

1 **Foliar phosphorus fractions reveal how tropical plants maintain photosynthetic**
2 **rates despite low soil phosphorus availability**

3 **Running Head:** Response of foliar traits to N and P addition

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22

23 **Summary**

- 24 1. Nitrogen (N) and phosphorus (P) are essential nutrients for plant metabolism and their
25 availability often limits primary productivity. Whereas the effects of N-availability on
26 photosynthetic capacity are well established, we still know relatively little about the
27 effects of P availability at a foliar level, especially in P-limited tropical forests.
- 28 2. We examined photosynthetic capacity, leaf mass per area (LMA), and foliar P fractions in
29 five woody plant species after six years of N and P fertilization in a lowland tropical forest.
- 30 3. Foliar N:P ratios indicated P limitation of the unfertilized plants; accordingly
31 photosynthetic P-use efficiency (PPUE) and LMA decreased with P addition, and foliar N
32 and P concentrations increased, whereas N addition had little effect on measured foliar
33 traits. However, P addition enhanced photosynthetic capacity only in one species, and not
34 in other four species. We then assessed plant acclimation to low P availability by
35 quantifying four fractions of foliar P representing different functional pools: structural P,
36 metabolic P (including inorganic P), nucleic acid P, and residual P. We found that P addition
37 enhanced the concentrations of metabolic, structural, and nucleic acid P fractions in all
38 species, but the magnitude of the effect was species-specific.
- 39 4. Our findings indicate that tropical species acclimate to low P availability by altering
40 allocation of foliar P to meet the demand of P for photosynthesis. Importantly, species
41 typical of lowland tropical forests in East Asia maintained their photosynthetic rate under
42 low P availability. We conclude that P limitation of leaf photosynthetic capacity may not
43 be as common as previously assumed due to plant acclimation mechanisms in low-P
44 tropical forests. Species-specific strategies to allocate P to different foliar fractions
45 represent a potentially important adaptive mechanism for plants in P-limited systems.

46 **Keywords:** Fertilization, Nitrogen deposition, P fractions, P limitation, Photosynthesis, PNUE, PPUE,
47 Secondary forest

48 **Introduction**

49 Nitrogen (N) and phosphorus (P) are essential nutrients for photosynthetic carbon assimilation,
50 and the most common nutrients limiting net primary productivity in terrestrial ecosystems.
51 Nitrogen availability constrains plant productivity in many temperate and boreal forests by limiting
52 leaf initiation and expansion (Vos & Biemond 1992) and the synthesis of Rubisco and other
53 photosynthetic proteins (Evans 1989). However, tropical forests were generally regarded as P
54 limited rather than N limited, because soil P availability generally declines with bedrock weathering
55 and soil age (Walker & Syers 1976). Quaternary glaciation exposed fresh bedrock over a large area
56 of temperate and boreal regions, but not in the tropics (Vitousek 1984). In accordance,
57 biogeochemical theory indicates that old, strongly weathered lowland tropical forests should be P
58 limited (Vitousek 1984; Vitousek & Howarth 1991; Vitousek *et al.* 2010). However, tropical forests
59 maintain the greatest plant biomass and the fastest rates of many biological processes (i.e.
60 decomposition, N transformation) on Earth (Tanner *et al.* 1990). Therefore, identifying the
61 strategies that tropical plants have evolved to use P efficiently under low soil P availability is an
62 important topic in plant ecology (Hidaka & Kitayama 2009; Reed *et al.* 2011; Hidaka & Kitayama
63 2013; Lambers *et al.* 2015).

64 Shifting P allocation in leaves is an important mechanism for plants to acclimate to low soil P
65 availability (Hidaka & Kitayama 2011). Foliar P is functionally divided into four major fractions: 1)
66 metabolic P, including low-molecular-weight phosphate esters (e.g., ADP, ATP, and sugar
67 phosphates), and inorganic phosphate (P_i); 2) nucleic acid P, most of which is contained in
68 ribosomal RNA; 3) structural P in membrane phospholipids; and 4) residual P in phosphorylated
69 proteins and unidentified residues (Kedrowski 1983; Hidaka & Kitayama 2011; Lambers *et al.* 2011).
70 Of these, metabolic P is of particular interest for studying P limitation, because P-containing
71 metabolites have key roles in the Calvin-Benson cycle, and insufficient metabolic P could limit
72 maximum photosynthetic rates (Ågren, Wetterstedt & Billberger 2012). Nucleic acid P generally
73 represents 40–60% of the organic P pool in leaves (Veneklaas *et al.* 2012). Over 85% of nucleic acid
74 P is contained in RNA, especially ribosomal RNA (rRNA), and a high P allocation to rRNA sustains

75 rapid protein synthesis, which is required for growth and photosynthesis (Hidaka & Kitayama 2011).
76 Therefore, there is generally a positive correlation between rRNA content and protein content, as
77 well as growth rates, over a range of taxa (Elser *et al.* 2000). Structural P accounts for 10-20% of all
78 foliar P (Veneklaas *et al.* 2012), and is contained mainly in phospholipids, which are an essential
79 component of plasmalemma and organelle membranes. Finally, residual P can represent 20% of
80 total foliar P in tropical trees (Hidaka & Kitayama 2011) and probably mostly comprises
81 phosphorylated proteins. Generally, the concentration of the residual P fraction is relatively
82 constant, because phosphorylated proteins are essential for many metabolic processes. However,
83 under extremely P-limiting conditions, phosphatases may also dephosphorylate phosphorylated
84 proteins (Schlüter *et al.* 2013), leading to a reduction in residual P concentrations.

85 Under P deficiency, photosynthesis is generally reduced (Turnbull, Warren & Adams 2007) due to
86 feedback inhibition resulting from reduced leaf growth (Dissanayaka *et al.* 2018) or because
87 orthophosphate (Pi) in the cytosol becomes limiting (Warren 2011). Plants also tend to increase
88 their leaf mass per unit area (LMA; Chiera, Thomas and Rufty (2002), and increase their
89 photosynthetic P-use efficiency (Hidaka & Kitayama 2009) when P supply is limited. Ellsworth *et al.*
90 (2015) suggest that P-limitation of photosynthetic capacity is likely common in the field. However,
91 since photosynthesis involves a series of chemical and physical processes that are dependent on
92 nutrient supply, some degree of acclimation to P availability can be expected for plants growing on
93 low-P soils. There is evidence that tree species growing on P-poor soils in tropical montane forests
94 reduce their overall need for foliar P by decreasing the metabolic P fraction (Hidaka & Kitayama
95 2011). However, it is unclear whether such a reduction in metabolic P affects leaf photosynthetic
96 capacity, because plant vacuoles serve as a reservoir of Pi (Mimura 1995), which buffers direct Pi
97 restriction of photosynthesis. Although generally a short-term acclimation to low P availability, this
98 buffering effect has been observed in many species (Rao & Terry 1995; Schachtman, Reid & Ayling
99 1998; Warren 2011). Another important P-saving mechanism under low P availability involves the
100 replacement of phospholipids (structural P) in membranes by sulfolipids and galactolipids
101 (Rouached, Arpat & Poirier 2010; Lambers *et al.* 2012; Veneklaas *et al.* 2012), which can maintain

102 foliar P metabolite concentrations at very low P availability. However, this mechanism appears to
103 be an evolutionary adaptation to low P availability, and has so far only been reported in Proteaceae
104 from severely P-impooverished soils in Australia (Lambers *et al.* 2012).

