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The P limitation of Pinus massoniana reforestation increases with stand maturity: Evidence from plants, leaf litter, and soil

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Research Article

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1	The P limitation of <i>Pinus massoniana</i> reforestation increases with stand maturity: Evidence
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3	
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32 Abstract

33	Background and Aims: Soil phosphorus (P) regulates plant productivity. However, the medium to
34	long-term time-scale effects of reforestation on the P recycle of plants, litter, and soil continuum,
35	thus regulating soil P storage, especially deep-soil P fractions dynamics, are unclear.
36	Methods: We determined soil (0~100 cm) P fractions, root and leaf N/P ratio, leaf-litter P
37	concentration, and biogeochemical drivers in 32-, 45-, and 60-year-old Pinus massoniana
38	reforestations in southwest China.
39	Results: The storages of soil labile, moderately labile, and occluded P in the 45- and 60-year stands
40	were lower than the 32-year stand. The concentrations of NaHCO ₃ -Pi, NaHCO ₃ -Po, and total labile
41	P in top-soil were lower while those in sub- and bottom-soil were not significantly in the 45- and
42	60-year stands compared to the 32-year stand. The concentrations of all soil layer NaOH-Po and
43	total moderately labile P were lower in the 45- and 60-year stands than the 32-year stand. The
44	concentrations of all soil layers C.HCl-Pi and total occluded P were lower in the 60-year stand than
45	the 32-year stand. The leaf N/P ratio and top-soil C/P ratio and acid phosphatase were higher and
46	the leaf-litter P concentration was lower in the older stands.
47	Conclusions: P. massoniana secreted more acid phosphatase and increased leaf P resorption to
48	compensate for the drop in soil P-availability with stand age, which in turn decreased leaf-litter P

input and thus resulted in a depletion of soil P. Overall, our results highlighted that P limitation
increased with stand maturity in *P. massoniana* reforestations.

51 Keywords Phosphorus limitation; Soil phosphorus fractions; Stand development; Stoichiometry;
52 *Pinus massoniana*.

54 **1. Introduction**

55 Phosphorus (P) is present in molecules from DNA to membrane lipids to the compounds that shuttle 56 energy in cells (Némery and Garnier 2016) and plays key roles in promoting plant net primary 57 productivity (Barbieri et al. 2021; Peñuelas et al. 2020), regulating soil carbon (C) sequestration 58 (Ellsworth et al. 2022; Wright 2022), and maintaining ecosystem sustainability (Chen et al. 2022b; Peñuelas and Sardans 2022). Soil is the main source of P absorbed by plants, storing more than 90% 59 60 of the total P stocks in terrestrial natural ecosystems (Zhang et al. 2021a). However, it is estimated that 43% of the land area on Earth is associated with P limitation, and another 39% area is co-limited 61 62 alongside nitrogen (N) (Du et al. 2020). The trade-off between maintaining soil P storage and 63 improving plant productivity, therefore, has become one of the important drivers for inspiring global 64 political disputes in recent years (Barbieri et al. 2021; Langhans et al. 2021; Tonini et al. 2019; 65 Withers 2019). Among them, exploring plant P utilization strategies (Turner et al. 2018; Vallicrosa 66 et al. 2023; Yu et al. 2022), clarifying the fate and patterns of releasing P from litter (Strukelj et al. 67 2017; Tie et al. 2023), and elucidating soil P-fractions and their transformation processes 68 (Brandtberg et al. 2010; Liu et al. 2021; Vincent et al. 2013) are the fundamental scientific questions. 69 Tropical and subtropical forest ecosystems are the key to driving global C and nutrient cycles 70 (Wieder et al. 2015; Yu et al. 2022). However, these forests are often associated with P limitation 71 due to strong soil weathering (Tie et al. 2023; Yang et al. 2021). In addition, the subtropical and 72 tropical forests grow fast and have high net primary productivity, thus P can be taken up from soil 73 and transported to plants (e.g., leaves, trunks, roots) quickly, which potentially results in a loss of 74 soil P and thus reduced soil P concentration (Peñuelas et al. 2011; Vallicrosa et al. 2023). For 75 example, prior studies reported that soil total P concentration and its availability decreased with

stand development across tropical and subtropical forests (Huang et al. 2013; Izquierdo et al. 2013; Selmants and Hart 2010). However, some studies have reported that the soil total P increased (Brandtberg et al. 2010; Zhu et al. 2021) or had a neutral response (Liu et al. 2021; Peng et al. 2023) with stand development due to the increase in vegetation diversity via natural succession. Overall, we found inconsistencies in the literatures that reported the effect of forest stand development on the soil total P concentration dynamics across tropical and subtropical forests.

