


## LETTER

# Substrate stoichiometry determines nitrogen fixation throughout succession in southern Chinese forests

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### Abstract

The traditional view holds that biological nitrogen (N) fixation often peaks in early- or mid-successional ecosystems and declines throughout succession based on the hypothesis that soil N richness and/or phosphorus (P) depletion become disadvantageous to N fixers. This view, however, fails to support the observation that N fixers can remain active in many old-growth forests despite the presence of N-rich and/or P-limiting soils. Here, we found unexpected increases in N fixation rates in the soil, forest floor, and moss throughout three successional forests and along six age-gradient forests in southern China. We further found that the variation in N fixation was controlled by substrate carbon(C) : N and C : (N : P) stoichiometry rather than by substrate N or P. Our findings highlight the utility of ecological stoichiometry in illuminating the mechanisms that couple forest succession and N cycling.

### Keywords

Asymbiotic nitrogen fixation, forest succession, nitrogen richness, phosphorus limitation, stoichiometry, substrate.

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## INTRODUCTION

Biological nitrogen (N) fixation, an important pathway of new N inputs from the atmosphere to terrestrial and aquatic ecosystems, has ecological significance in global biogeochemical cycling (Cleveland *et al.* 1999; Reed *et al.* 2011; Zehr 2011). Commonly, N fixation can be performed by microorganisms (e.g. *Rhizobia* and *Frankia*) occupying plant root nodules, by heterotrophic bacteria living in soil and litter layers (Reed *et al.* 2011, 2013), canopy foliage (Moyes *et al.* 2016), and marine sediment (Currin *et al.* 1990), or by cyanobacteria that builds mutualistic relationships with terrestrial epiphytes (Menge & Hedin 2009; Reed *et al.* 2011) and ocean phytoplankton (Hutchins *et al.* 2015). Importantly, N-fixing microorganisms provide available N to plants and microbes and alleviate N limitation in many biomes, such as forests (e.g. Reed *et al.* 2007a; DeLuca *et al.* 2008; Menge & Hedin 2009; Perakis *et al.* 2017), grasslands (e.g. Reed *et al.* 2007b), tundra (e.g. Rousk & Michelsen 2016), deserts (e.g. Ramond *et al.* 2018), and oceans (e.g. Zehr 2011). As N can constrain net primary productivity (NPP) widely across ecosystems (LeBauer & Treseder 2008; Zehr 2011), N inputs via biological fixation increase ecosystem C sequestration and NPP and help constrain atmospheric CO<sub>2</sub> concentrations (Zehr 2011; Dynarski & Houlton 2018; Ramond *et al.* 2018). Despite the importance of N fixation in Earth's ecosystems, our knowledge of how N fixation varies with

succession and/or ecosystem development and the potential controls remains poor, which limits our ability to estimate and predict global C–N cycling and NPP in a changing world (Vitousek *et al.* 2013; Meyerholt *et al.* 2016).

The traditional view in succession theory holds that biological N fixation (e.g. in legumes, epiphytes and decaying wood) exhibits the highest rates in early- or mid-successional ecosystems and declines with ecosystem maturity (Gorham *et al.* 1979; Vitousek & Howarth 1991; Batterman *et al.* 2013a). This view is established based on the biogeochemical theories that N constrains plant growth and high N retention occurs in early-successional terrestrial ecosystems (nutrient-retention hypothesis, Vitousek & Reiners 1975; Vitousek & Howarth 1991), while late-successional ecosystems rich in soil N (due to chronic N fixation and/or deposition) have fewer exogenous N demands (N saturation theory; Aber *et al.* 1998). Theoretically, N fixers are faced with the risks of being outcompeted by non-N fixers under N-rich conditions (Crews 1999) because fixing atmospheric N<sub>2</sub> is energetically expensive (Gutschick 1981; Vitousek & Field 1999). Many studies have found that legumes and N-fixing microbes down-regulate fixation rates throughout succession (e.g. Nohrstedt 1985; Hope & Li 1997; Crews *et al.* 2000; Son 2001; Pearson & Vitousek 2001; Barron *et al.* 2011; Batterman *et al.* 2013a), although there are some exceptions (Zackrisson *et al.* 2004; Menge & Hedin 2009; annual review, Reed *et al.* 2011).