105 Lowland tropical forest trees have experienced long-term low P status; adaptations to P limitation
106 thus likely evolved in these species, but field-based evidence for this is lacking. A recent study in a
107 Panamanian tropical forest showed that P limitation of plant growth is species-specific, but does
108 not translate into a community-wide response, because some species are adapted to low P
109 availability and able to grow rapidly, despite low soil P availability (Turner, Brenes-Arguedas &
110 Condit 2018). The finding redefined our understanding of P limitation in species-rich tropical
111 forests; however, we do not fully understand the mechanisms by which tropical species are able to
112 acclimate to low-P soils and maintain rapid growth.

113 Given the importance of photosynthetic capacity for plant performance, a better understanding of
114 species-specific foliar P-allocation patterns and their relationship with photosynthetic capacity in
115 low-P tropical forests is needed. We investigated this within an existing fertilization experiment in
116 lowland tropical forest in China, using five woody plant species with distinct distributions: three
117 species are limited to the lowland tropics (stenotopic), one species occurs in both tropical and
118 subtropical forests of East Asia, and is also found in temperate regions (eurytopic; Fig. S1). To
119 understand how plants acclimate to low-P soils through shifts in foliar traits and P-allocation
120 pattern, we measured leaf photosynthesis, photosynthetic P-use efficiency (PPUE), LMA, leaf N and
121 P concentrations, and leaf P fractions after six years of N- and P fertilization. We hypothesized that:

122 1) Fertilisation with P would have a much stronger effect on foliar traits (i.e. photosynthetic capacity,
123 LMA, N and P concentrations) than fertilisation with N.

124 2) The response of photosynthetic capacity to P fertilization would be species-specific, whereby
125 the maximum photosynthetic rate of stenotopic tropical species would be unaffected by P addition,
126 as they are adapted to the low soil P availability.

127 3) Species-specific changes in the allocation of P to different leaf fractions (structural P, metabolic

128 P, nucleic acid P, and residual P) explain the maintenance of photosynthetic capacity under low P
129 availability.

130 **Material and methods**

131 *Site description*

132 The study was carried out at Xiaoliang Research Station for Tropical Coastal Ecosystems of the
133 Chinese Academy of Sciences (21° 27'N, 110°54'E), located in the southwest of Guangdong
134 Province, China. This region is characterized by a tropical monsoon climate with a mean
135 annual temperature of 23°C. Annual rainfall ranges from 1400 to 1700 mm, with dry season
136 from November to March. The soil is classified as a latosol developed from granite (Wang *et al.*
137 *et al.* 2014). Annual wet N deposition in the region was c. 40 kg N ha⁻¹ in 2011 and 2012 (Mo *et al.*
138 *et al.* 2015; Chen *et al.* 2016).

139 The study site was located in secondary broad-leaf mixed forest on coastal land (c. 5 km from
140 the coast) with a very small slope. The forest was restored from *Eucalyptus exserta* plantation
141 by introducing 312 plant species between 1964 and 1975. Thereafter, natural colonization
142 during succession displaced almost all of the planted species, resulting in a typical secondary
143 evergreen tropical forest with the biodiversity and structural complexity of natural forest (Mo
144 *et al.* 2015; Chen *et al.* 2016).

145 *Experimental design*

146 An N- and P-fertilization experiment was established in a randomized block design within the
147 secondary tropical forest in September 2009 (Zhao *et al.* 2014; Chen *et al.* 2016). Four 10-m ×
148 10-m plots were established within each of five replicate blocks; adjacent blocks were
149 separated by 50 m. Four treatments, N-addition (+N), P-addition (+P), N- and P-addition (+NP),
150 and a control treatment (CT, no addition of mineral nutrients) were assigned randomly to the
151 four plots within each block. The edges of each plot were trenched to a depth of 20-cm and

152 surrounded by a 2-m wide buffer. Since most fine roots are distributed in surface soils, the
 153 trenches largely inhibited the transfer of nutrients among treatments, as evidenced by clear
 154 differences between fertilized and unfertilized treatments in extractable soil P after six years
 155 of fertilization in 2015 (Table 1). Fertilizers were applied every two months from 2009 - 2015
 156 to give total amounts of N and P equivalent to 100 kg ha⁻¹ yr⁻¹. Briefly, for every fertilizer
 157 application, 476.6 g NH₄NO₃ (equal to 166.6 g N) and/or 808 g Na₂HPO₄ (equal to 166.6 g P)
 158 were dissolved in 30 L groundwater and then applied to the corresponding plots uniformly
 159 using a backpack sprayer near the soil surface; 30 L of groundwater were also applied to
 160 control plots (Wang *et al.* 2014; Li *et al.* 2015). The amount of added water in each plot was
 161 equivalent to 0.08% and 0.35% of rainfall inputs in the wet and dry seasons, respectively (Mo
 162 *et al.* 2015).

163 **Table 1** General soil chemical properties (0-10 cm) in fertilization treatments in a secondary coastal
 164 forest in China before the start of fertilizer application in September 2009 and after six years of
 165 fertilization treatments in May 2015; where CT is control, and +N, +P or +NP denote fertilization
 166 with nitrogen (N), phosphorus (P) or both nutrients, respectively; means ±SE are shown for *n*=5.
 167 Different superscript letters indicate significant differences among treatments at *p*<0.05. TP: total
 168 P concentration, TN: total N concentration, AP: available phosphorus; data for 2009 are reported
 169 in Li *et al.* (2015).

Date	Variables	CT	+N	+P	+NP
Sep. 2009	pH (H ₂ O)	4.0±0.06	4.0±0.05	4.0±0.05	4.0±0.09
	AP(mg kg ⁻¹)	4.10±0.56	3.79±0.42	4.06±0.37	3.70±0.60
	NO ₃ ⁻ -N (mg kg ⁻¹)	2.88±0.35	2.72±0.11	2.68±0.31	2.35±0.33
	NH ₄ -N(mg kg ⁻¹)	2.12±0.12	1.85±0.13	1.81±0.11	2.03±0.17
	TN (g kg ⁻¹)	2.71±0.15	2.34±0.21	2.66±0.10	2.68±0.19
	TP (g kg ⁻¹)	0.40±0.03	0.38±0.02	0.42±0.02	0.43±0.03
May. 2015	pH (H ₂ O)	3.8±0.02	3.8±0.06	3.9±0.06	3.9±0.06
	AP (mg kg ⁻¹)	4.60 ^c ±0.57	5.60 ^c ±0.69	71.4 ^a ±2.8	53.2 ^b ±9.15
	NO ₃ ⁻ -N (mg kg ⁻¹)	6.02±0.75	7.42±1.58	3.11±0.46	6.04±1.85
	NH ₄ -N(mg kg ⁻¹)	3.55±0.36	4.11±0.46	4.19±0.73	4.16±0.50
	TN (g kg ⁻¹)	1.31±0.05	1.36±0.04	1.25±0.09	1.50±0.12

TP (g kg ⁻¹)	0.35 ^b ±0.02	0.39 ^b ±0.01	0.65 ^a ±0.02	0.58 ^a ±0.05
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170 *Measurement of foliar traits*