82 Moreover, the P recycle (e.g., plants P returns to soil via litter-fall) and its balance among plants, 83 litter, and soil continuum controls the dynamics of soil P fractions (Peñuelas et al. 2011; Strukelj et 84 al. 2017; Zhu et al. 2021), which increases the unpredictability of soil P responses to stand 85 development. Multiple potentially biogeochemical mechanisms can explain the dynamics of soil P 86 during stand development, including: i) the accumulative plant P uptake increases with stand 87 development due to the increase in plant biomass, resulting in a lower soil P concentration (Vu et al. 88 2007; Zhu et al. 2021); ii) the changes in litter production and its P concentration during forest stand 89 development alters litter P input (Brandtberg et al. 2010; Francos et al. 2023; Richardson et al. 2008); 90 iii) phosphatases secreted by plants and microorganisms stimulates soil mineralization, which 91 affects the transformation among soil P fractions (Celi et al. 2013; Peng et al. 2023); iv) soil acidity 92 increases with stand development, so soil P is fixed with iron (Fe), aluminum (Al), and/or calcium 93 (Ca) and thus transformed P into more stable forms (Selmants and Hart 2010; Zhu et al. 2021); and 94 v) P deposition in some areas with high level can directly increase P input and thus alter soil P 95 concentration (Wang et al. 2017). Nevertheless, most prior studies only focused on the dynamics of top-soil (e.g., \leq 20 cm) P during stand development. In fact, it is estimated that more than 70% of 96 97 soil P stocks in terrestrial ecosystems are stored in deep-soil (e.g., 20 - 100 cm) (He et al. 2021).

98 Recent studies highlighted that the biogeochemistry cycle of deep-soil is different from that of top-99 soil due to the distinction of microbial diversity and root distribution (Henneron et al. 2022; Yang 100 et al. 2021), which implies that soil P fractions and their transformation processes are most likely 101 varied with soil depth. However, the dynamic of P fractions in deep-soil, especially those deeper 102 than 60 cm during stand development of tropical and subtropical forests, and the specific 103 biogeochemical transformation mechanism are still unclear.

104 South China is an important case region in the subtropics, as it has 71% of total subtropical forest areas worldwide including one of the largest areas of reforestation (Fang et al. 2001; Peng et 105 106 al. 2014; Tong et al. 2020; Zhu et al. 2021). It is estimated that more than 20 million hectares of 107 fast-growing tree species were reforested from the 1960s to 2015 due to the rapid increase in demand 108 for wood (Peng et al. 2014; Tong et al. 2020). Among them, 20% to 30% of the total areas are Pinus 109 massoniana (one of the typical fast-growing pioneer tree species) where stand ages mainly vary 110 from 30 to 60 years including near-mature stands (31 to 40 years), mature stand (41 to 50 years), and over-mature stand (51 to 60 years) (Bai et al. 2023; Zhu et al. 2021). Soil P storage and its 111 112 availability are crucial for the productivity and forest sustainability of extensive reforested P. 113 massoniana, since soil P likely cannot be replenished in a timely manner in fast-growing subtropical pure forests (Chen et al. 2022a; Liu et al. 2021). Notwithstanding, prior studies have primarily 114 115 reported the differences in top-soil P availability among P. massoniana reforestation, mixed P. 116 massoniana, and broadleaf forest, and evergreen broadleaf forest in south China (Huang et al. 2013; Zhu et al. 2021). However, limited information is available on the dynamics of soil (especially deep-117 118 soil) P fractions and its relationship with P recycle among plants and litters during stand maturity (e.g., transition from near-mature stand to over-mature stand) of *P. massoniana* reforestation. 119

120	Here, we determined the P fractions at the top- $(0 - 20 \text{ cm})$, sub- $(21 - 60 \text{ cm})$, and bottom-
121	soil (61 – 100 cm) layers using sequential extraction procedure and calculated soil P storage in P.
122	massoniana reforestations located in southwest China with stand age of 32-, 45-, and 60-year. The
123	potential biogeochemical drivers, e.g., root and leaf N/P ratios, litter P concentration, soil acid
124	phosphatase activity, soil pH, and soil stoichiometric ratios, were also determined in this study. We
125	aim to: i) study the dynamics of soil P fractions at top-soil and specially sub- and bottom-soil layers
126	across different stand maturity classes of P. massoniana reforestations; and ii) study the variations
127	of plant P uptake (e.g., root and leaf N/P ratios), leaf-litter P input (e.g., leaf-litter P concentration),
128	soil P mineralization (e.g., soil acid phosphatase activity), soil P fixation (e.g., acidification), and
129	soil stoichiometry ratios, thus improving the understanding of soil P biogeochemical transformation
130	mechanism. Since P. massoniana is a fast-growing tree species, the following hypotheses were
131	tested:
132	H1: Although P. massoniana roots can secrete acid phosphatase to activate insoluble phosphate, the
133	concentrations and storage of soil labile and moderately labile P will decrease due to accumulated
134	plant's P uptake and reduced leaf-litter P input, thus aggravating P limitation in the older stands.
135	H2: The decrease of P availability with stand development will be larger in top-soil compared to
136	deep-soil, because the fine roots of <i>P. massoniana</i> and its mediated P uptake mainly occurred in top-
137	soil.
138	H3: The storage of soil occluded P will decrease with stand development, since some of them are

139 transferred to availability soil P fractions.

