar N:P ratio has previously been associated with physiological growth strategies in both invasive and native species (Hidaka and Kitayama, 2011). Alleviation of P addition was greater in the two invasive species than in the native species and involved increases in foliar P concentrations in the invasive species. This effect, however, was weakened by elevated [CO_2] and N addition, which was consistent with a previous finding

346 that elevated [CO_2] and N addition increased the foliar N: P ratio and exacerbated plant P
347 limitation (Zhang et al., 2016). Therefore, P addition significantly decreased foliar N:P ratios,
348 and increased plant biomass.

349 The shifts in the foliar P fractions with increasing amounts of P addition under conditions
350 of elevated [CO_2] and N addition also provide clues to the underlying adaptive mechanisms
351 that explain the success of invasive species (Hidaka and Kitayama, 2011). Invasive plants
352 may regulate the balance between the levels of phosphorylated intermediates and inorganic P
353 in order to maintain photosynthetic rates when soil P availability is deficient (Wang et al.,
354 2019). In our study, although P addition increased the concentrations of P fractions
355 concentrations of invasive species, P addition did not affect the LMA. These results indicate
356 that P addition greatly changed foliar P allocation, rather than LMA so as to maintain stable
357 photosynthetic rates when plant growth is limited by phosphorus. Plants store a large amount
358 of Pi in their vacuoles, which could provide sufficient triose phosphates for chloroplasts and
359 photophosphorylation for plant photosynthesis (Mo et al., 2019). In the current study, P
360 addition significantly increased foliar Pi (the largest proportion of foliar P) for *M. micrantha*
361 and *C. odorata*; among foliar P fractions, the increase was not highest for leaf Pi, because Pi
362 in the leaf is generally diverted to other P fractions for photosynthesis to meet the demand of
363 plant growth (Ostertag, 2010). The concentration of the metabolic P fraction in all species
364 was low but increased more than the other P fractions in response to P addition. The largest
365 increase in metabolic P indicated that plant metabolic intermediates (e.g., phytate) increased
366 in response to P addition, which consequently increased carbon metabolism and nucleotides

(Ostertag, 2010; Veneklaas et al., 2012).

Consistent with our third hypothesis, the increases in Pi and metabolic P fractions with the increasing amounts of P addition were greater in the invasive species than in native species, but elevated [CO_2] and N addition slightly weakened this effect. A possible explanation is that the Pi and metabolic P fractions are transformed into membrane phospholipids under P addition, and that this transformation is weakened by elevated [CO_2] and N addition (Lambers et al., 2015). Previous studies found that the status of Pi in the cytosol strongly affected photosynthetic rates (Schachman et al., 1998), and that the interaction of elevated [CO_2] and N addition supported stable photosynthetic rates by maintaining Pi and metabolic P in invasive species in P-poor soils (Lambers et al., 2015). Other studies have reported that, to maintain photosynthesis when soil P availability is low, plants can use P from lipids or nucleic acids to maintain foliar P in the form of Pi and metabolic P in the cytosol (Ostertag, 2010; Mo et al., 2019; Prodhan et al., 2019). In contrast, we observed that P addition increased the concentrations of all foliar P fraction, and that the increases were greatest for metabolic P and Pi in plant cell. These results further indicate that invasive plants can alter the balance between foliar Pi or metabolic P and other fractions (lipid P and nucleic P) in order to maintain a high photosynthetic capacity in soils with low P availability.

Relationships between foliar traits and P fractions

In our study, the concentrations of foliar P fractions (Pi, metabolic P, lipid P, and nucleic

387 acid P) were negatively correlated with LMA under conditions of different levels of P, N, and
388 [CO_2]. When growing in soil with low P availability in subtropical forest, P-limited invasive
389 plants develop thick and tough leaves with a high LMA in order to prolong leaf life span
390 (Ellsworth and Reich, 1996; Hidaka and Kitayama, 2011). Increased LMA may lead to a
391 decrease in photosynthetic capacity (A_{area} and A_{mass}) because of the increased resistance to
392 CO_2 diffusion. In the current study, the decrease in the foliar P concentration for invasive
393 plants growing in soil without P addition was associated with an increase in LMA and seemed
394 to indicate a reduced demand for P. PPUE was negatively correlated with structural P for the
395 invasive species growing in soil with low P availability, which indicated that the invasive
396 species were able to slightly increase LMA without sacrificing PPUE by decreasing
397 concentrations of foliar P fractions (Pi, metabolic P, lipid P, and nucleic acid P), as suggested
398 by Mo et al., (2019).