171 We used previous vegetation surveys at the study site to identify the 10 most common
172 understory species, and selected five common species that occurred in sufficient numbers in
173 all experimental treatments. *Clerodendrum cyrtophyllum* Turcz. is a eurytopic shrub species,
174 which is distributed widely from temperate forest to tropical forest in East Asia (Fig. S1).
175 *Schefflera octophylla* (Lour.) Harms is a small tree that can grow to 15 m height and can be
176 found in subtropical and tropical forests (He & Zeng 1978). *Syzygium bullockii* Hance. and
177 *Psychotria rubra* (Lour.) Poir. are shrubs or small trees (Chen & Pie 1982; Chen 1984; Chen
178 1999), typical of lowland tropical forests (i.e. stenotopic species). *Uvaria microcarpa* Champ.
179 Ex Benth is a shrub only found in tropical forests (i.e. a stenotopic species, Jiang and Li (1979).
180 We selected individuals with a height of c. 2 m for consistency of *in situ* foliar level
181 measurements. We measured foliar photosynthetic rates, leaf mass per area (LMA) and foliar
182 N and P concentrations on mature leaves of all five species after six years of fertilization with
183 N and/or P in 2015. Leaf photosynthetic rates were measured using a portable open-system
184 infrared gas analyzer (LI-6400, LI-COR Biosciences, Lincoln NE, USA). Measurements were
185 carried out during seven days in August 2015 between 8:00 AM and 12:00 PM on fully
186 expanded healthy sun-exposed mature leaves. Three to seven individuals per species were
187 measured in each treatment. Rates of CO₂ exchange were measured *in situ* under controlled
188 cuvette conditions set to 1000 μmol m⁻² s⁻¹ PAR, 400 μmol CO₂ mol⁻¹, a chamber temperature
189 of 25 ±1°C and a vapor pressure deficit of 0.5-1.0 kPa. The photosynthetic photon flux density
190 of 1000 μmol m⁻² s⁻¹ was the same as previous studies in tropical forests (Hidaka & Kitayama
191 2009; Hidaka & Kitayama 2013), and is generally a saturating light value for photosynthesis in
192 these understory species. Leaves measured for CO₂-assimilation rates were subsequently
193 collected to determine LMA using a portable leaf area meter (LI-3000A, LI-COR Biosciences)

194 and a second leaf sample was collected from the same branch for chemical analyses. All leaf
195 samples were stored on ice, returned to the lab and washed within 6 h. After determination
196 of leaf area, the first leaf samples were dried to constant weight at 50°C for 72 h and weighed
197 to determine dry mass and LMA (g m^{-2}). The second leaf sample was freeze-dried for analysis
198 of foliar N and P concentrations (mg g^{-1} dry weight) and P fractions. Leaf samples were ground
199 after removing petioles and main veins, and foliar P concentrations were measured
200 spectrophotometrically after digestion with sulfuric acid (H_2SO_4); foliar N concentrations were
201 determined using the Kjeldahl method (Wang *et al.* 2013). Photosynthetic nutrient use
202 efficiency for N (PNUE) and P (PPUE) was defined as the rate of net photosynthesis per unit N
203 or P expressed on a leaf dry mass basis, and photosynthetic capacity is given on dry mass basis
204 (A_{mass} ; $\text{nmol CO}_2\text{g}^{-1}\text{s}^{-1}$) and leaf area basis (A_{area} ; $\text{umol CO}_2\text{m}^{-2}\text{s}^{-1}$).

205 *Measurement of P fractions*

206 Foliar P was partitioned into four fractions: structural P, metabolic P (including P_i), nucleic acid
207 P and residual P, using sequential extraction (Kedrowski (1983) with modifications (Hidaka and
208 Kitayama (2011)). First, c. 0.5 g of freeze-dried leaf sample was weighed into a 50-ml centrifuge
209 tube (tube 1); it was then extracted twice with 7.5 ml 12:6:1 CMF (chloroform, methanol,
210 formic acid, v/v/v). The liquid extract was transferred into a clean tube (tube 2). The residue
211 from the initial extraction was then extracted twice with 9.5 ml 1:2:0.8 CMW (chloroform,
212 methanol, water, v/v/v), and the liquid solvent was also added to tube 2. The remaining
213 residue was then mixed with 9.5 ml water-washed chloroform and the supernatant was also
214 transferred to tube 2, and mixed thoroughly. The upper phase of the extract in tube 2 was
215 transferred to a clean tube (tube 3) and the lower lipid-rich phase was used to determine
216 structural P.

217 All liquid extracts in tube 3 were mixed with those remaining in tube 1, and 5 ml methanol
218 (85% v/v) was added; the tube was then placed in a vacuum dryer for 48 h to remove residual

219 chloroform and methanol. The extract was refrigerated (4°C) for 1 h. First, 1 ml TCA
220 (trichloroacetic acid, 100%) and then 10 ml TCA was added and the extract was shaken for 1
221 h before being centrifuged at 3000 g. The supernatant was analyzed for metabolic P.

222 Finally, the residue was mixed with 35 ml TCA (2.5%, w/v), extracted for 1 h in a hot water
223 bath (95°C), and centrifuged at 3000 g after cooling to room temperature. The liquid layer was
224 measured for nucleic acid P and the residue from this final extraction was analyzed for residual
225 P. All foliar P fractions were measured spectrophotometrically after digestion with sulfuric
226 acid (H₂SO₄) and are expressed on a dry mass basis (mg g⁻¹).

227 *Data analyses*

228 All data analyses were conducted in R version 3.1.0 (R Core Team 2017), using the nlme
229 package (Pinheiro *et al.* 2016) for linear mixed effects models and the FactoMineR package
230 (Le, Josse & Husson 2008) for multivariate analyses. As our experiment used a randomized
231 block design, we first used linear models (Analysis of Variance) to assess the block effect on
232 soil nutrients before fertilization (Table 1), and found no significant block effect on soil
233 extractable N or P. We then used linear mixed effects models (*lme* function) to examine the
234 effects of N-addition, P-addition and species identity on leaf parameters. In the initial model,
235 N-addition, P-addition, species and their interaction were considered fixed effects, and plots
236 within blocks as the random effect. We compared nested models using likelihood ratio tests
237 and AICs to check for model improvement (Pinheiro & Bates 2000). There was generally a
238 better model fit (lower AIC values) with interaction terms; we thus selected the full factor
239 model and assessed the significance of each fixed effect using the *anova* function. As there
240 were significant interactions between treatments and species (Table 2), we also investigated

241 species-specific responses to N- and P addition using separate linear models with block as an
242 error term (Table S1). Where models for individual species were significant, post-hoc tests
243 (Tukey's HSD) were conducted for multiple comparisons among treatments. Results are
244 reported as significant at $p < 0.05$.

245 **Results**

246 All measured foliar traits varied strongly among the five study species and the responses of
247 foliar traits to nutrient additions were often species-specific (Table 2), indicating different
248 nutrient-use or allocation strategies in our study species.