2. Materials and methods

142	The study site was located at the National Long-term Scientific Research Site of Guizhou University
143	(106°43' E, 26°43' N; 1200~1300 m a.s.l.; Fig S1A), with a subtropical plateau humid monsoon
144	climate. The mean annual temperature is 13.6 °C and the mean annual precipitation is 1156 mm. In
145	the study site, approximately 50, 100, and 80 hectares of <i>P. massoniana</i> were reforested in 1963
146	(60-year-old), 1978 (45-year-old), and 1991 (32-year-old), respectively. The original vegetation
147	type in the study site was mixed evergreen and deciduous broadleaf forests before reforesting P.
148	massoniana. The P. massoniana reforestations at our study site were thinned when the canopy
149	density exceeded 0.9, with a thinning intensity of less than 20% at each time. The logging residues
150	(e.g., deadwood, branches, and leaves) were also removed at each thinning. The stand density of the
151	60-, 45-, and 32-year reforested stands were 225 \pm 35.4, 246 \pm 30.9, and 720 \pm 95.3 individual
152	ha-1, respectively. The soil was old alluvial yellow loam classified as a Ferralsol according to the
153	World Reference Base for Soil Resources (WRB 2015), and the depth to bedrock was approximately
154	120 cm. The annual average atmospheric P deposition in the study site was approximately 0.10 g P
155	$m^{-2} y^{-1}$ (Zhu et al. 2016).

156 2.2. Experimental design

Fifteen plots of 20×20 m² were established from 15 to 30 August 2022 with five replicates for each age group (Fig. S1A – C). The distance of each plot was at least 500 m. The vegetation diversity in each plot was investigated, and except for *P. massoniana*, the other vegetation species come from natural succession. The results can be found in Table S1. In addition, the DBH of *P. massoniana* trees in each plot was measured, and the average DBH of the 60-, 45-, and 32-year stands were 34.9 162 \pm 3.88 cm, 27.6 \pm 2.89 cm, and 20.1 \pm 1.03 cm, respectively.

163 2.2.1. Leaf and root sampling

164 Three trees that represent the average stand-level DBH were selected from each plot. The mature 165 leaves of the upper part of selected trees' canopy were collected and then mixed to form a composite 166 sample according to each study plot. The fine roots (≤ 2 mm) of the selected trees from top-soil (0 167 - 20 cm) were collected and mixed to form a composite sample. In the laboratory, the leaf and root 168 samples were oven-dried (65 °C for 96 h) and were then passed through a 0.1-mm sieve prior to 169 determining the concentrations of total N and total P.

170 2.2.2. Leaf-litter sampling

The prior study reported that approximately 90% amount of the annual litter production in *P. massoniana* reforestation was leaf litter (Bai et al. 2023), so the present study mainly focused on this type of litter. Five leaf-litter collection boxes (the length, width, and height were 50 cm; made of nylon with 0.1 mm mesh size) were arranged in each studied plot from 15 to 30 August 2022 (see Fig S1B). Fresh fallen leaves within the boxes were collected and then mixed to form a composite sample. The composite leaf-litter samples were transported to the laboratory and were oven-dried (65 °C for 96 h) and passed through a 0.1-mm sieve prior to measuring the concentration of total P.

178 2.2.3. Soil sampling

Five soil profile subplots were arranged at the midpoint and quarter of the diagonal of the study plot
(see Fig S1B and C). Mosses and litters were removed from the soil surface, and then soil samples

at the 0 - 20 cm, 21 - 60 cm, and 61 - 100 cm layers were collected from each profile and were mixed to form a composite soil sample. The composite soil samples were transported to the laboratory and were air-dried and passed through 2 mm and 0.149 mm sieves prior to measuring the activity of acid phosphatase, concentrations of soil organic carbon (SOC), total N, P fractions, and pH. In addition, cutting rings were used to collect 0 - 20 cm, 21 - 60 cm, and 61 - 100 cm layers of soil samples from each profile and then transported to the laboratory prior to measuring the soil bulk density.

188 2.2.4. Chemical analyses

Leaf and root samples were digested with 10 mL of a 5:1 mixture of sulfuric acid and perchloric acid prior to determining the concentrations of total N and total P using an automatic discontinuous chemical analyzer (Smart-Chem 200, Paris, France) (Cleveland 2002). Leaf-litter samples were also digested with 10 mL of a 5:1 mixture of sulfuric acid and perchloric acid prior to determining the total P concentration.

194 The soil bulk density was determined using the cutting rings method (Chen et al. 2015). The 195 soil pH was determined in a mixture of soil and deionized water for removing CO₂ (soil: water = 1: 196 2.5) using a glass electrode in aqueous extracts (Allen et al. 1974). The activity of acid phosphatase 197 (E.C. 3.1.3.2) was determined using para-nitrophenyl phosphate as the orthophosphate monoester 198 analog substrate (Schinner et al. 1996; Tabatabai 1982). The SOC concentration was determined using dichromate oxidation-ferrous sulfate titration (Schinner et al. 1996). The soil total N 199 200 concentration was determined by Kjeldahl digestion, and the soil total P concentration was 201 determined by molybdenum-antimony colorimetry (Allen et al. 1974).