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Two mechanistic hypotheses support the view that N fixation declines throughout succession. First, forest development results in soil N richness (Aber *et al.* 1998; Crew *et al.* 2001; Mo *et al.* 2003), which is adverse to N fixers (Dynarski & Houlton 2018; Zheng *et al.* 2019) (*N richness hypothesis*). Because fixing atmospheric N<sub>2</sub> is energetically more expensive than soil N uptake (Gutschick 1981), facultative N fixers often down-regulate fixation rates when soil N is sufficient, while obligate N fixers that fix N<sub>2</sub> constantly regardless of soil N richness are replaced by non-N fixers (Menge *et al.* 2009; Barron *et al.* 2011; Menge & Crews 2016). Second, forest development (e.g. primary and secondary succession) results in soil P depletion (Walker & Syers 1976; Hedin *et al.* 2003; Mitchell & Ruess 2009; Vitousek *et al.* 2010), which constrains N fixation (e.g. Crews *et al.* 2000; Vitousek & Hobbie 2000; Reed *et al.* 2007a) (*P limitation hypothesis*). Phosphorus is important for the growth of N fixers (e.g. the biosynthesis of ribosomes, proteins, and phospholipids) and the generation of adenosine triphosphate (ATP), which supports N fixation (Alberly 2005). Yet, because of biological uptake (Walker & Syers 1976), hydrologic losses (Hedin *et al.* 2003), the conversion to occluded forms (Crews *et al.* 1995), and soil acidification caused by chronic N deposition (Matson *et al.* 1999), soil P becomes limiting over time and thereby constrains N fixation. Although these hypotheses support the view of declining N fixation throughout succession, they cannot explain the phenomenon that N fixers (i.e. N-fixing microbes and epiphytes) remain active in old-growth forests that are rich in soil N (Reed *et al.* 2008; Menge & Hedin 2009) or scarce in soil P (Zheng *et al.* 2018a). This phenomenon indicates our incomplete understanding of the mechanisms controlling N fixation throughout forest succession.

Resource stoichiometry plays a critical role in the growth and function of microorganisms (Redfield 1958; Sterner & Elser 2002; Scott *et al.* 2012; Hessen *et al.* 2013). Compared to single resources (e.g. N), resource stoichiometry (e.g. C : N or N : P) may sometimes better predict N fixation rates (Reed *et al.* 2011). A typical example is from a case study in a 120 000-year chronosequence in New Zealand where N fixation in the litter, bryophytes, and cyanolichens was not affected by the soil N pool and exhibited a 'non-nitrostatic dynamic' (i.e. N fixers maintained high fixation rates despite soil N richness; Menge & Hedin 2009). One potential mechanism is that canopy and litter N fixers are decoupled from soil N pool (Menge & Hedin 2009), which creates N-poor conditions (plant tissues have high C : N ratios relative to decomposers) that favor N fixation despite soil N richness (Reed *et al.* 2008; Barron *et al.* 2009; Menge & Hedin 2009). Moreover, several previous studies have found leaf litter with a high labile C content but low total N content yielding resources with higher C : N that favored N fixers in the litter layer (Vitousek & Hobbie 2000; Pérez *et al.* 2010). Although these important findings and assumptions indicate that resource C : N ratios control N fixation across vertical gradients (spatial scale), our knowledge regarding the resource C : N effects on N fixation throughout succession dynamics (temporal scale) remains relatively limited. Given that forest succession not only changes substrate C and N (Crews *et al.* 2001) but also P availability (Walker & Syers 1976; Crews

*et al.* 1995), and that the biomass of N fixers and process of N fixation require the involvement of C, N, and P (Alberly 2005; Reed *et al.* 2011), a variable that incorporates C, N, and P may govern N fixation rates throughout succession (*stoichiometry hypothesis*).

Our study aims to address how forest succession regulates N fixation and explore the mechanisms underlying such regulation (Fig. S1). Because forest succession involves species replacement and associated changes (e.g. increases in resources, biomass, and stand age; Horn 1974; Peet 1981), we conducted experiments in three successional forests (where tree species replacement occurred; *Experiment 1*) and in six age-gradient forests (where soil C and N resources and microbial and plant biomass increased; *Experiment 2*) in southern China (Fig. S11). We measured the C, N, and P concentrations and stoichiometry and N fixation rates in the soil, forest floor, and moss (N-fixing trees are rare in the tropics of Asia, including in southern China; Menge *et al.* 2019). Given that plant and soil N concentrations may increase (via N deposition and fixation) throughout succession, which may change substrate C and P availability and stoichiometry, we applied long-term (3–13 years) N-addition treatments (0–150 kg N ha<sup>-1</sup> year<sup>-1</sup>) to explore whether resource availability or stoichiometry control N fixation. We hypothesised that (H1) N fixation would decline throughout succession given that N fixers have less competitive advantages in late-successional ecosystems; (H2) single nutrient (i.e. N or P) availability could explain the variation in N fixation given that forest succession leads to soil N richness (*N richness hypothesis*) and/or P limitation (*P limitation hypothesis*); and (H3) resource stoichiometry (i.e. C : N or C : (N : P); see Fig. S2 for the definitions of both variables) might predict N fixation better than a single resource because N fixation may be co-regulated by C, N (*C : N stoichiometry hypothesis*), and P (*C : (N : P) stoichiometry hypothesis*).