399 The results in this study were used to develop a conceptual framework for the
400 mechanism of P maintenance in low P soil availability under elevated CO_2 and N addition
401 (Fig. 7). Elevated CO_2 and N addition exacerbated the P demand of invasive species, which
402 was indicated by increased ratios of foliar N:P and foliar N: fractions P, and decreased foliar P
403 concentration after elevated CO_2 and N addition. Therefore, the transformation of
404 non-metabolic P (lipid P and nucleic acid P) to metabolic P and phosphate was enhanced.
405 This pathway was essential to meet the increased P requirement for the growth of invasive
406 species in low soil P availability under elevated CO_2 and N addition in the subtropical forest
407 ecosystem.

Conclusions

In the current study, elevated [CO_2] more than N addition allowed invasive plants to adjust their foliar traits and acclimate to low soil P availability; the acclimation was substantially greater in the two invasive species than in the native species. Plant biomass significantly increased under P addition, and the foliar N: P ratio >20 of the invasive species indicated P limitation of its growth. Rather than decreasing their LMA, the invasive species acclimated to low soil P availability under elevated [CO_2] and N addition by greatly reducing their allocation of P to non-metabolic foliar P fractions (nucleic acid P and lipid P); conversely metabolic P and Pi were not reduced, and this may have allowed maintenance of a high photosynthetic capacity. These adaptive responses help explain the success of invasive plants under conditions of rising atmospheric [CO_2] and N deposition in soil with low P availability. This knowledge may prevent them from rapid proliferation with global climate changes.

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Author's contributions

L.Z. and D.W. conceived and designed the research; L.Z., G.Z., N.L., X.Z. and M.X. performed the research; L.Z. and X.L. analyzed and interpreted the data; L.Z. and X.L. wrote the paper, H.L. revised the manuscript.

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Table 1 Effects of species (S), phosphorus (P) addition, elevated [CO_2], nitrogen (N) addition, and their interaction on the foliar traits of invasive and native species as determined by multi-way ANOVA (*F* values are shown in the table). A_{area} is photosynthetic rates per unit area; A_{mass} is photosynthetic rate per unit mass; LMA is leaf mass per unit area; PNUE is photosynthetic N-use efficiency and PPUE is photosynthetic P-use efficiency; [N] is N concentration, [P] is P concentration.

Fixed effect	A_{area}	A_{mass}	LMA	PNUE	PPUE	[N]	[P]	N:P ratios	Phosphate	Metabolic P	Nucleic acid P	Lipid P	Residual P	Biomass
S	**39.5**	**134**	**97.5**	**36.4**	2.8	**128**	**343**	**16.3**	**212.9**	**57.8**	**190**	**157**	**32.9**	**61.4**
P	0.9	1.5	**9.1**	**14.6**	**32.8**	2.4	**55.4**	**55.0**	**13.4**	**14.8**	**10.3**	**57.3**	**3.9**	**14.4**
CO_2	**147**	**41.1**	**5.6**	**36.4**	2.8	1.6	3.7	**11.2**	0.3	**8.5**	**15.4**	1.4	**6.6**	**27.6**
N	**48.0**	**12.1**	0.1	1.4	**26.0**	**27.4**	3.4	**20.4**	0.6	2.7	0.02	0.4	2.5	**37.0**
S × P	0.6	1.1	**4.4**	1.4	**4.5**	**3.8**	**3.7**	**7.6**	1.6	**4.5**	1.3	**3.9**	**2.9**	1.7
S × CO_2	**3.2**	0.6	**5.8**	**8.8**	**19.4**	**5.6**	**11.6**	**5.7**	**24.4**	0.8	**3.8**	0.8	**6.5**	0.9
CO_2 × P	1.3	1.5	0.8	**6.0**	**9.9**	0.5	**5.0**	**6.8**	2.2	2.9	2.4	**5.9**	**2.0**	2.4
S × P× CO_2	1.4	1.0	**4.6**	2.1	**2.5**	0.6	0.7	1.6	1.1	1.6	1.5	1.7	**4.8**	0.3
S × P× CO_2×N	**4.1**	2.5	**4.5**	**11.9**	**15.6**	1.0	0.3	0.4	0.2	1.1	0.8	**3.8**	**7.6**	1.2

Significant *F* values (p<0.05) are in bold.

Table 2 Ratios of total foliar nitrogen (N) to phosphorus (P) in P-containing leaf fractions.