202	The sequential extraction procedure developed by Hedley et al. (1982) and modified by Sui et
203	al. (1999) and Tiessen and Moir (2008) has been widely used, becoming a key means of studying
204	soil P cycle (Brandtberg et al. 2010; Chen et al. 2015; Langhans et al. 2021; Liu et al. 2021). This
205	modified sequential extraction methodology divides soil P into nine different forms (Hedley et al.
206	1982; Sui et al. 1999; Tiessen and Moir 2008). As labile P fractions, H ₂ O-Pi (inorganic P extracted
207	using H ₂ O) is a form that can be directly absorbed by plants, while NaHCO ₃ -Pi (inorganic P
208	extracted using NaHCO ₃) and NaHCO ₃ -Po (organic P extracted using NaHCO ₃) are forms that can
209	be desorbed and dissolved into soil available P in a short period of time. In contrast, it takes a longer
210	time to transform NaOH-Pi (inorganic P extracted using NaOH), NaOH-Po (organic P extracted
211	using NaOH), and D.HCI-P (extracted using dilute hydrochloric acid) into soil available P, thus they
212	are divided into moderately labile P fractions. Concentrated HCI extraction of P (C.HCI-Pi, C.HCI-
213	Po) and total residual P (Residual Pt) are considered as occluded P fractions because these forms of
214	P are encapsulated by minerals and are relatively difficult to utilize by plants and microorganisms
215	(Hedley et al. 1982; Sui et al. 1999; Tiessen and Moir 2008).
216	Thus, the soil P fractions in this study were determined using the sequential extraction

IJ Ч 217 procedure developed by Hedley et al. (1982) and modified by Sui et al. (1999) and Tiessen and Moir (2008). Specifically, 0.5 g of dried soil (through a 2-mm sieve) was repeatedly determined by 218 219 increasing chemical strength. Soil P was divided into nine fractions, i.e., H₂O-Pi (extracted with 30 220 mL deionized water), NaHCO3-P (including NaHCO3-Pi and NaHCO3-Po; extracted with 30 mL 0.5 mol L⁻¹ NaHCO₃ solution), NaOH-P (including NaOH-Pi and NaOH-Po; extracted with 30 mL 221 0.1 mol L⁻¹ NaOH solution), D.HCl-Pi (extracted with 30 mL 1 mol L⁻¹ dilute HCl), C.HCl-P 222 223 (including C.HCl-Pi and C.HCl-Po; extracted with 30 mL concentrated HCl), and Residual-Pt (extracted with 5 mL 6:1 HClO₄ and HNO₃ + 5 mL concentrated H₂SO₄). More detailed information can be found in Fig. S2.

226 2.3. Data calculation

The soil, root, and leaf stoichiometric ratios in this study are mass-based, e.g., soil C/P ratio is the ratio of SOC to total P concentrations (g kg⁻¹); soil, root, and leaf N/P ratio are the ratio of total N to total P concentrations (g kg⁻¹). Soil P storage (g P m⁻³) was calculated by the following equation (Zhang et al. 2021a):

231 Soil P storage =
$$\sum \rho_i \times d_i \times c_i$$

232 Where ρ_i and *ci* are the bulk density (g cm⁻³) and the concentration of soil P fractions (mg kg⁻ 233 ¹) of the *i*th layer of soil, respectively; d_i is the depth of the *i*th layer of soil (m).

235 The Shapiro-Wilk test and Levene's test were first applied to test the normality and homogeneity 236 of variance of each response variable. The Box-Cox method was then used to transform the variables 237 with nonnormal distributions or unequal variances (i.e., NaHCO₃-Po, NaOH-Pi, and D.HCl-Pi 238 concentrations) to normal distribution. Linear mixed effects models employing the restricted 239 maximum likelihood (REML) estimation method were used to determine the effects of stand age, 240 soil depth, and their interaction, as fixed independent factors, on the soil P fraction concentrations, 241 soil stoichiometric ratios, soil acid phosphatase, soil pH, and soil bulk density; and to determine the 242 effects of stand age on the percentage of soil P fractions to total P, soil P storage, root and leaf N/P ratios, and leaf-litter P concentration. The plot was taken as an independent random factor. The
Bonferroni adjustment method was applied to the confidence intervals and significance values to
account for multiple comparisons. Statistical analyses were performed using SPSS 25.0 for
Windows (SPSS Inc., Chicago, USA).

247 **3. Results**

- 248 3.1. Concentrations of soil P fractions
- 249 The main effect of stand age, soil depth, and their interactions on the concentrations of NaHCO₃-Pi, NaHCO₃-Po, and total labile P was significant (Fig. 1B - D). The main effect of stand age on the 250 251 concentrations of NaOH-Pi, NaOH-Po, total moderately labile P, C.HCl-Pi, and total occluded P 252 and the main effect of soil depth on the concentration of NaOH-Po was significant (Fig. 2A-B, D 253 and Fig.3A and D). Specifically, compared to the 32-year plots, the concentrations of top-soil NaHCO₃-Pi, NaHCO₃-Po, NaOH-Po, and total labile P were significantly lower in the 45-year and 254 255 60-year plots; and the concentration of all soil layer total moderately labile P was significantly lower 256 in the 45-year and 60-year; and the concentration of all soil layer NaOH-Pi, C.HCl-Pi, and total 257 occluded P were also significantly lower in the 60-year plots.
- 258 3.2. Contribution of soil P fractions to total P

259 In the top-soil, the contribution of labile P and moderately labile P were significantly lower (Fig. 4)

- and the contribution of occluded P was significantly higher in the 45-year and 60-year plots than in
- the 32-year plots. In the sub- and bottom-soil, the contribution of occluded P was only significantly
- higher in the 45-year plots than in the 32-year plots, while it was insignificant between the 60-year

264 3.3. Storage of soil P fractions

The soil total P storage significantly decreased with the increase of stand age (Fig. 5). Specifically, soil labile P storage was significantly lower by 20.9 g P m⁻³ and 24.5 g P m⁻³, respectively, and the moderately labile P storage was significantly lower by 213.8 g P m⁻³ and 248.6 g P m⁻³, respectively, in the 45-year and 60-year plots than that in the 32-year plots (the storage of soil labile P and moderately labile P were 43.4 ± 3.67 g P m⁻³ and 453.5 ± 40.5 g P m⁻³, respectively). Soil occluded P storage was significantly lower by 273.8 g P m⁻³ in the 60-year plots than in the 32-year plots (712.2 ± 76.9 g P m⁻³).