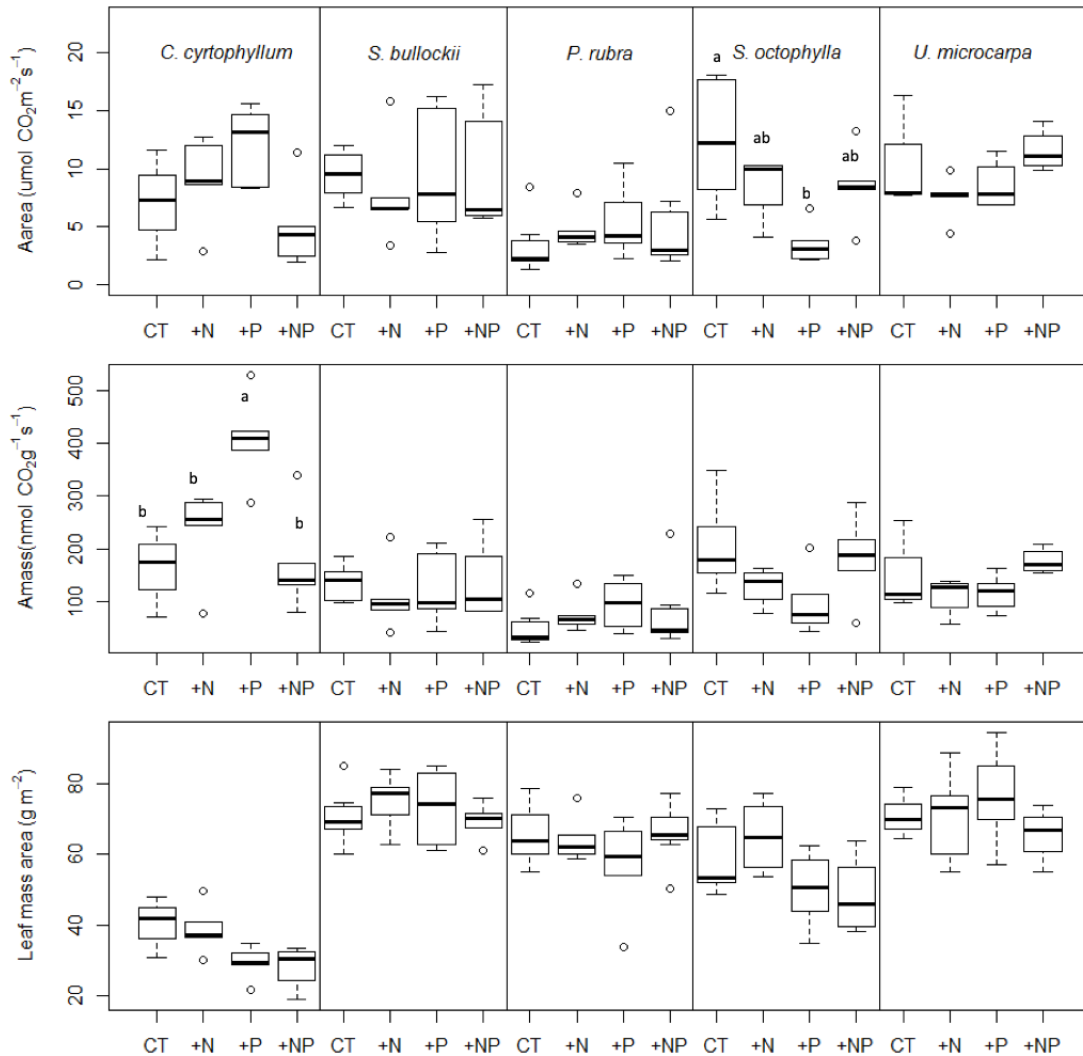
249 *Photosynthesis and leaf mass per area (LMA)*

250 Although there was no overall effect of N- and/or P addition on photosynthetic rate,
251 significant interactions indicated species-specific responses to N and P addition (Table 2). Both
252 of A_{area} and A_{mass} showed the similar patterns among species and fertilization treatments (Fig.1
253 & Table 2). Analyses for each species individually showed that A_{mass} was affected by the
254 interaction of N and P in *C. cyrtophyllum* and *S. octophylla* (Table S1). A_{mass} in *C. cyrtophyllum*
255 (i.e. the eurytopic species) increased only when P was added alone, resulting in significantly
256 higher A_{mass} in the +P treatment than in the +NP, +N or control treatments (Fig. 1). By contrast,
257 A_{area} in *S. octophylla* was significantly lower in the +P treatment than in the control, but the
258 negative effect of P addition was ameliorated by the addition of N (Fig. 1).

259 Leaf mass per area (LMA) in the control ranged from 40 g m^{-2} to 71 g m^{-2} (Fig. 1; Table 2), and
260 decreased significantly with P addition (Table 2), with the strongest reduction in *C.*

261 *Cyrtophyllum* and *S. octophylla* ($p=0.006$ and $p=0.02$, respectively; Fig 1, Table S1). There was

262 no effect of N addition on LMA for any species (Fig. 1; Table S1).



263

264 **Fig. 1** Box-plots of area-based and mass-based photosynthetic rates (A_{area} and A_{mass}) and leaf mass

265 per unit area (LMA) for five woody species in a tropical forest following six years of fertilization with

266 nitrogen (+N), phosphorus (+P) or both nutrients (+NP) compared to controls (CT). Different letters

267 within panels indicate post-hoc significant differences among treatments at $p < 0.05$.

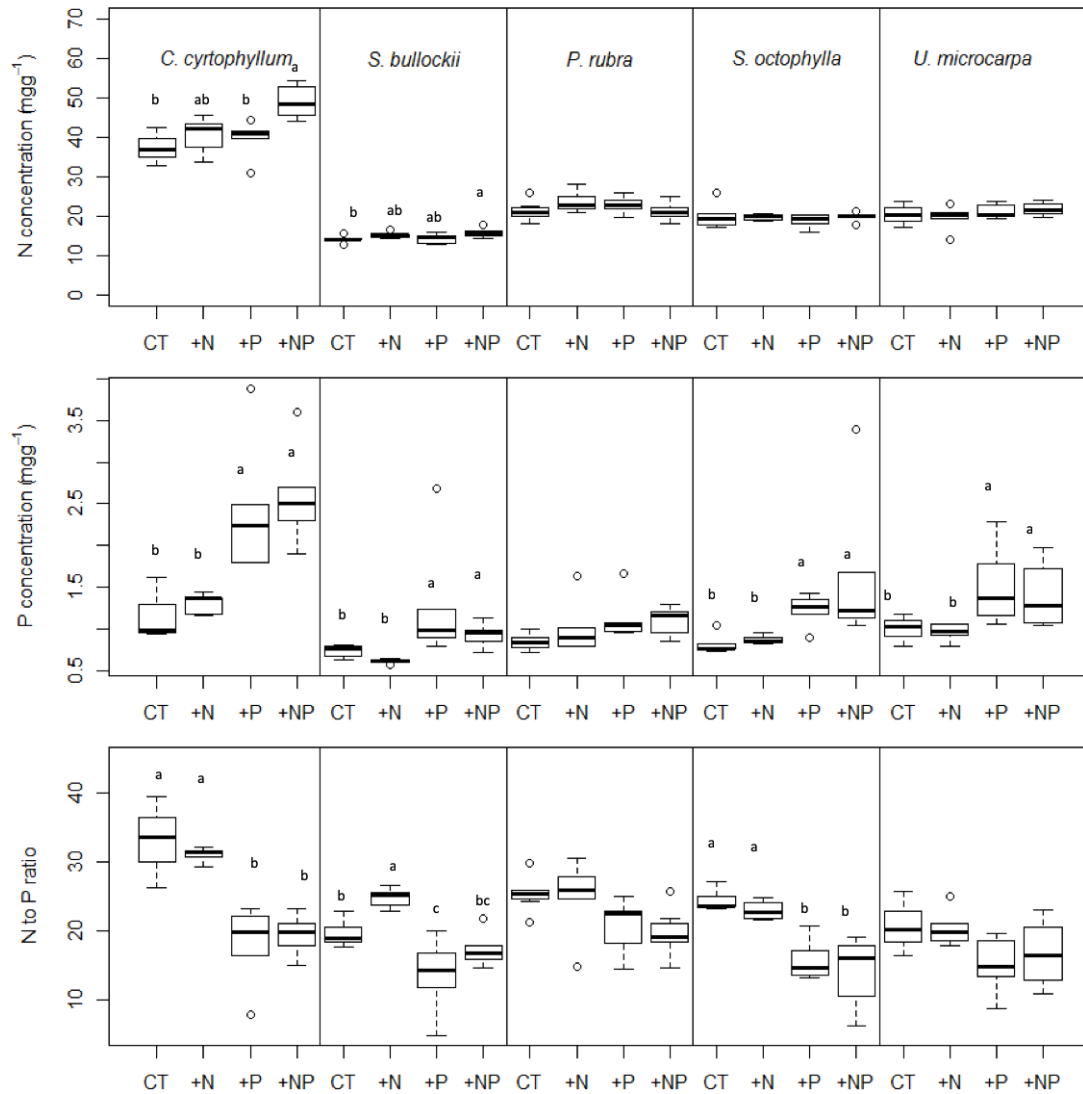
268 **Table 2** Significance (p-values) of terms from linear mixed model analysis for foliar traits, nutrient concentrations and phosphorus fractions of five woody species in a
 269 tropical forest following six years of fertilization with nitrogen (N) and phosphorus (P), where S is species; N is N-addition; P is P-addition; A_{area} is photosynthetic rates
 270 per unit area; A_{mass} is photosynthetic rates per unit mass; LMA is leaf mass per unit area; PNUE is photosynthetic N-use efficiency and PPUE is photosynthetic P-use
 271 efficiency.