Species and parameters		Ambient CO_2						Elevated CO_2					
		control	1/2P	1P	2P	N	NP	control	1/2P	1P	2P	N	NP
M.	Foliar N : total P	16.2±2.0ab	18.5±0.5a	14.6±0.8bc	11.6±0.8c	21.4±1.0c	15.7±1.9b	13.6±0.3a	15.4±1.4a	14.3±1.6a	14.4±1.7a	19.5±1.5b	15.5±1.1a
micrantha	Foliar N : phosphate	72.3±11.3a	74.5±8.4a	70.1±3.1a	63.9±6.8a	104.2±2.9b	66.8±12.5a	67.6±5.8	67.3±4.1	57.2±9.4	65.2±11.4	66.0±9.5*	52.5±4.0
	Foliar N : metabolic P	151±29.2abc	130±24.6ab	116±9.7a	114±21a	230±43c	195±3.1bc	109±22.2a	203±31.1abc	165±39.8ab	97.4±17.9a	312±58.2c	244.2±13.5bc
	Foliar N : nucleic acid P	53.1±4.3ab	53.6±2.8a	44.5±2.9ab	41.7±3.8b	63.1±6.9c	48.0±3.6ab	45.2±3.3a	48.6±1.9a*	56.2±5.3a	53.9±5.1a	63.1±4.8b	50.2±2.7a
	Foliar N : lipid P	91.7±11.1a	94.1±10.4a	65.8±6.6ab	55.8±7.9b	93.0±4.7b	72.1±9.7ab	68.4±7.4a	74.2±8.9a	73.8±3.4a	74.2±10.2a	105.8±9.5b	79.6±10.0a
	Foliar N : residue P	89.3±5.9a	108±15.7ab	140±10.3b	112±12.2ab	154±9.4c	140±1.9b	95.9±7.8a	132±15.6a	107±12.2a	104±14.9a	104±4.5a	119±14.1a
C.	Foliar N : total P	26.4±1.7a	19.7±1.1b	15.6±0.7c	11.4±0.6d	38.7±2.6e	18.4±1.3c	23.8±1.3a	22.9±2.3a	15.9±1.4b	13.3±0.3b	33.3±4.5c	18.4±0.9ab
odorata	Foliar N : phosphate	131.2±17.1c	100.9±6.6b	76.7±6.9ab	57.1±5.9a	163.2±12.8d	93.9±7.7b	139.8±26.9a	138.1±6.8a	99.9±8.4a	91.4±7.0a*	222±34.8b	116±6.4a
	Foliar N : metabolic P	8050±2434b	183.6±28.9a	95.9±3.5a	92.2±11.5a	917±250a	156±18.2a	694±234d*	378±109bc	174±22.8ab	126±12.5a	530±99.9cd	206±13.1ab
	Foliar N : nucleic acid P	81.2±7.5a	63.5±5.7b	51.2±2.0bc	41.2±2.6c	109±9.1d	66.3±4.1b	76.3±8.2a	86.6±9.1a	51.6±3.2b	55.2±3.6b	110±16.4c	56.4±3.0a
	Foliar N : lipid P	164±19.9a	92.2±4.3b	61.9±1.6c	52.3±4.5c	198±17.6c	74.0±7.2ab	112±17.0a*	104±12.6a	70.3±6.6b	65.2±4.0b	148±27.2c	78.9±4.8a
	Foliar N : residue P	117±4.5ab	137±9.0a	99.9±4.0bc	91.8±5.8c	201±12.9d	124±10.1bc	83.8±9.3a*	88.3±9.3a*	82.8±5.1a	72.4±3.7a	153±18.3b	98.3±5.7a*
	Foliar N : total P	23.0±1.0a	18.9±1.2a	11.7±1.8b	14.0±0.9b	21.4±0.4a	14.4±2.7b	20.0±1.9a	14.5±0.8b*	13.9±0.9bc	11.0±0.6c	24.4±1.9a	18.2±1.9ab
P.	Foliar N : phosphate	110±6.3b	77.1±10.9a	66.2±7.1a	64.3±6.3a	198±13.3c	111±10.6b	109±19ab	81.0±4.7ab	126±46ab*	50.9±4.2a	243±59.3c	144±13.0b
scandens	Foliar N : metabolic P	894±417a	2207±1530b	171±39.4a	170±30a	367±24.0a	203±32a	776±157a	320±94a*	359±66a	363±95.0a	11826±5415b*	350±29.8a
	Foliar N : nucleic acid P	72.9±4.6a	55.8±5.7bc	41.7±5.4c	57.3±3.4b	75.3±3.8a	58.8±13.3b	67.9±5.6c	55.9±1.1ab	56.4±4.5bc*	45.5±2.7a*	80.8±6.6d	79.1±9.8cd
	Foliar N : lipid P	123±16.7c	84.4±12.6b	52.2±5.6a	70.2±1.9ab	153±14.8c	69.8±13.1a	93.8±14.7a*	64.8±5.2b	46.1±3.7b	44.9±3.4b*	97.9±7.6a	62.8±4.7b
	Foliar N : residue P	110±8.8a	65.2±7.8b	57.0±6.3b	65.3±3.3b	76.0±3.9b	63.9±9.4b	74.1±9.4b*	59.5±2.6b	69.8±4.7b	42.7±2.0a*	92.5±7.4c	85.1±1.9bc

Values are means ± SE(n=6). Means in a row and within each CO_2 treatment followed by different lowercase letters are significantly different (p<0.05); means between ambient and elevated CO_2 followed by asterisk (*) are significantly different (p<0.05).