272 3.4. Dynamics of soil main properties

The main effect of stand age on soil bulk density and pH was insignificant (Fig. 6A - B), while the main effect of stand age, soil depth, and their interactions on soil C/P and N/P ratios and acid phosphatase were significant (Fig. 6C - E). Specifically, the C/P ratio and acid phosphatase in the top-soil were significantly higher in the 45- and 60-year plots than in the 32-year plots, while they in the sub- and bottom-soil were insignificant among all the stands. The top- and sub-soil N/P ratios were significantly higher in the 60-year plots than in the 32-year plots.

- 279 3.5. Dynamics of root and leaf N/P ratios and leaf-litter P concentration
- 280 The root N/P ratio in the 60-year plots (22.8 ± 3.27) was significantly higher than that in the 32-year
- 281 plots (16.0 \pm 1.99; Fig. 6F). The leaf N/P ratio in the 45- and 60-year plots were 19.9 \pm 1.77 and

 19.8 ± 2.18 , respectively (Fig. 6G), which were significantly higher than that in the 32-year plots (15.8 ± 1.55) . The leaf-litter P concentration in the 45- and 60-year plots was 0.87 ± 0.07 g kg⁻¹ and 0.86 ± 0.08 g kg⁻¹, respectively (Fig. 6H), which were significantly lower than that in the 32-year 285 plots $(1.10 \pm 0.12$ g kg⁻¹).

286 4. Discussion

Our results highlighted that although the top-soil acid phosphatase activity was higher in the 45-287 288 and 60-year stands than in the 32-year stand (Fig. 7A and B), the top-soil total labile P and total 289 moderately labile P concentrations (irrespective of soil layers) were lower in the older stands, which 290 resulted in an exacerbating P limitation based on the increased leaf and/or root N/P ratio. This result 291 supported our first hypothesis. The concentrations of NaHCO₃-Pi, NaHCO₃-Po, and total labile P at 292 top-soil were lower in the older stands than in the 32-year stand while the differences were neutral 293 at sub- and bottom-soil, which was consistent with our second hypothesis, highlighting a larger 294 decrease of soil P availability at top-soil. Moreover, the top-, sub-, and bottom-soil total occluded P 295 concentrations and their storage were lower in the 60-year stand, which was consistent with our 296 third hypothesis predicting the soil occluded P would decrease with stand maturity.

297 4.1. P limitation in *P. massoniana* reforestation increased with stand maturity

Nitrogen and P are the main limiting nutrients in terrestrial ecosystems, and the ecosystems can be divided into N limitation, P limitation, and N and P co-limitation (Du et al. 2020). Several methods are often used to infer nutrient limitations, including the threshold for leaf N/P ratio and nutrient reabsorption (Du et al. 2020). For example, prior studies have highlighted that the leaf N/P ratio was 302 higher than 16 (in mass basis), which indicates that the plant is limited by P, while the ratio less than 303 16 indicates a N and P co-limitation (from 14 to 16) or N limitation (less than 14) (Koerselman and 304 Meuleman 1996). In this study, the leaf N/P ratio was significantly increased from 15.8 ± 1.55 in the 32-year stand to 19.9 ± 1.77 and 19.8 ± 2.18 in the 45- and 60-year stands, respectively (Fig. 305 306 6G), suggesting that P limitation exacerbated during the stand maturity (Elser et al. 2010; Sardans 307 et al. 2021). Nutrient reabsorption is the process of nutrient transportation from senescent leaves to 308 other organs, which leads to a decrease in nutrient concentrations in fallen leaves but relatively 309 stable nutrient concentrations in newly grown organs (Richardson et al. 2008; Yuan and Chen 2015). 310 This phenomenon is a strategy for plant's response to nutrient scarcity, and it is used to identify 311 nutrient limitations (Du et al. 2020; Yuan and Chen 2015). Our results showed that leaf-litter P 312 concentration was lower in the 45- $(0.87 \pm 0.07 \text{ g kg}^{-1})$ and 60-year $(0.86 \pm 0.08 \text{ g kg}^{-1})$ stands than 313 in the 32-year $(1.10 \pm 0.12 \text{ g kg}^{-1})$ stand (Fig. 6H), which furtherly confirmed that P limitation 314 increased with stand development of P. massoniana due to an increase in leaf P reabsorption 315 (Richardson et al. 2008).

4.2. Top-soil labile P storage decreased more than that at deep-soil during the stand maturity



al. (2013) and Zhu et al. (2021). This phenomenon, in the present study, was because the
concentrations of top-soil NaHCO₃-Po and NaHCO₃-Pi were lower in the 45- and 60-year stands
than that in the 32-year stand, resulting in a lower total labile P concentration (Fig. 1B – D and Fig.
7).