Fixed effect	A_{area}	A_{mass}	LMA	N concentration	P concentration	N:P ratios	PNUE	PPUE	Metabolic P	Nucleic P	Structural P	Residual P
S	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
N	ns	ns	ns	<0.001	ns	ns	ns	ns	ns	ns	ns	0.009
P	ns	ns	0.001	0.001	<0.001	<0.001	ns	0.005	<0.001	<0.001	<0.001	<0.001
N×S	0.020	ns	ns	0.001	ns	ns	ns	ns	ns	ns	ns	0.001
P×S	ns	ns	ns	0.001	0.001	0.005	ns	0.038	0.006	0.016	ns	<0.001
N×P	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	0.004
N×P×S	<0.001	0.005	ns	0.038	ns	ns	0.002	0.040	ns	ns	ns	0.027

273 *Foliar N and P concentrations and N:P ratios*

274 The response of foliar N concentrations (hereafter 'foliar [N]') to fertilizations was strongly
275 influenced by species identity and treatment, but overall foliar [N] was slightly but significantly
276 higher in treatments with added N (Table 2). Foliar P concentrations (hereafter foliar [P])
277 increased markedly with P addition in all species (Fig. 2; Table 2). Foliar [P] increased by 33%
278 - 107% in +P treatments compared with controls, and by 27% - 121% in +NP treatments. Foliar
279 N:P ratios in control plots indicated P limitation at the study site: *C. cyrtophyllum* had the
280 highest foliar N:P ratio of 33; *P. rubra* and *S. octophylla* had similar intermediate N:P ratios (25
281 and 24, respectively), whereas the foliar N:P ratios in *S. bullockii* and *U. microcarpa* were
282 somewhat lower (20 and 21, respectively). A decline in foliar N:P ratios was observed in all
283 species as a result of foliar P accumulation in the +P and +NP treatments (Fig. 2). Nitrogen
284 addition did not affect foliar N:P ratios in any species, except *S. bullockii* (Fig. 2, Table S1),
285 which had a higher foliar N:P ratio with N addition as a result of increased foliar [N].

286



287

288 **Fig. 2** Box-plots of foliar nitrogen (N) and phosphorus (P) concentrations and N to P ratio for five
 289 species in a tropical forest following six years of fertilization with N (+N), P (+P) or both nutrients
 290 (+NP) compared to controls (CT). Different letters within panels indicate significant post-hoc
 291 differences among treatments at p<0.05.

292 *Photosynthetic phosphorus- and nitrogen-use efficiency*

293 PNUE was not affected by N addition, whereas PPUE decreased with P addition (Table 2; Fig.
 294 S2). PPUE was 9% to 56% lower in the +P treatment and 18% to 51% lower in the +NP
 295 treatment compared to the controls. Species identity modified the response of PNUE and
 296 PPUE to N and P additions (Table 2), but individual analyses only showed significantly higher

297 PNUE in *C. cyrtophyllum* in the +P treatment, and lower PPUE in *C. cyrtophyllum* and *S.*
 298 *octophylla* in the +NP and +P treatments, respectively (Fig. S2).

299 *Foliar phosphorus fractions*

300 Overall, only residual P increased with N addition (Table 2) and this result was largely due to
 301 an increase in residual P in *C. cyrtophyllum* in the +NP treatment (Table 3). There was a strong
 302 increase in all P fractions with P-addition (Tables 2 and 3), and species identity significantly
 303 influenced the response of metabolic P, nucleic acid P and residual P, and marginally influenced
 304 the response of structural P (P × species interactions; Table 2). Metabolic P (which includes P_i)
 305 increased the most in response to P-addition, followed by structural P and nucleic acid P (Table
 306 3 & Table S2). The pattern for residual P was less clear: there was a significant increase of
 307 residual P in *C. cyrtophyllum* and *P. rubra* in the +P and +NP treatments, but not in any other
 308 species (Table 3).

309 **Table 3** Concentrations of foliar phosphorus (P) fractions (mg g⁻¹ dry weight) of five woody plant
 310 species in a tropical forest following six years of fertilization with nitrogen (+N), phosphorus (+P) or
 311 both nitrogen and phosphorus (+NP) compared with controls (CT); means ±S.E. are given. Different
 312 superscript letters indicate significant differences among treatments at p<0.05.

Species	Treatment	Metabolic P	Nucleic acid P	Structural P	Residual P
<i>Clerodendrum cyrtophyllum</i>	CT	0.49±0.14	0.24 ^b ±0.06	0.32±0.03	0.12 ^b ±0.03
	+N	0.49±0.05	0.33 ^{ab} ±0.01	0.35±0.05	0.14 ^b ±0.01
	+P	1.36±0.25	0.44 ^{ab} ±0.06	0.54±0.08	0.27 ^b ±0.09
	+NP	1.23±0.26	0.47 ^a ±0.04	0.42±0.06	0.49 ^a ±0.27
<i>Syzygium bullockii</i>	CT	0.15 ^a ±0.02	0.24 ^{ab} ±0.02	0.09±0.05	0.24±0.02
	+N	0.13 ^a ±0.01	0.18 ^b ±0.01	0.10±0.04	0.19±0.02
	+P	0.43 ^a ±0.20	0.30 ^a ±0.03	0.28±0.08	0.24±0.01
	+NP	0.23 ^{ab} ±0.05	0.26 ^{ab} ±0.03	0.21±0.02	0.23±0.01
<i>Psychotria rubra</i>	CT	0.12±0.02	0.18 ^a ±0.01	0.13±0.03	0.42 ^b ±0.01
	+N	0.20±0.07	0.19 ^a ±0.03	0.16±0.02	0.46 ^{ab} ±0.02