Table 3 Correlations between foliar phosphorus (P) fractions and foliar traits in the two invasive species. A_{area} is photosynthetic rates per unit area; A_{mass} is photosynthetic rates per unit mass; LMA is leaf mass per unit area; PNUE is photosynthetic N-use efficiency; PPUE is photosynthetic P-use efficiency; [N] is N concentration, [P] is P concentration. Values are correlation coefficients. * and ** indicates significance at $p<0.05$ and $p<0.01$, respectively.

P fraction	A_{area}	A_{mass}	LMA	PNUE	PPUE	[N]	[P]	N:P ratios
Phosphate	0.33**	0.58**	-0.55**	0.24**	0.01	0.47**	0.80**	-0.36**
Metabolic P	0.12	0.32*	-0.42**	0.03	-0.13	0.39**	0.70**	-0.32**
Nucleic acid P	0.35**	0.61**	-0.61**	0.26**	-0.05	0.66**	0.88**	-0.24**
Lipid P	0.29**	0.53**	-0.59**	0.30**	-0.20*	0.49**	0.89**	-0.43**
Residual P	0.36*	0.39**	-0.20*	0.28**	-0.04	0.29**	0.54**	-0.30**

Figure captions

Figure 1 Plant biomass for the invasive species (*Mikania micrantha* and *Chromolaena odorata*) and the native species (*Paederia scandens*) as affected by P addition rate and the combined addition of P and N under ambient or elevated [CO_2]. The six treatments listed along X axis are as follows: Control (neither P nor N added); 1/2P, 1P, and 2P (0.75, 1.5, and 3 g P m^{-2} $yr^{-1,}$ respectively); N (6.25 g N m^{-2} yr^{-1}); NP (6.25 g N m^{-2} yr^{-1}+1P). For each species and each CO_2 treatment, means with different lowercase letters are significantly different at $p<0.05$. For each species and within each P or N addition treatment, means with different uppercase letters are significantly different at $p<0.05$. In all cases, the absence of lowercase or uppercase letters indicates the absence of statistical significance.

Figure 2 Leaf mass per unit area (LMA), photosynthetic rates per unit area (A_{area}), and photosynthetic rates per unit mass (A_{mass}) for the invasive species (*M. micrantha* and *C. odorata*) and the native species (*P. scandens*) as affected by P addition rate and the combined addition of P and N under ambient or elevated [CO_2]. Treatments and statistical comparisons are described in Figure 1.

Figure 3 Photosynthetic nutrient-use efficiency for nitrogen (PNUE) and phosphorus (PPUE) for invasive species *M. micrantha* and *C. odorata* and the native species *P. scandens* as affected by P addition rate and the combined addition of P and N under ambient or elevated [CO_2]. Treatments and statistical comparisons are described in Figure 1.

Figure 4 Foliar nitrogen (N) and phosphorus (P) concentrations of the invasive species *M. micrantha* and *C. odorata* and the native species *P. scandens* as affected by P addition rate and the combined addition of P and N under ambient or elevated [CO_2]. Treatments and statistical

comparisons are described in Figure 1.

Figure 5 Concentrations of foliar inorganic P (phosphate, Pi) and organic P (metabolic P, nucleic acid P, structural P, and residual P) of the invasive species *M. micrantha* and *C. odorata* and the native species *P. scandens* as affected by P addition rate and the combined addition of P and N under ambient or elevated [CO_2]. Treatments and statistical comparisons are described in Figure 1.

Figure 6 Correlation between foliar phosphorus (P) fractions concentration and plant biomass. The data from three target species and six treatments. R^2 values for linear trend lines are shown on each plot.

Figure 7 A conceptual framework of a noval pathway to sustain P demand of invasive species under elevated CO_2 and N addition in low soil P availability.