327 As one of the key biochemical factors driving soil P cycling, soil phosphatase can promote the mineralization of soil organic P into inorganic P forms (Celi et al. 2013; Sardans et al. 2021). 328 329 Therefore, the higher soil phosphatase activity, the stronger soil mineralization; and vice versa (Liu et al. 2021; Sardans et al. 2021). In our study, top-soil acid phosphatase activity in the 45- and 60-330 331 year stands were higher by 30.3% and 56.9%, respectively, than that in the 32-year stand (Fig. 7), 332 which strongly suggests that soil P mineralization was greater in the older stands, thus resulting in a lower NaHCO₃-Po concentration (Celi et al. 2013; Liu et al. 2021; Sardans et al. 2021). 333 334 Interestingly, the top-soil NaHCO₃-Pi concentration also decreased with stand development (Fig. 335 1B). This result can be explained although microorganisms and roots can mineralize soil organic P 336 to inorganic P by secreting more acid phosphatase, the mineralization rate is probably lower than 337 plant P uptake rate. For example, Zhu et al. (2021) reported that although increased soil (< 30 cm) 338 acid phosphatase activity stimulated P mineralization, soil inorganic P concentration overall 339 decreased with stand development due to the increased plant P use efficiency (PUE). An additional 340 reason can also explain the decrease of top-soil labile P concentrations and its storage during the 341 stand maturity in the present study. The pattern of P release from leaf-litter depends on whether 342 microorganisms are limited by P or by other nutrients, e.g., N (Bani et al. 2018; Duarte et al. 2010). 343 In the cases of P limitation, the leaf-litter P releases slowly or even accumulates due to fungal mycelium transferring P from top-soil to the leaf-litter (Liu et al. 2021; Sardans et al. 2023; Tie et 344

al. 2023). The P limitation was greater in the older stands in our study, as we discussed above, which
most likely leads to more P transfers from soil to decomposed leaf-litter via fungal mycelium (Bani
et al. 2018; Sardans et al. 2023), thus further reducing the top-soil labile P concentration. However,
more studies are needed to better explain this dynamic.

349 The concentration of sub- and bottom-soil NaHCO₃-Pi, NaHCO₃-Po, and total labile P showed a decreasing trend with the maturation of *P. massoniana* reforestations, but not significantly (Fig. 1 350 351 B – D). This can be explained by the fact that *P. massoniana* fine roots (diameter ≤ 2 mm) and their 352 mediated plant P uptake mainly occur in top-soil (< 20 cm) rather than deep-soil (Brandtberg et al. 353 2010; Zhu et al. 2021). For example, Cheng et al. (2012) reported that P. massoniana roots with 354 diameters $\leq 2 \text{ mm}$ and $\leq 1 \text{ mm}$ were mainly distributed in the 0 - 20 cm and 0 - 10 cm soil layers, respectively. In addition, the study reported by Yang et al. (2021) also highlighted that plant root P 355 356 uptake during forest stand development resulted in a decrease of soil P concentration in 0-20 cm 357 layer but not in 20 - 30 cm layer, which also supported our inference.

Taken together, the concentrations and storage of NaHCO₃-Pi, NaHCO₃-Po, and total labile P in top-soil decreased with stand development while they were neutral in sub- and bottom-soil, which highlighted that there was a larger decrease of P availability in top-soil than that in deep-soil. Therefore, we strongly suggest that the differences in the dynamics of soil labile P among top- and deep-soil should be considered for managing *P. massoniana* reforestations.

4.3. The storage and concentration of soil moderately labile P were lower in the older stands

In this study, the storage of soil moderately labile P was lower in the older *P. massoniana* reforestations (Fig. 5), which was consistent with the findings of Izquierdo et al. (2013). The lower

366	soil NaOH-Po concentration in the 45- and 60-year stands than that in the 32-year stand (Fig. 2B)
367	can explain the decrease of soil moderately labile P storage. On the one hand, rock weathering and
368	litter P input are the two major sources of soil P (Brandtberg et al. 2010; Celi et al. 2013; Zhang et
369	al. 2021b). For example, P released from litter can integrate into soil organic P pool (Brandtberg et
370	al. 2010; Francos et al. 2023). Nonetheless, in old forests, litter P input is key for maintaining soil P
371	concentration (Celi et al. 2013; Peng et al. 2023). The leaf-litter P concentration of the 45- and 60-
372	year stands decreased by 20.9% and 21.9%, respectively, compared to the 32-year stand (Fig. 7A
373	and B), which most likely resulted in a lower leaf-litter P input, thus reducing soil organic P storage
374	(Yang et al. 2021; Zhu et al. 2021). On the other hand, although this study did not determine plant
375	biomass P storage, the logging residues (e.g., deadwood, branches, and leaves) were removed at
376	each thinning. This can directly reduce plant biomass P storage, leading to a decrease in soil organic
377	P concentration (Brandtberg et al. 2010; Yang et al. 2021). In addition, the top-, sub-, and bottom-
378	soil NaOH-Pi concentration showed a decreasing trend during the stand maturity (Fig. 2A), which
379	can also at least partially explain the lower soil moderately labile P storage in the older stands. Last
380	but not least, the decrease of soil moderately labile P storage can be explained by the increase of the
381	accumulated amount of plant P uptake during stand development (Vu et al. 2007; Zhu et al. 2021).
382	However, Zhu et al. (2021) reported that soil moderately labile P concentration increased with
383	stand development, which is contrary to our results. Two mechanisms can explain this difference
384	between our study and the study reported by Zhu et al. (2021). First, the leaf-litter P input decreased
385	with stand development due to a lower leaf-litter P concentration in our study (as discussed above),
386	while it increased in the study forests of Zhu et al. (2021), which may have led to a differential
387	response between the two studies. Second, soil moderately labile P cycle can be controlled by soil