	+P	0.30±0.07	0.24 ^a ±0.02	0.16±0.02	0.51 ^a ±0.03
	+NP	0.19±0.03	0.21 ^a ±0.02	0.15±0.03	0.53 ^a ±0.02
<i>Schefflera octophylla</i>	CT	0.21 ^b ±0.02	0.17 ^b ±0.01	0.35±0.03	0.10±0.01
	+N	0.24 ^b ±0.01	0.16 ^b ±0.01	0.36±0.03	0.10±0.00
	+P	0.47 ^a ±0.02	0.26 ^a ±0.01	0.47±0.03	0.10±0.01
	+NP	0.90 ^a ±0.28	0.33 ^a ±0.05	0.52±0.18	0.11±0.01
<i>Uvaria microcarpa</i>	CT	0.27±0.06	0.27±0.04	0.21±0.04	0.25±0.03
	+N	0.27±0.02	0.27±0.00	0.25±0.03	0.22±0.01
	+P	0.66±0.15	0.33±0.03	0.31±0.03	0.23±0.02
	+NP	0.60±0.15	0.27±0.02	0.31±0.04	0.21±0.00

313 Discussion

314 It is thought that the productivity of lowland tropical forests is limited by P, rather than N
315 (Thomas, Montagu & Conroy 2006; Pasquini & Santiago 2012). However, experimental
316 manipulations have failed to detect a consistent response to P addition in species-rich lowland
317 tropical forests (Cleveland *et al.* 2011; Wright *et al.* 2018). A recent study indicated that P
318 limitation is widespread at the level of individual species, but not at the community level,
319 because some species grow relatively rapidly on infertile soils, despite extremely low P
320 availability (Turner, Brenes-Arguedas & Condit 2018). Our study showing altered allocation to
321 foliar P fractions after P addition provides a potential mechanism to explain how tropical
322 species can maintain photosynthetic capacity under low P availability.

323 *Fertilization effects on foliar traits and P fractions*

324 Increased LMA has been widely reported as a sign of N or P deficiency in crop species (Chiera,
325 Thomas & Rufty 2002; Assuero, Mollier & Pellerin 2004) and although we observed increased
326 foliar [N] in response to N-addition in two species (*C. cyrtophyllum* and *S. bullockii*), we
327 measured no corresponding change in LMA. By contrast, LMA declined markedly with P

328 addition in all five species, especially in *C. cyrtophyllum* and *S. octophylla* (Fig. 1), suggesting
329 that P addition alleviated P deficiency in these species. This supports our first hypothesis that
330 foliar traits would show a greater response to P than N addition. Species-specific responses of
331 foliar nutrients and LMA to fertilization (Table 2, Fig. 1) may reflect differences in their life
332 history strategies and resource requirements (Sayer & Banin 2016). Overall, these results
333 provide evidence for P limitation of tree growth in the studied forest.

334 Despite several lines of evidence suggesting P limitation in tropical forests (Vitousek *et al.*
335 2010; Turner, Brenes-Arguedas & Condit 2018; Wright *et al.* 2018), photosynthetic capacity
336 was only increased by P addition in the eurytopic species *C. cyrtophyllum* which is consistent
337 with our second hypothesis and demonstrates that species growing on low-P soils can
338 maintain photosynthetic capacity. Similarly, there was no response of photosynthetic rate to
339 N and P fertilization in understory species of a tropical plantation (Zhu, Lu & Mo 2014) or in
340 response to P addition in lowland tropical forest understory seedlings after 10 years of P
341 fertilization (Pasquini & Santiago 2012). The results, combined with LMA results, also agree
342 with studies of several crop species which demonstrated that photosynthesis is far less
343 sensitive to fertilization than leaf growth (Chiera, Thomas & Rufty 2002; Assuero, Mollier &
344 Pellerin 2004; Dissanayaka *et al.* 2018).

345 Various adaptive strategies allow plants to acclimate to low P availability. Generally, plants on
346 P-impooverished soils have evolved physiological mechanisms for maximizing soil P acquisition,
347 such as increasing root surface area, carboxylate exudation, and phosphatase release (Reed
348 *et al.* 2011; Zemunik *et al.* 2015). Our results show that the low P availability in tropical forests
349 does not necessarily affect rates of photosynthesis. PPUE decreased with P addition in our
350 study, showing that the unfertilized plants have efficient P-use strategies and maintain
351 functions despite low availability of soil P. Increased PPUE has been observed in response to
352 decreasing P availability along a natural soil fertility gradient, where plants were able to

353 maintain rates of photosynthesis despite a remarkable decline in foliar [P] (Hidaka & Kitayama
354 2009). Changes in foliar P allocation may help explain how plants can maintain stable rates of
355 photosynthesis when P availability is low.

356 Vacuoles in plant cells serve as a reservoir of P_i (Mimura 1995), which is required for export of
357 triose phosphates from chloroplasts and for photophosphorylation. Accordingly, we observed
358 large increases in metabolic [P] (most of which is likely to be $[P_i]$; (Veneklaas *et al.* 2012), with
359 P addition in all species. The ability of plants to take up and store additional P_i or a relatively
360 low capacity to down-regulate P_i uptake (Ostertag 2010), may explain the observed large
361 changes in metabolic [P]. However, since photosynthetic capacity was unaffected by P
362 addition in the stenotopic species, we expect these plants were able to maintain sufficient
363 metabolic [P], despite low soil P status at the study site.

364 The mechanism by which plants maintain sufficient metabolic P concentrations to support
365 stable rates of photosynthesis has been reported in crop plants (Schlüter *et al.* 2013) and
366 Proteaceae from severely P-impoorished soils in Australia (Lambers *et al.* 2015), but has never
367 been reported for tropical lowland forests. In this study, the metabolic P fraction includes P_i ,
368 and when P availability decreases, excess P_i stored in vacuoles (Veneklaas *et al.* 2012) is
369 released to maintain the $[P_i]$ in the cytosol, followed by release of P from membrane
370 phospholipids (Mimura 1995; Schachtman, Reid & Ayling 1998; Lambers *et al.* 2012). As plant
371 growth rates decrease, nucleic acid P will eventually also be used to maintain the cytosolic $[P_i]$
372 in cells. Rates of photosynthesis will therefore only be affected when the cytosolic $[P_i]$ can no
373 longer be maintained (Schachtman, Reid & Ayling 1998). Hence, under low soil P availability,
374 a certain proportion of foliar P is allocated to metabolic [P] (including P_i) to maintain
375 photosynthesis which could draw P from structural P (Hidaka & Kitayama 2013), although we
376 found little evidence for this link in our study, possibly because the plants constitutively
377 function at low phospholipid concentrations, as in Proteaceae (Lambers *et al.* 2012). By

378 contrast, we observed the expected increases in all foliar P fractions with P-addition, with the
379 largest increase in [P_i] due to luxury uptake and storage of P_i (Ostertag 2010).

380 Our current knowledge of the residual P fraction is insufficient to explain why P-addition
381 resulted in a negative relationship between photosynthetic rates and residual [P] in this study
382 (Fig. 4); further work is needed to clarify the functional role of the residual P fraction.