## MATERIALS AND METHODS

### Site description

We conducted the first experiment (forest succession experiment; *Experiment 1*) in Dinghushan (DHS) biosphere reserve located in Guangdong Province of southern China (112°10' E, 23°10' N). There are three forests at different successional stages: a coniferous forest (early-succession), a mixed coniferous and broadleaved forest (hereafter, the coniferous/broadleaved forest; mid-succession) and a broadleaved forest (late-succession). The coniferous forest is dominated by the pioneer tree species *Pinus (P.) massoniana*. Because of natural regeneration and invasion by broadleaved species, the coniferous/broadleaved forest, which was originally dominated by *P. massoniana*, is now co-dominated by coniferous (*P. massoniana*) and broadleaved species (*Castanopsis (C.) chinensis* and *Schima (S.) superba*) (Mo *et al.* 2003). The broadleaved forest experiences long-term succession and is dominated completely by native broadleaved species, e.g. *C. chinensis*, *S. superba*, *Machilus chinensis*, and *Cryptocarya chinensis* (Fang *et al.* 2005). Our forest successional series was characterised by native species replacement (from coniferous to broadleaved species; Table 1).

**Table 1** General characteristics of the studied forest sites

Site	Experiment type	Forest type	Location	Plots/Replicates/ Established	Main tree species	Mean DBH of main tree species (cm)	Soil N (mg g <sup>-1</sup> )	Soil C (mg g <sup>-1</sup> )	Soil P (mg g <sup>-1</sup> )	Soil MBC (mg kg <sup>-1</sup> )	MAT (°C)	MAP (mm)
DHS1	Forest succession	Coniferous forest ( <i>Early succession</i> )	112°10' E, 23°10' N	Control, +N150/n = 5/2007	<i>Pinus massoniana</i>	19.3§	1.5	26.8	0.26	449§	21†	1927†
		Mixed coniferous and broadleaved forest ( <i>Middle succession</i> )	112°10' E, 23°10' N	Control, +N150/n = 5/2007	<i>Pinus massoniana</i> <i>Schima superba</i> <i>Castanopsis chinensis</i>	20.5§ 4.2§	2.1	34.7	0.29	327§	21†	1927†
		Broadleaved forest ( <i>Late succession</i> )	112°10' E, 23°10' N	Control, +N150/n = 5/2007	<i>Schima superba</i> <i>Castanopsis chinensis</i> <i>Machilus chinensis</i>	26.0§ 14.8§ 18.3§	2.8	41.2	0.38	564§	21‡	1927‡
			112°50' E, 22°34' N	Control, +N50/n = 3/2010	<i>Acacia auriculiformis</i>	15.0*	1.6*	22.1*	0.29	254*	22.5*	1534*
HSSMTDHS2	Forest age-gradient	Eucalypt plantation (30 years)	113°05'– 24°	Control, +N50/n = 3/2013	<i>Cryptocarya chinensis</i> <i>Syzygium rehderianum</i> <i>Eucalyptus urophylla</i>	12.9§ 11.1*	1.5*	15.5*	0.27	288*	22.5*	1534*
		Acacia plantation (30 years)	112°50' E, 22°34' N	Control, +N50/n = 3/2010	<i>Acacia auriculiformis</i>	18.6** (means of all species available)	1.9	25.4	0.35	334	20.8***	2364***
		Broadleaved forest (50 years)	112°10' E, 23°10' N	Control, +N50/n = 3/2003	<i>Cryptocarya chinensis</i> <i>Schima superba</i> <i>Machilus chinensis</i> <i>Castanea henryi</i>	17.5#	1.4†	23.3†	0.30†	410ℓ	21†	1927†
			112°10' E, 23°10' N	Control, +N50/n = 3/2003	<i>Pinus massoniana</i> <i>Pinus massoniana</i> <i>Schima superba</i> <i>Castanopsis chinensis</i> <i>Schima superba</i> <i>Castanopsis chinensis</i> <i>Machilus chinensis</i> <i>Cryptocarya chinensis</i> <i>Syzygium rehderianum</i>	22.0# 6.4# 23.5# 8.6# 10.3# 20.6# 29.4#	2.0†	32.1†	0.25†	450ℓ	21†	1927†
	Broadleaved forest (> 400 years)	112°10' E, 23°10' N	Control, +N50/n = 3/2003	<i>Pinus massoniana</i> <i>Pinus massoniana</i> <i>Schima superba</i> <i>Castanopsis chinensis</i> <i>Schima superba</i> <i>Castanopsis chinensis</i> <i>Machilus chinensis</i> <i>Cryptocarya chinensis</i> <i>Syzygium rehderianum</i>	40.0‡	2.9‡	40.0‡	0.34‡	590ℓ	21‡	1927‡	