388	pH, because P can be rapidly fixed by Al and Fe in acid soils with pH lower than 5.0 and 3.5,
389	respectively (Vu et al. 2007). In the study by Zhu et al. (2021), the soil pH decreased from 4.9 \pm
390	0.21 to 4.5 \pm 0.12 during stand development. However, our results highlighted that the soil pH
391	(higher than 5.1; Fig. 6B) did not significantly decrease during the stand development. These results
392	implied that the soil P fixation was different between our study and the prior study (Zhu et al. 2021),
393	resulting in different dynamics of soil moderately labile P with stand development.

4.4. The soil occluded P relative contribution increased while its storage decreased during the stand maturity

396 Soil occluded P is a main P pool in acidic soils, storing more than half of the total soil P (Selmants 397 and Hart 2010; Zhang et al. 2021b). In this study, the relative contribution of top-, sub-, as well as 398 bottom-soil occluded P to total P were both higher in the 45-year stand (ranging from 64.3% to 74.6%) than in the 32-year stand (ranging from 51.1% to 63.7%) (Fig. 4), which was consistent with 399 prior studies (Izquierdo et al. 2013; Zhang et al. 2021b). The soil's moderately labile P can be 400 401 transformed and absorbed by plants within a few months or years (Tiessen and Moir 2008). In 402 contrast, the soil occluded P is encapsulated by minerals, so its transformation and absorption take 403 decades or even hundreds of years (Izquierdo et al. 2013). Consequently, the relative contribution of soil occluded P was higher in the older stands. In short, our results highlighted that not only top-404 soil (0-20 cm) but also deep-soil (21-60 cm and 61-100 cm) occluded P fractions gradually 405 dominated the soil total P pool with the stand maturity. 406 407 Interestingly, the soil total occluded P storage showed a decreasing trend with the stand

408 maturation, and the difference was significant between the 60-year stand and 32-year stand, which

409	is similar compared to an earlier study which reported a decrease of soil occluded P concentration
410	with vegetation development (Chen et al. 2015). The lower top-, sub, and bottom-soil C.HCl-Pi
411	concentrations in 60-year stand were the key reason for the decrease of soil total occluded P storage
412	in the present study (Fig. 3A). Although soil occluded P fractions are relatively stable, it can also
413	provide a large amount of P to plants and microorganisms (Liu et al. 2021). For example, prior
414	studies reported that soil C.HCl-Pi concentration significantly decreased with vegetation succession
415	because it was transferred into soil available P and then absorbed by plants (Garcia-Montiel et al.
416	2000; Zhang et al. 2021b). As we discussed above, the P limitation of P. massoniana in the 60-year
417	stand was greater than that in the 32-year stand, thus more soil occluded P fractions may have been
418	transferred to available P in order to meet plant demand (Garcia-Montiel et al. 2000; Zhang et al.
419	2021b), which resulted in a lower soil occluded P storage. In addition, in this study, the difference
420	of soil occluded P storage between the 45- and 32-year stands was not significant, which strongly
421	suggests that soil occluded P transformation is a long-term process. Therefore, a longer time scale
422	is needed to examine the dynamics of soil P fractions with the stand development of P. massoniana
423	reforestations in the future.

424 **5.** Conclusion

Our results showed that not only the soil labile P and moderately labile P storages but also the soil occlude P storage decreased with stand development. Moreover, the top-soil acid phosphatase and leaf N/P ratio were higher and the leaf-litter P concentration was lower in the 45- and/or 60-year plots than in 32-year plots. These results indicated that although *P. massoniana* can compensate for the drop in soil P availability through secreting more acid phosphatase and increasing leaf P 430 resorption, the leaf-litter P input decreased with the stand maturity, which resulted in an imbalanced 431 P recycle among plants, leaf-litter, and soil and thus a depletion of soil P. Therefore, we strongly 432 suggest increasing the input of litter and promoting the release of P from litter in P. massoniana reforestations. For example, the logging residues (e.g., deadwood, branches, and leaves) should be 433 434 stored at the surface as additional litter when implementing the thinning measure. On the other hand, our results showed that there were multiple broadleaf tree species under P. massoniana 435 436 reforestations (Table S1), indicating that it is feasible to cultivate mixed coniferous and broadleaf 437 forests through natural succession. Thus, it is also recommended to preserve naturally regenerated 438 broadleaf tree species (as early as possible), not only because the remaining *P. massoniana* trees can 439 provide shade to the seedlings of these naturally grown species, but also because mixed forests can 440 generally promote the decomposition of *P. massoniana* leaf-litter and can increase soil P availability 441 (Bai et al. 2023; Chen et al. 2022b). In addition, due to top-soil labile P concentration decreasing 442 more than that at deep-soil during the stand maturity of *P. massoniana* reforestations, applying P fertilizer in top-soil is an economical and recommended forest management measure. Nonetheless, 443 444 the decrease of deep-soil moderately labile and occlude P should also be considered for managing 445 P. massoniana reforestations. Overall, our study improved the understanding of the medium to longterm time-scale effects of reforestation on deep-soil P fractions as well as provided evidences for 446 447 maintaining soil P storage and even for sustainable management of P. massoniana reforestation in 448 south China.