383 *Insights into species-specific acclimation to P availability through foliar P allocation*

384 The distinct responses of foliar traits among the five study species revealed different P
385 requirements and allocation. It is noteworthy that *C. cyrtophyllum*, a eurytopic species with a
386 wide distribution from temperate to tropical zones, not only had the highest foliar [P] of all
387 species, regardless of treatment (Fig. 2), but was also the only species showing increased
388 photosynthetic capacity with P addition. The substantial increase in nucleic acid [P], combined
389 with the decline in LMA, suggests that leaf growth increased with P addition in this species,
390 but we did not measure this directly. These results, combined with the increased PNUE with
391 P addition, suggest that *C. cyrtophyllum* had the highest P requirement of all the studied
392 species, and its photosynthetic capacity was limited by low P availability in this tropical forest.
393 The high plasticity of *C. cyrtophyllum* for re-allocating P in response to P availability may also
394 help to explain its wide distribution in temperate and tropical forests (Fig. S1).

395 *Schefflera octophylla*, which is distributed in subtropical and tropical forest, also showed lower
396 LMA with P addition as well as increased concentrations of foliar P and all P fractions, except
397 residual P, but its photosynthetic capacity actually declined in the +P treatment. The distinct
398 responses of foliar traits in *S. octophylla* to individual nutrients compared with the +NP
399 treatment suggests that leaf area in this species is potentially P-limited, but that maintaining
400 foliar N:P stoichiometry may be more important for photosynthesis than increases in either
401 nutrient alone.

402 *Syzygium bullockii*, which only occurs in tropical forest, showed the greatest increase in
403 structural [P] in response to P addition, whereas LMA and photosynthetic capacity were not
404 affected by fertilization (Fig. 2). This suggests that *S. bullockii* utilizes more P from
405 phospholipids to maintain photosynthetic rates and leaf area when P availability is low.
406 Increased nucleic acid and structural P fractions with P fertilization in this species also suggest
407 that foliar growth is P limited. Finally, the lack of changes in photosynthetic capacity or LMA
408 in *P. rubra* and *U. microcarpa* following fertilization suggest that they are well adapted to low-
409 P soils. Both species only occur in tropical forests and *P. rubra* in particular showed no
410 evidence of P limitation, whereas small increases in metabolic and nucleic acid [P] in *U.*
411 *microcarpa* (Table S2), suggest that some foliar processes may be P-limited.

412 **Conclusions**

413 In tropical forests on P-poor soils, plants exhibit morphological, physiological, molecular, and
414 biochemical adaptations to low P availability. Although we found some evidence for P limitation
415 in this tropical forest, four species in this study maintained their photosynthetic capacity,
416 despite low P availability. Our measurements of foliar P fractions demonstrate that the study
417 species acclimated to low P availability by reducing P allocation to non-metabolic foliar P
418 fractions to meet their demand for metabolic P to maintain photosynthetic capacity. We
419 conclude that P limitation of leaf photosynthetic capacity may not be as common as previously
420 assumed for lowland tropical forests, and some species have a high capacity to maintain their
421 photosynthetic rate in low-P soils. The species-specific strategies to allocate P to different
422 foliar fractions represents a potentially important adaptation mechanism for plants in P-
423 limited systems.

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430 **Author contributions:**

431 F.W. and Z.L. planned and designed the research. Q.M., B.Z., Y.L. and S.Y. conducted field work.
432 F.W., Q.M. and E.S. analyzed data. F.W., Q.M., E.S., H.L., J.T., M.H., Z.L. and X.Z. wrote the
433 manuscript.

434 **Data accessibility:**

435 All data associated with this manuscript will be archived for public access should the
436 manuscript be accepted.

437 **Conflicts of interests:**

438 The authors declare that they have no conflict of interests.

439

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563

564 **Supplementary materials**

565 **Table S1** Species-specific linear mixed model analysis (*p*-values) for leaf traits, nutrient concentrations and phosphorus (P) fractions of five study species in a tropical
 566 forest following six year fertilization with nitrogen (N) and phosphorus (P), where LMA is leaf mass per area; PNUE is photosynthetic nitrogen use efficiency; PPUE is
 567 photosynthetic phosphorus use efficiency, and [N] and [P] are foliar nitrogen and phosphorus concentrations, respectively.

Species	Sources	A _{mass}	LMA	[N]	[P]	N:P ratios	PNUE	PPUE	Metabolic P	Nucleic P	Structural P	Residual P
<i>Clerodendrum cyrtophyllum</i>	N	0.026	ns	0.020	ns	ns	0.010	ns	ns	ns	ns	0.043
	P	ns	0.006	0.027	<0.001	<0.001	ns	ns	0.006	0.001	0.049	<0.001
	N×P	0.006	ns	ns	ns	ns	0.007	ns	ns	ns	ns	0.06
<i>Syzygium bullockii</i>	N	ns	ns	0.014	ns	0.006	ns	ns	ns	ns	ns	ns
	P	ns	ns	ns	0.012	<0.001	ns	ns	0.022	0.004	ns	ns
	N×P	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
<i>Psychotria rubra</i>	N	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	P	ns	ns	ns	0.051	0.005	ns	ns	ns	ns	ns	0.002
	N×P	ns	ns	0.05	ns	ns	ns	ns	ns	ns	ns	ns
<i>Schefflera octophylla</i>	N	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	P	ns	0.02	ns	0.022	<0.001	ns	0.013	0.021	0.001	0.019	ns
	N×P	0.021	ns	ns	ns	ns	0.029	ns	ns	ns	ns	ns

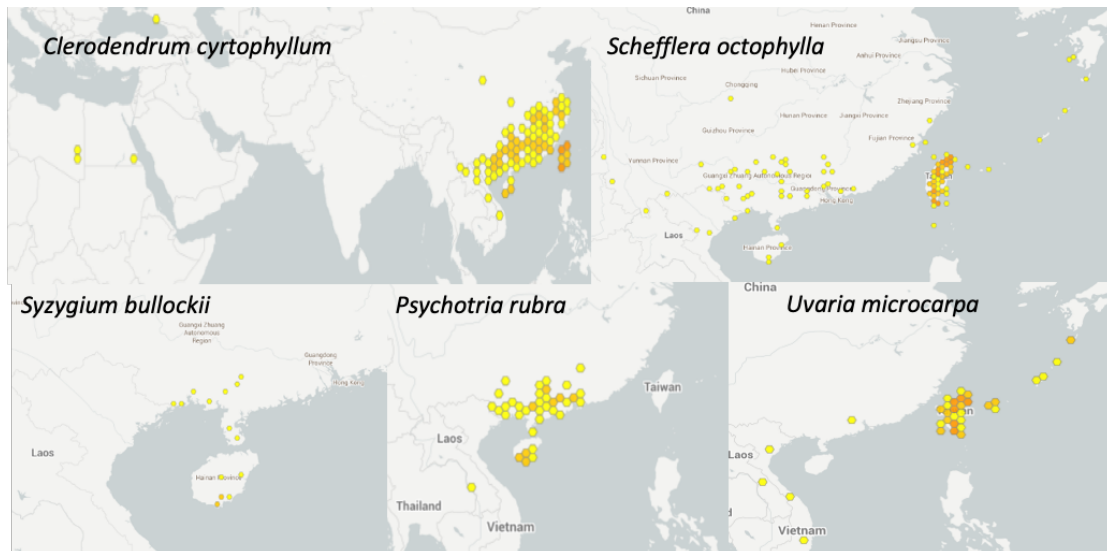
<i>Uvaria microcarpa</i>	N	<i>ns</i>	<i>ns</i>	<i>ns</i>	<i>ns</i>	<i>ns</i>	<i>ns</i>	<i>ns</i>	<i>ns</i>	<i>0.04</i>	<i>ns</i>	<i>ns</i>
	P	<i>ns</i>	<i>ns</i>	<i>ns</i>	<i>0.025</i>	<i>0.05</i>	<i>ns</i>	<i>ns</i>	<i>0.016</i>	<i>0.07</i>	<i>0.05</i>	<i>ns</i>
	N×P	<i>ns</i>	<i>ns</i>	<i>ns</i>	<i>ns</i>	<i>ns</i>	<i>0.045</i>	<i>ns</i>	<i>ns</i>	<i>ns</i>	<i>ns</i>	<i>ns</i>

568

569 **Table S2** Relative proportions (mean % \pm S.E.) of foliar phosphorus (P) fractions in five study species
 570 in a tropical forest following six years of fertilization with nitrogen (+N), phosphorus (+P) or both
 571 nutrients (+NP), compared to controls (CT); different superscript letters indicate significant
 572 differences among treatment at $p < 0.05$.