Notes Data with the label of \*, †, ‡, #, §, \*\*, †, ‡, #, §, ℓ were from the tables or figures of Zhang *et al.* (2012), Zheng *et al.* (2017), Zheng *et al.* (2018a), Fang *et al.* (2005), Zhang *et al.* (2015), Liu *et al.* (2012), and Wang *et al.* (2009), respectively. N: nitrogen; C: carbon; P: phosphorus; MBC: microbial biomass carbon; DBH: diameter at breast height; MAT: mean annual temperature; MAP: mean annual precipitation; DHS: Dinghushan biosphere reserve; HS: Heshan research station; SMT: Shimantai nature reserve. DHS1 and DHS2 represent different study sites in the Dinghushan biosphere reserve. †N50 and †N150 represents N addition at a rate of 50 and 150 kg ha<sup>-1</sup> year<sup>-1</sup>, respectively.

We conducted the second experiment (forest age-gradient experiment; *Experiment 2*) at three sites in Guangdong Province of southern China: Heshan (HS) national field research station (112°50' E, 22°34' N), Shimentai (SMT) national nature reserve (113°05'–113°31' E, 24°22'–24°31' N) and DHS biosphere reserve (112°10' E, 23°10' N). The three sites experience the same monsoon climate and have similar hydrothermal conditions (Table 1). At the HS site, we selected a 30-year-old eucalyptus (*Eucalyptus urophylla*) plantation and a 30-year-old acacia (*Acacia auriculiformis*) plantation, which were established on degraded grasslands in 1984 (Zheng *et al.* 2016). At the SMT site, we selected a 50-year-old evergreen broadleaved forest that was originally a limestone site in 1965 (Zhang *et al.* 2015). At the DHS site, we selected an 80-year-old coniferous forest and an 80-year-old coniferous/broadleaved forest, which were established on degraded lands in 1930, and we selected a > 400-year-old broadleaved forest that has been protected since 1633 (Brown *et al.* 1995) (the DHS forest sites selected for this experiment were near those for *Experiment 1*). To distinguish the different study sites between *Experiment 1* and *Experiment 2*, we labeled them as DHS1 and DHS2, respectively (Table 1). Soil resources (e.g. C and N), microbial biomass (e.g. MBC), and plant biomass (e.g. DBH) increased along forest age-gradient (from 30 to > 400 years; Table 1).

### Experimental design

*Experiment 1* was initiated in February 2007 at the DHS1 site with two levels of N addition (each in five replicates): 0 and 150 kg N ha<sup>-1</sup> year<sup>-1</sup>. Each 5 × 5 m<sup>2</sup> plot was surrounded by a 5-m wide buffer strip, and all plots were randomly laid out within each forest. Solutions of NH<sub>4</sub>NO<sub>3</sub> were sprayed below the canopy bimonthly from February 2007 to July 2016 using a backpack sprayer. Fertiliser was mixed with 5 L of water for each N-addition plot, and each control plot received 5 L of water.

*Experiment 2* was initiated in July 2003, August 2010, and April 2013 at the DHS2, HS, and SMT sites, respectively, with two levels of N addition (each in three replicates): 0 and 50 kg N ha<sup>-1</sup> year<sup>-1</sup>. Each square plot (10 × 10 mand 10 × 20 mat the HS and DHS2 sites, respectively) or circular plot (17 m in semidiameter with an area of 907 m<sup>2</sup> at the SMT site) was surrounded by a more than 10-m wide buffer strip. At the SMT site, NH<sub>4</sub>NO<sub>3</sub> solutions equivalent to 3 mm of precipitation were applied below the canopy monthly during the growing season (April–October) from April 2013 to July 2016 in the N-addition plots (Zhang *et al.* 2015; Zheng *et al.* 2018b). Fertilisers (NH<sub>4</sub>NO<sub>3</sub>) mixed with 10 L and 20 L of water were sprayed below the canopy using a backpack sprayer in the N-addition plots at the HS (bimonthly from August 2010 to July 2014) and DHS2 (monthly from July 2003 to July 2015) sites, respectively, and an equivalent volume of water was sprayed in control plots.

### Sampling

Field sampling was performed in July 2016 at the DHS1 site and in July 2014, 2015, and 2016 at the HS, DHS2, and SMT

sites, respectively. Three to five forest floor samples, including fine woody tissues and fresh and decomposed leaves, were randomly collected from each plot using a metal frame (20 × 20 cm<sup>2</sup>). Mineral soil underneath the forest floor was collected to a depth of 10 cm using a 2.5-cm soil corer. N-fixing mosses distributed at tree trunk bases or on stone surfaces were collected by scraping three to 12 5 × 5 cm<sup>2</sup> pieces in each plot. All samples were weighed, and portions were oven-dried to determine the moisture content.