Fig. 1

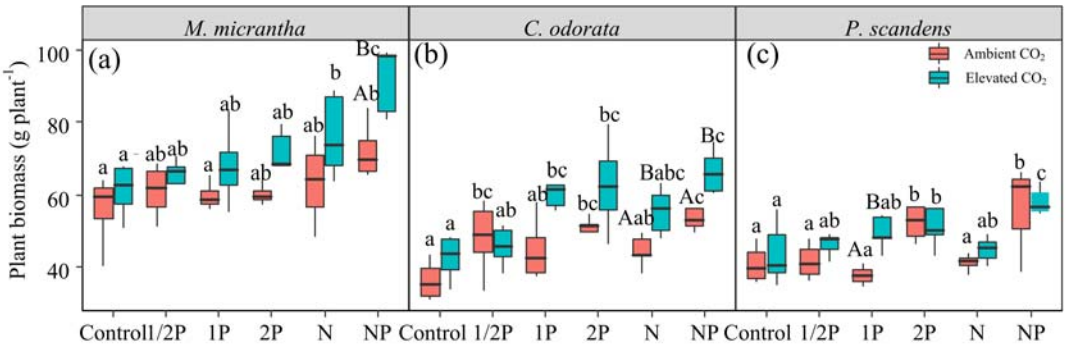


Fig. 2

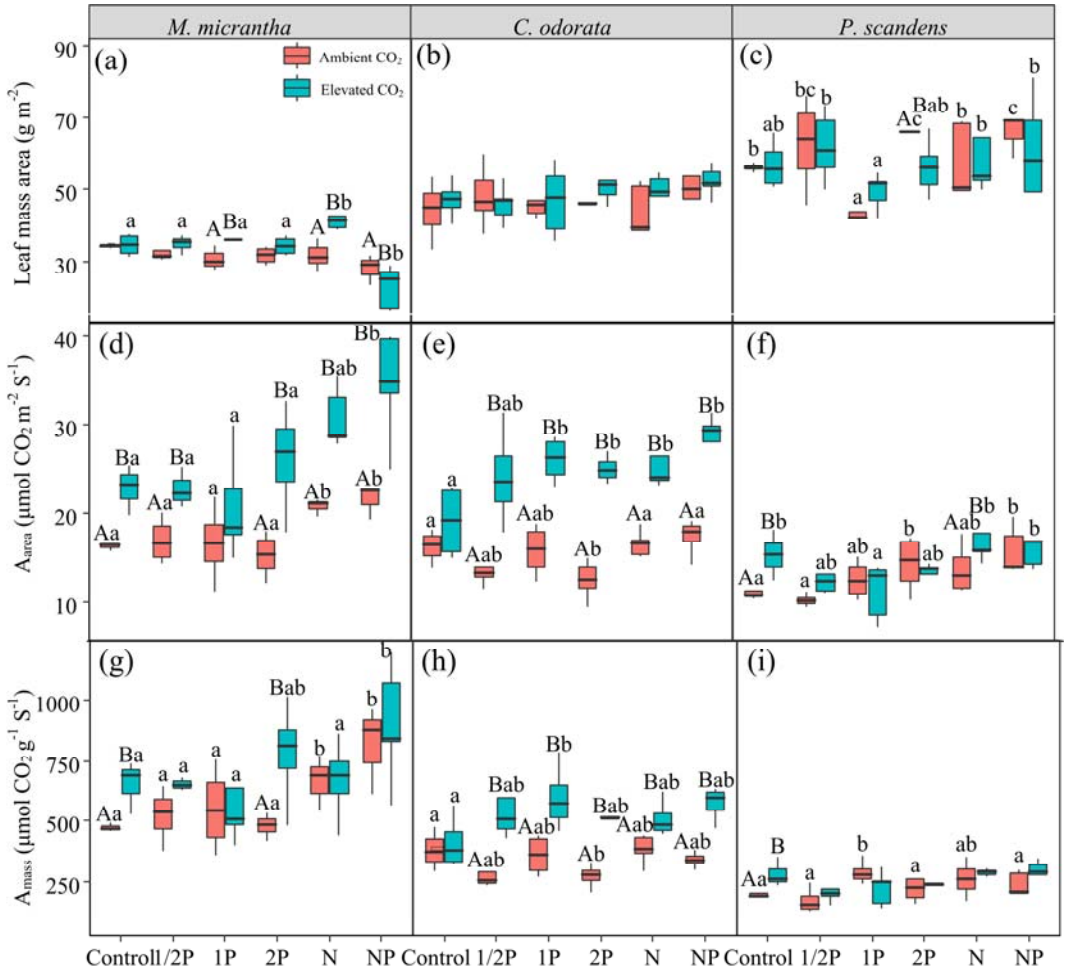


Fig. 3

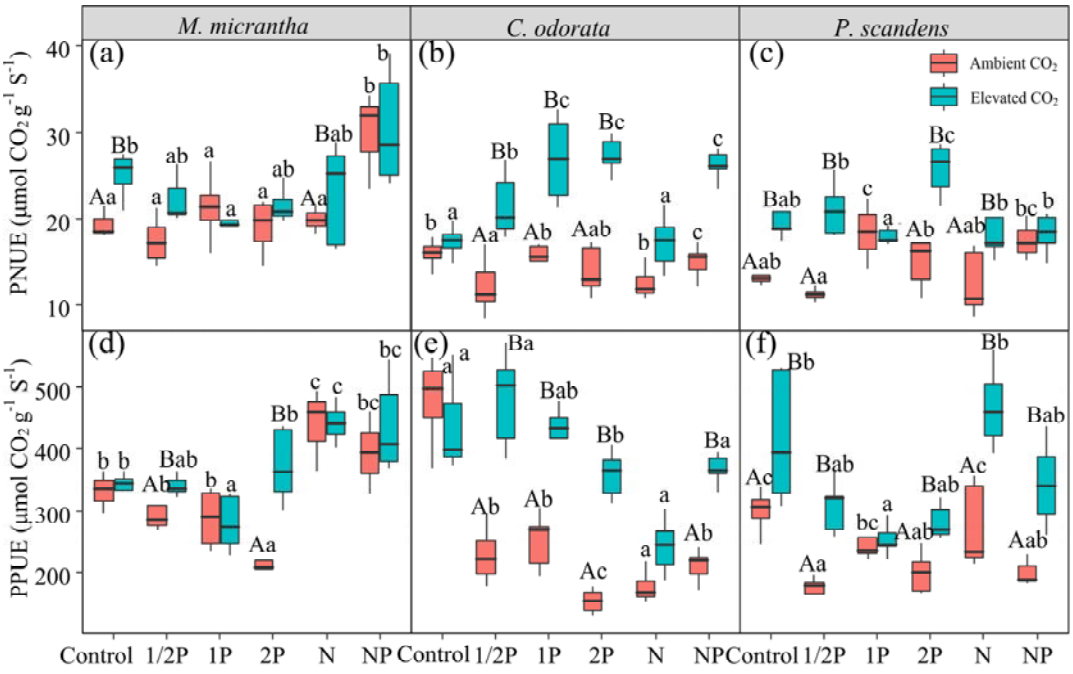


Fig. 4

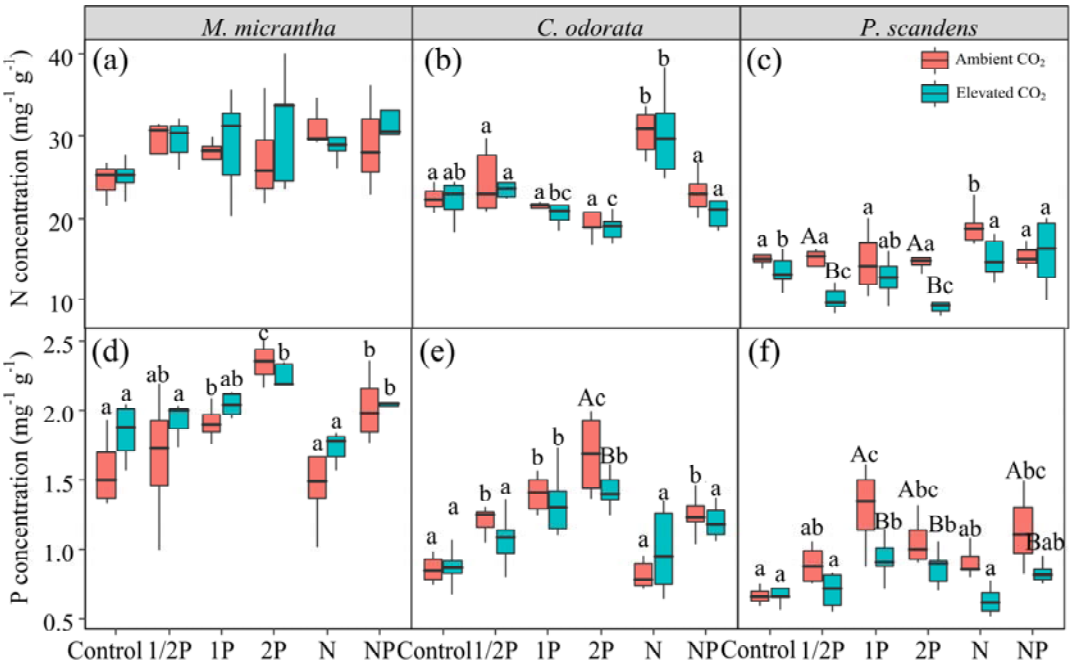


Fig. 5

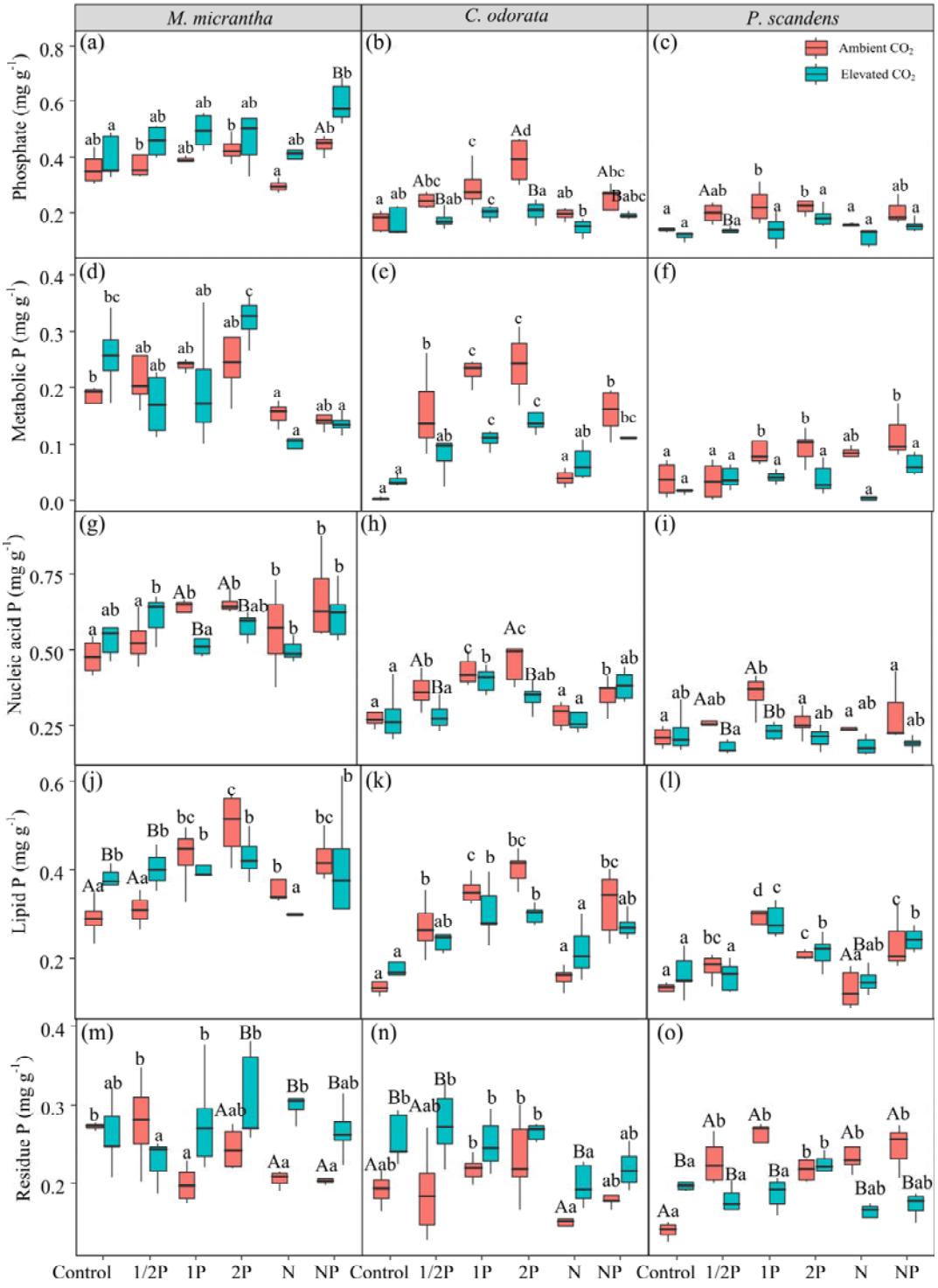


Fig. 6

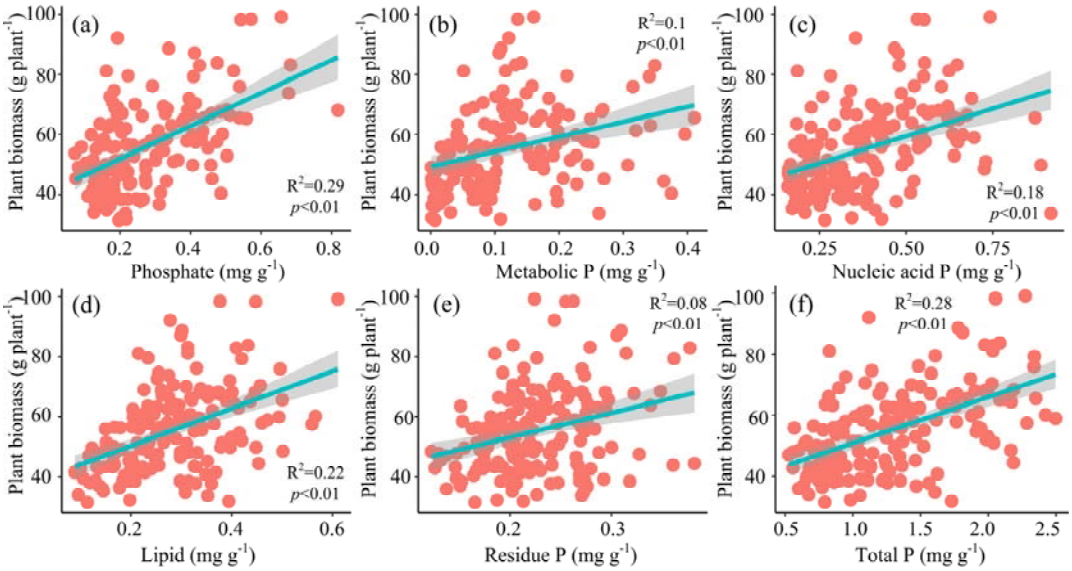