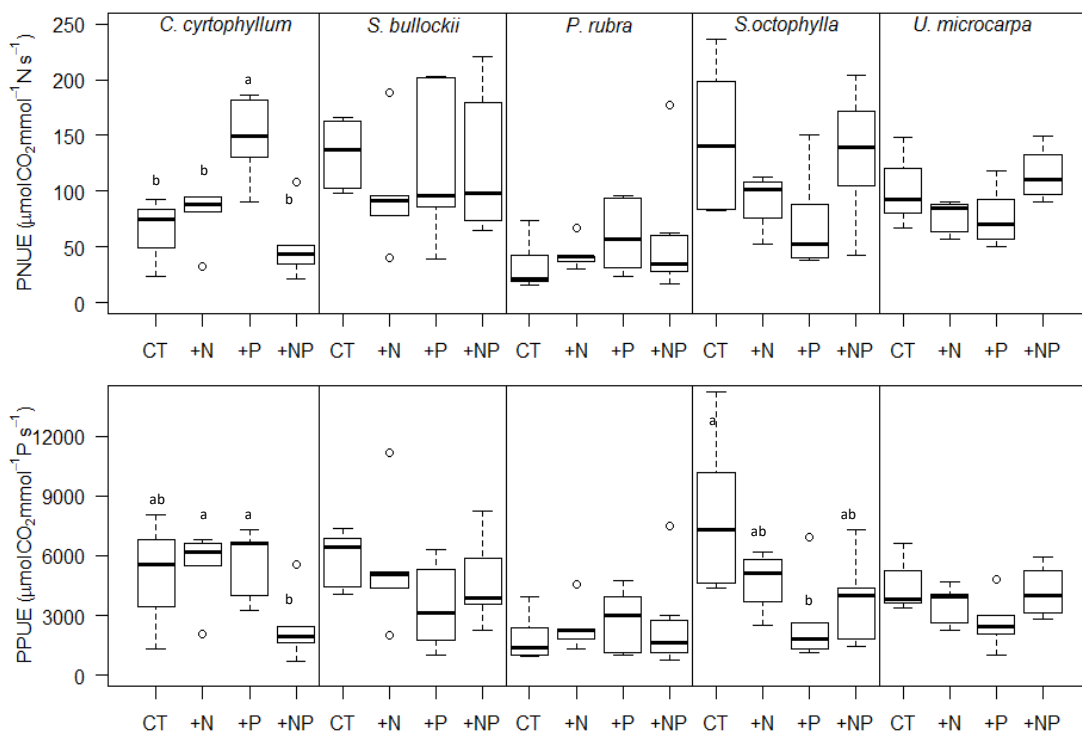
Species	Treatment	Metabolic P	Nucleic acid P	Structural P	Residual P
<i>Clerodendrum cyrtophyllum</i>	CT	40.6 \pm 5.07	20.3 \pm 1.42	29.4 \pm 5.40	9.76 ^b \pm 0.66
	+N	37.7 \pm 3.85	25.4 \pm 1.40	26.2 \pm 2.94	10.7 ^b \pm 1.02
	+P	48.8 \pm 2.95	18.6 \pm 2.26	23.2 \pm 3.40	9.41 ^b \pm 1.28
	+NP	45.5 \pm 4.14	18.2 \pm 1.19	16.8 \pm 2.51	19.5 ^a \pm 2.46
<i>Syzygium bullockii</i>	CT	19.1 ^b \pm 2.49	31.4 \pm 2.81	16.5 \pm 6.89	32.7 \pm 2.37
	+N	21.9 ^{ab} \pm 1.47	29.8 \pm 1.81	17.1 \pm 5.83	31.2 \pm 2.76
	+P	28.6 ^a \pm 7.07	26.8 \pm 3.39	22.7 \pm 1.55	23.1 \pm 3.51
	+NP	24.0 ^{ab} \pm 4.57	28.0 \pm 1.48	21.6 \pm 2.41	25.4 \pm 2.11
<i>Psychotria rubra</i>	CT	13.7 ^b \pm 1.77	21.7 \pm 1.79	14.3 \pm 2.98	50.4 \pm 3.04
	+N	17.6 ^{ab} \pm 3.12	19.0 \pm 0.65	15.7 \pm 0.57	47.8 \pm 3.14
	+P	21.6 ^a \pm 2.93	20.1 \pm 0.54	13.2 \pm 1.73	45.2 \pm 1.66
	+NP	17.7 ^{ab} \pm 2.11	19.5 \pm 1.27	13.4 \pm 2.34	49.4 \pm 2.14
<i>Schefflera octophylla</i>	CT	24.8 ^b \pm 1.38	20.3 \pm 0.81	42.1 \pm 0.74	12.9 \pm 1.57
	+N	28.4 ^{ab} \pm 1.71	18.7 \pm 1.10	41.4 \pm 1.54	11.5 \pm 0.36
	+P	34.7 ^a \pm 2.24	20.2 \pm 0.36	37.4 \pm 2.13	7.70 \pm 1.11
	+NP	44.0 ^a \pm 5.82	19.0 \pm 2.25	27.6 \pm 7.09	9.42 \pm 2.58
<i>Uvaria microcarpa</i>	CT	26.5 ^b \pm 2.95	26.8 ^a \pm 1.11	21.8 \pm 4.62	25.0 ^a \pm 1.60
	+N	27.8 ^b \pm 0.95	23.7 ^{ab} \pm 0.90	25.9 \pm 1.86	22.6 ^{ab} \pm 0.76
	+P	41.5 ^a \pm 3.63	22.0 ^{ab} \pm 1.07	20.8 \pm 1.00	15.7 ^b \pm 1.77
	+NP	41.4 ^a \pm 4.35	20.3 ^b \pm 1.81	22.4 \pm 1.05	15.9 ^b \pm 1.96

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574

575 Fig. S1. The distribution ranges of the five study species in the tropical forest of south China. The data
 576 was extracted from Global Biodiversity Information Facility data base (www.gbif.org), the yellow point
 577 indicates the occurrence of each species by observations or specimen.



578

579 **Fig. S2.** Box-plots of photosynthetic nitrogen (N) and phosphorus (P) -use efficiency for five species
 580 in a tropical forest following six years of fertilization with N (+N), P (+P) or both nutrients (+NP)
 581 compared to controls (CT). Different letters within panels indicate significant post-hoc differences

582 among treatments at $p < 0.05$.

583