### Acetylene reduction assay

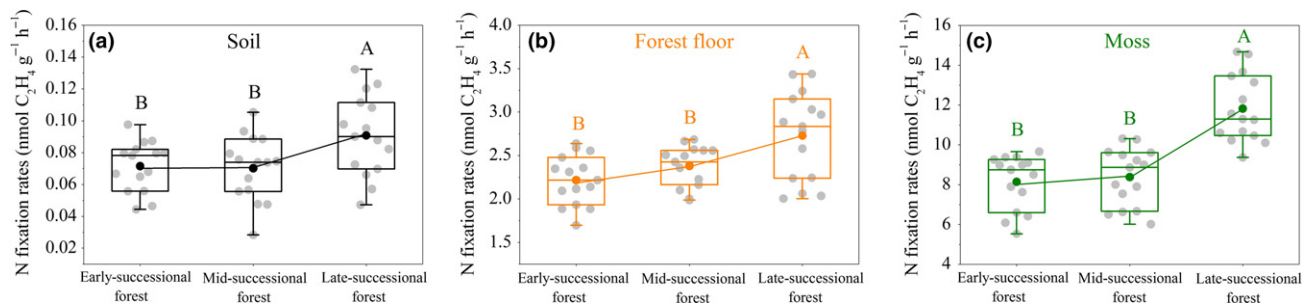
Nitrogen fixation rates were measured using an acetylene reduction assay (Hardy *et al.* 1968). Specifically, fresh samples (5–6 g forest floor, 10–13 g soil, or 3–4 g moss) were sealed into 120-mL gas-tight glass jars with 10% of the headspace replaced with pure C<sub>2</sub>H<sub>2</sub> (99.99%). Samples were incubated *in situ* for 7–24 h to approximate the ambient light and temperature conditions. After incubation, the headspace gas from each jar was sampled and stored in a 12-mL evacuated Exetainer (Labco, High Wycombe, UK) and returned to the laboratory for analysis. The C<sub>2</sub>H<sub>4</sub> concentrations of each gas sample were measured using a gas chromatograph (Shimadzu GC14, Tokyo, Japan). The background C<sub>2</sub>H<sub>4</sub> concentrations in the pure C<sub>2</sub>H<sub>4</sub> gas and the C<sub>2</sub>H<sub>4</sub> concentrations naturally produced by the samples were measured and subtracted. Nitrogen fixation rates per unit mass were expressed as the C<sub>2</sub>H<sub>4</sub> production rates (nmol C<sub>2</sub>H<sub>4</sub> g<sup>-1</sup> dry weight h<sup>-1</sup>; Table S1), and N fixation rates per unit area were scaled up using the C<sub>2</sub>H<sub>4</sub> production rates, standing stock, and conversion ratio (see Table S2–S3).

### Substrate chemical properties

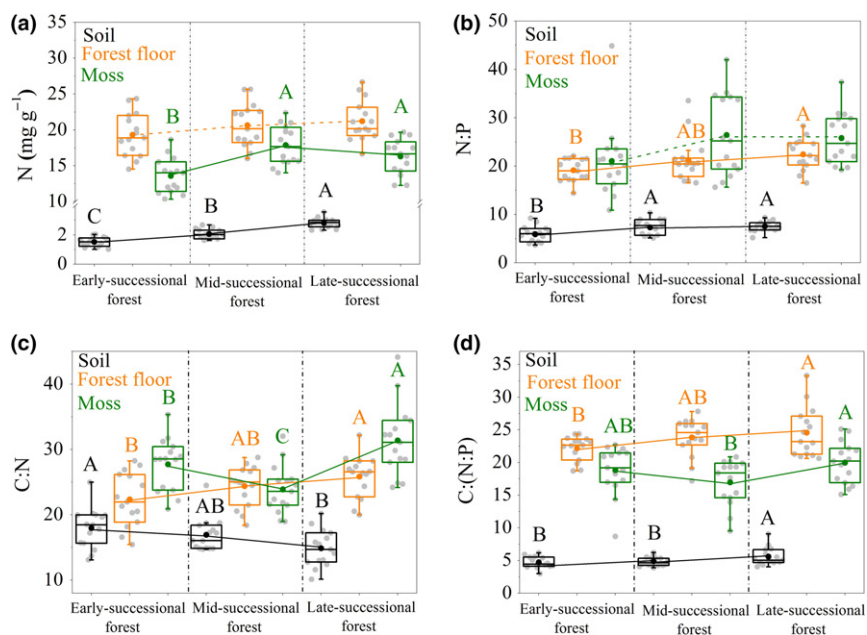
Total C concentration was measured by potassium dichromate oxidation titration with an Fe<sup>2+</sup> solution (Liu 1996). Total N and total P concentrations were measured by micro-Kjeldahl digestion followed by indophenol blue and Mo-Sb colorimetric methods, respectively, using a spectrophotometer (Shanghai UV-8000, Metash Instruments Co., Shanghai, China) (Liu 1996).