Fig. 7

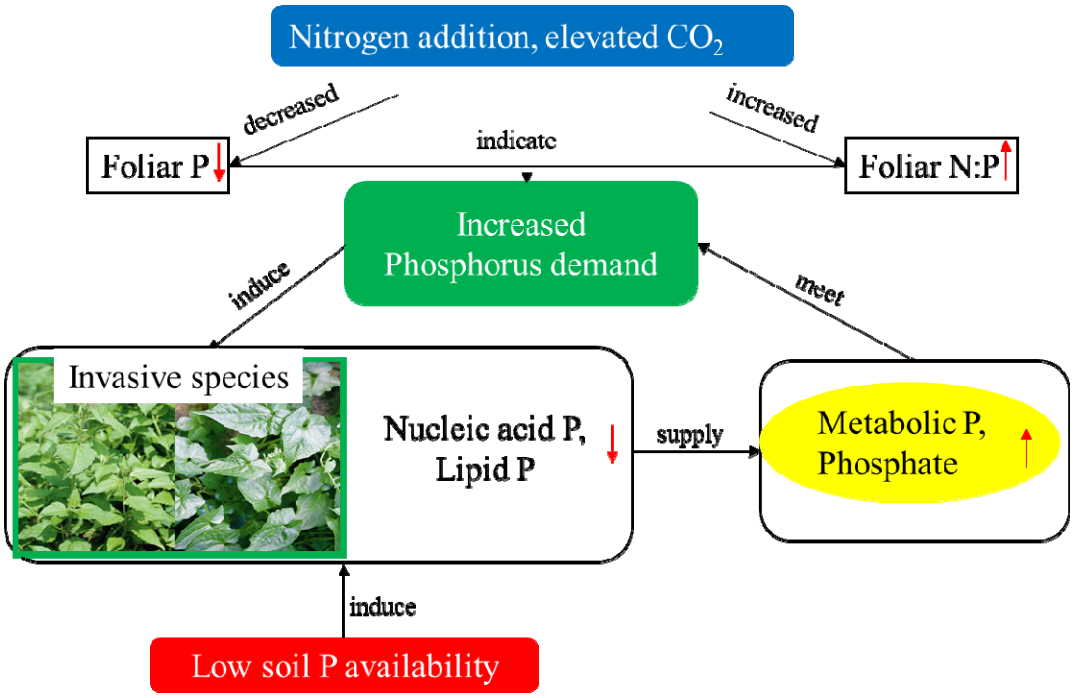
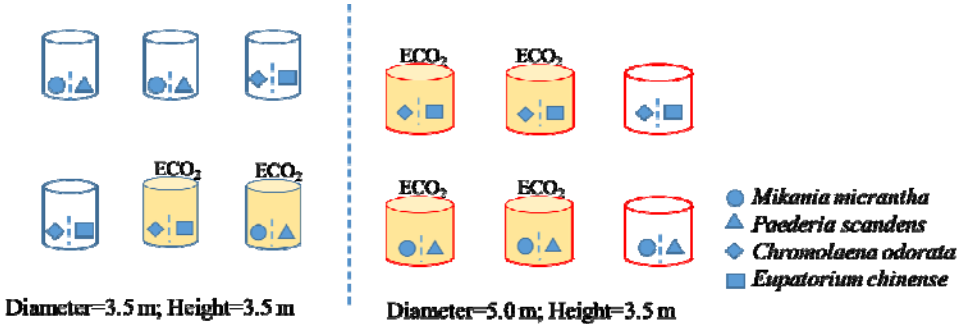


Figure S1 Diagram of species planted in open-top chambers. Chambers labeled ECO_2 were treated with elevated [CO_2]. The other chambers were treated with ambient [CO_2].



Introduce: To clearly discriminate the response of invasive species to the joint effects, two indigenous co-occurring species (*Paederia scandens* and *Eupatorium chinense*) with similar morphology to invasive species were together collected. *Mikania micrantha*, *Chromolaena odorata* and *P. scandens* were collected in South China Botanical Garden, *E. chinense* was collected in Zhejiang Province. Most of the treated *E. chinense* dead in the cultivating process, as they could not adapt to the climate conditions in Guangzhou, China. So the foliar parameters of *E. chinense* couldn't be measured, and no data showed in the paper.