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458 **Competing interests**

467

459 The authors declare that they have no competing financial interests.

Contributions of the coauthors 460

461	Liehua Tie: Methodology, Formal analysis, Writing - original draft, Writing - review & editing.
462	Josep Peñuelas: Conceptualization, Results discussion, Writing - original draft, Writing - review
463	& editing. Congde Huang: Writing - original draft, Writing - review & editing. Jordi Sardans:
464	Writing - original draft, Writing - review & editing. Arun K. Bose: Writing - original draft, Writing
465	- review & editing. Shengnan Ouyang: Methodology, Writing - review & editing. Yixian Kong:
466	Methodology, Resources. Yan Guo: Methodology, Resources. Yujie Wu: Methodology, Resources.

Wei Cheng: Methodology, Resources. Guijie Ding: Methodology, Writing - review & editing.

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654

655 **Figure captions**

656

Fig. 1 Differences in soil labile P fractions among different stand ages and soil depths. Values are the means \pm standard deviations of five replicate plots. The Y, D, and Y*D represent main effects of stand age, soil depth, and their interactions, respectively. Different capital letters denote significant differences between stand ages at the same depth of soil (p < 0.05), and different lowercase letters denote significant differences between soil depths at the same age of stand (p < 0.05).

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Fig. 2 Differences in soil moderately labile P fractions among different stand ages and soil depths. Values are the means \pm standard deviations of five replicate plots. The Y, D, and Y*D represent the main effects of stand age, soil depth, and their interactions, respectively. Different capital letters denote significant differences between stand ages at the same depth of soil (p < 0.05), and different lowercase letters denote significant differences between soil depths at the same age of stand (p < 0.05).

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Fig. 3 Differences in soil occluded P fractions among different stand ages and soil depths. Values are the means \pm standard deviations of five replicate plots. The Y, D, and Y*D represent the main effects of stand age, soil depth, and their interaction, respectively. Different capital letters denote significant differences between stand ages at the same depth of soil (p < 0.05), and different lowercase letters denote significant differences between soil depths at the same age of stand (p < 0.05).

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Fig. 4 Percentage (%) of soil labile, moderately labile, and occluded P to total P among different stand ages and soil depths. Values are the means \pm standard deviations of five replicate plots. Different capital letters denote significant differences between stand ages (p < 0.05).

Fig. 5 Differences in soil P storage at 0 - 100 cm among different stand ages. Values are the means \pm standard deviations of five replicate plots. Different capital letters denote significant differences between stand ages (p < 0.05), ** denotes significant differences in soil total phosphorus storage (p < 0.01).

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Fig. 6 Differences in leaf and root N/P ratios, leaf-litter P concentration, and soil main properties during stand development. Values are the means \pm standard deviations of five replicate plots. The Y, D, and Y*D represent the main effects of stand age, soil depth, and their interactions, respectively. Different capital letters denote significant differences between stand ages (p < 0.05), and different lowercase letters denote significant differences between soil depths at the same age of stand (p < 0.05).

693

Fig. 7 The schematic graph of aboveground and underground P transfer during the stand
maturity in *P. massoniana* reforestations.













Stand age (Year)





717 Supplementary figures

Table S1 The main vegetation in the study *P. massoniana* reforestations. Except for *P. massoniana*,

720 the other vegetation species come from natural succession.

32-year-old stand	45-year-old stand	60-year-old stand
		Pinus massoniana, Styphnolobium
Pinus massoniana, Rhus chinensis,	Pinus massoniana, Koelreuteria	japonicum, Liquidambar formosana,
Albizzia julibrissin, Camellia	bipinnata, Liquidambar formosana,	Rhus chinensis, Mallotus japonicus,
oleifera, Rubus pirifolius,	Rhus chinensis, Camellia oleifera,	Quercus serrata, Albizzia julibrissin,
Parathelypteris glanduligera,	Rubus pirifolius, Albizzia julibrissin,	Camellia oleifera, Rubus pirifolius,
Dicranopteris dichotoma,	Parathelypteris glanduligera,	Parathelypteris glanduligera,
Capillipedium parviflorum,	Dicranopteris dichotoma, Adiantum	Dicranopteris dichotoma,
Adiantum capillus-veneris, Setaria	capillus-veneris, Capillipedium	Capillipedium parviflorum,
plicata	parviflorum	Adiantum capillus-veneris, Setaria
		plicata

722 Figure

723

Fig. S1 The study site and experimental photos. Panel A, a satellite image of the study site; Panel
B, the subplot of the soil profile and leaf-litter collection boxes in the study plot; Panel C, the stand

- characteristics of the study forests and a close-up image of the soil profile.



P. massoniana reforestation stand development

727



729 Fig. S2 Flow chart of the sequential extraction of soil P fractions in this study.