### Data analyses

Data were tested for normality (Kolmogorov–Smirnov test) and homoscedasticity (Levene's test). One-way analysis of variance (ANOVA) followed by Tukey's HSD test was used to determine the effects of forest succession and age-gradient on the substrate N fixation rates, N, N : P, C : N, and C : (N : P). Linear regression models were used to explore the relationships between N fixation rates and the substrate N, P, C : N, or C : (N : P) and between response ratios of N fixation and those of substrate N, P, C : N, or C : (N : P) following N addition (the calculation of response ratios is shown in Text S1). Structural equation models were used to quantify the relative importance of direct and indirect pathways of the forest-succession effects on N fixation (Grace 2006). ANOVA and linear regression models were performed using SPSS 19.0 (SPSS Inc., Chicago, IL, USA) and structural equation models



**Figure 1** Box figures of nitrogen fixation rates (per unit mass) in the soil (a), forest floor (b), and moss (c) throughout forest succession (from early- to late-successional forests). Each box represents the lower and upper quartiles with the medians and means shown as the central lines and solid circles, respectively. Different capital letters represent significant differences among the forests ( $n = 15$ ,  $P < 0.05$ ). Early-, mid-, and late-successional forests represent coniferous, mixed coniferous and broadleaved, and broadleaved forests in this study, respectively.



**Figure 2** Nitrogen (N), N : phosphorus (N : P), carbon : N (C : N), and C : (N : P) in different substrates (soil, forest floor, and moss) throughout forest succession (from early- to late-successional forests) (a–d). Each box represents the lower and upper quartiles with the medians and means shown as the central lines and solid circles, respectively. Different capital letters represent significant differences among the forests ( $n = 15$ ,  $P < 0.05$ ). Early-, mid-, and late-successional forests represent coniferous, mixed coniferous and broadleaved, and broadleaved forests in this study, respectively.

were performed using AMOS 21.0 (SPSS Inc.). Statistically significant differences were recognised at  $P < 0.05$ .

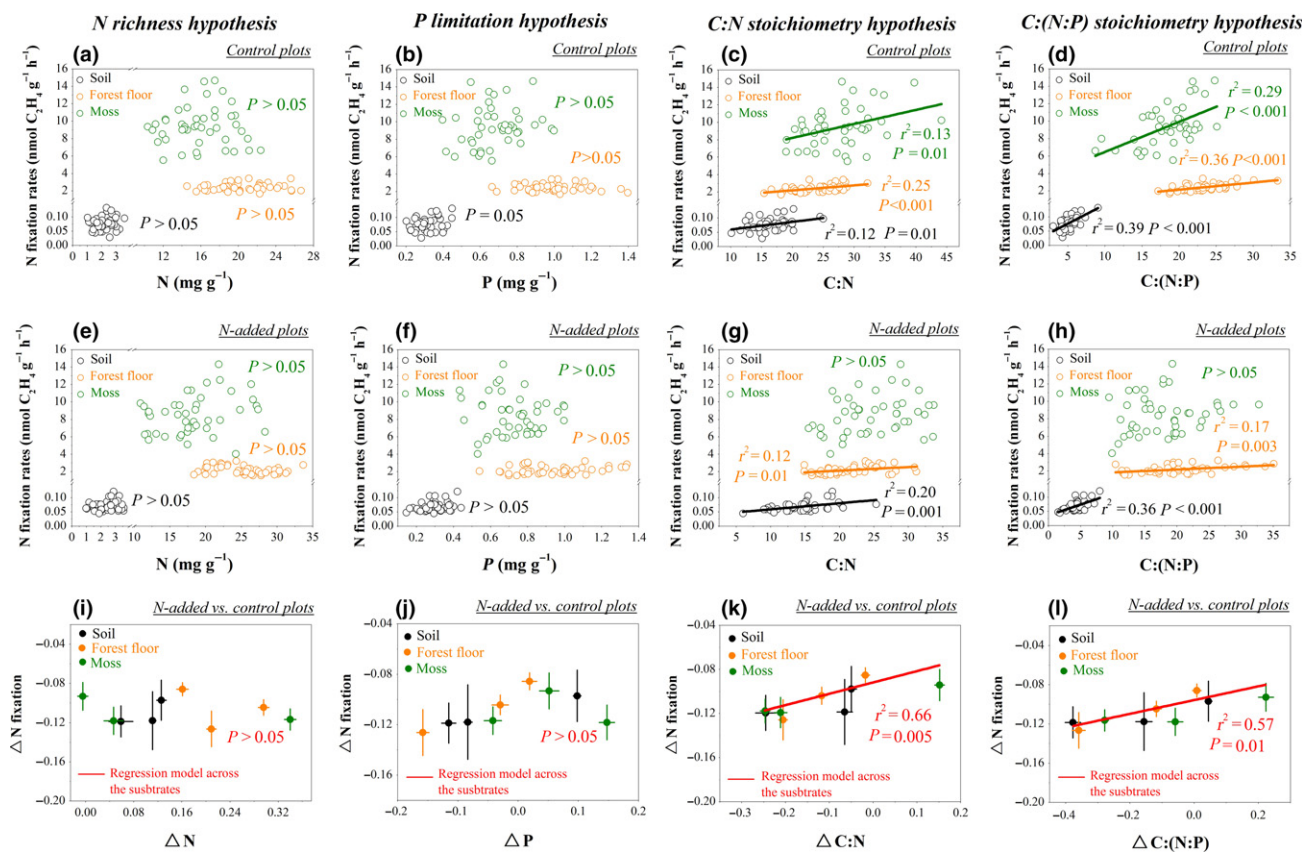
## RESULTS

### N fixation, substrate N and P, and stoichiometry throughout succession

Nitrogen fixation rates per unit mass were higher in the late-successional forest than in the early-successional and mid-successional forests ( $F_{2,42} = 4.58$   $P = 0.016$ ,  $F_{2,42} = 7.87$   $P = 0.001$ , and  $F_{2,42} = 27.85$   $P < 0.001$  for the soil, forest floor and moss, respectively; ANOVA; Fig. 1). Similarly, N fixation rates per unit area were higher in the late-successional forest than in the early-successional and mid-successional forests ( $F_{2,42} = 4.32$

$P = 0.020$ ,  $F_{2,42} = 3.94$   $P = 0.027$ , and  $F_{2,42} = 16.07$   $P < 0.001$  for the soil, forest floor, and moss, respectively; ANOVA; Fig. S8a). The late-successional forest had the higher total N fixation rates ( $2.9 \pm 0.2$  mg N m<sup>-2</sup> day<sup>-1</sup>) than the early-successional ( $2.2 \pm 0.1$  mg N m<sup>-2</sup> day<sup>-1</sup>) and mid-successional ( $2.3 \pm 0.1$  mg N m<sup>-2</sup> day<sup>-1</sup>) forests ( $F_{2,42} = 8.65$   $p = 0.001$ ; ANOVA; Fig. S8a).

Substrate N concentrations, N : P (i.e. total N concentrations divided by total P concentrations), C : N (i.e. total C concentrations divided by total N concentrations), and C : (N : P) (i.e. total C concentrations divided by the quotient of total N and total P concentrations) changed throughout succession (from coniferous to broadleaved forests; Fig. 2). Specifically, the soil and moss N concentrations increased ( $F_{2,42} = 57.62$   $P < 0.001$  and  $F_{2,42} = 10.88$   $P < 0.001$ ,



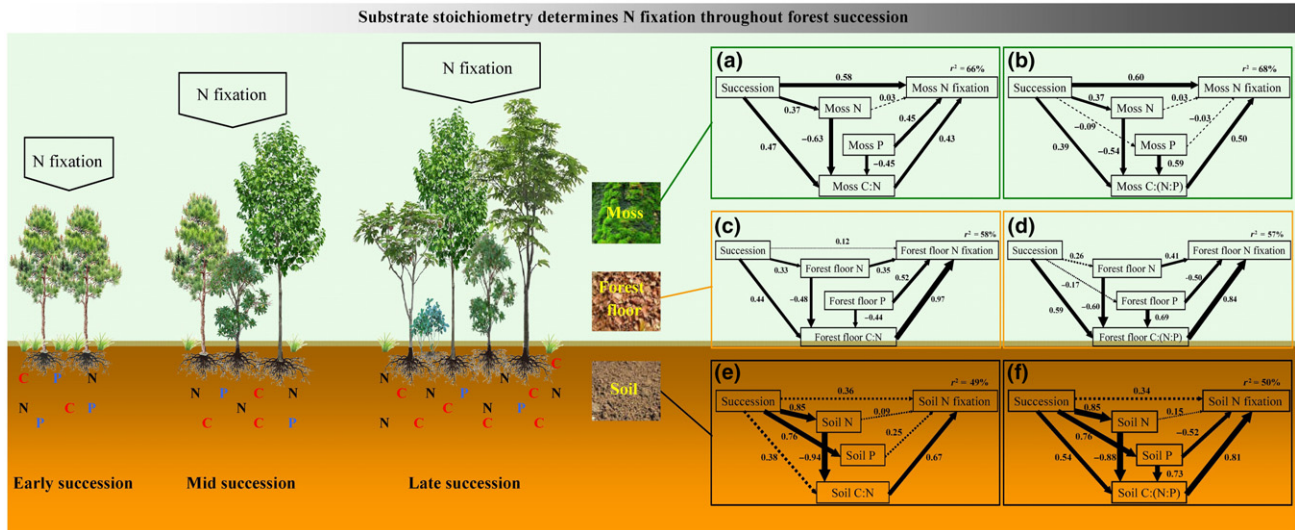
**Figure 3** Relationships between nitrogen (N) fixation rates (per unit mass) and N, phosphorus (P), carbon : N (C : N), or C : (N : P) in different substrates (soil, forest floor, and moss) throughout forest succession. Linear regression models of N fixation rates against N, P, C : N, or C : (N : P) within each substrate in control plots ( $n = 45$ ) (a–d). Linear regression models of N fixation rates against N, P, C : N, or C : (N : P) within each substrate in N-addition plots ( $n = 45$ ) (e–h). Linear regression models of response ratios of N fixation rates against those of N, P, C : N, or C : (N : P) following N addition (*note*: the three solid points (with error bars) within each substrate represent the three forests;  $n = 9$ ) (i–l). Each solid point and error bar represents mean response ratio and 95% confidence interval following N addition, respectively.

respectively; ANOVA), whereas the forest floor N concentrations did not change ( $F_{2,42} = 1.60$   $P = 0.214$ ; ANOVA) throughout succession. The forest floor and moss C : N increased ( $F_{2,42} = 3.70$   $P = 0.033$  and  $F_{2,42} = 10.55$   $P < 0.001$ , respectively; ANOVA) but the soil C : N decreased ( $F_{2,42} = 4.61$   $P = 0.015$ ; ANOVA) throughout succession. Substrate N : P ( $F_{2,42} = 5.51$   $P = 0.007$  and  $F_{2,42} = 3.35$   $P = 0.045$  for the soil and forest floor, respectively; ANOVA) and substrate C : (N : P) ( $F_{2,42} = 3.36$   $P = 0.044$ ,  $F_{2,42} = 3.79$   $P = 0.031$ ,  $F_{2,42} = 3.46$   $P = 0.041$  for the soil, forest floor, and moss, respectively; ANOVA) increased throughout succession, indicating that substrate C (particularly labile C, e.g., readily oxidizable C (ROC), MBC, and dissolved organic C (DOC); see Fig. S3) concentrations increased rapidly compared to substrate N : P ratios throughout succession (see Fig. S4). The increased substrate C : (N : P) also indicated that the potential effects of increasing C availability on N fixers might be larger than those of substrate N richness and P limitation, which might drive N fixation throughout succession (*stoichiometry hypothesis*; Fig. S2).

Neither substrate N nor P affected N fixation rates throughout succession, because linear regression models showed that neither N nor P explained the variation in N fixation rates

within each substrate in control or N-addition plots ( $P > 0.05$ ; Fig. 3). In contrast, substrate C : N and C : (N : P) explained 12–25% and 29–39% of the N fixation variation in control plots, respectively ( $P \leq 0.01$ ; Fig. 3c,d) and 12–20% and 17–36% of the N fixation variation in N-addition plots, respectively ( $P \leq 0.01$ ; Fig. 3g,h). Under N-addition treatment, although response ratios of substrate C : N ( $\Delta C : N$ ) and C : (N : P) ( $\Delta C : (N : P)$ ) varied throughout succession (Fig. S5), substrate  $\Delta C : N$  and  $\Delta C : (N : P)$  had positive relationships with  $\Delta N$  fixation ( $r^2 = 0.66$ ,  $P = 0.005$  and  $R^2 = 0.57$ ,  $P = 0.01$ , respectively; Fig. 3k,l).

Structure equation models indicated that forest succession controlled N fixation via substrate stoichiometry (Fig. 4). Specifically, forest succession directly stimulated moss N fixation (explained 58–60%,  $P < 0.001$ ) and indirectly stimulated moss N fixation by changing the moss C : N or C : (N : P) (19–20%,  $P \leq 0.002$ ), and forest succession indirectly inhibited moss N fixation by changing both the moss N and C : N (or C : (N : P)) (9–10%,  $P \leq 0.009$ ; Fig. 4a,b). Forest succession indirectly stimulated forest floor N fixation by changing the forest floor C : N and C : (N : P) (43–50%,  $P < 0.001$ ) and by changing the forest floor N (11%,  $P \leq 0.015$ ), and forest succession indirectly inhibited forest floor N fixation by



**Figure 4** Structural equation models disentangling the major pathways of forest succession controlling nitrogen (N) fixation in the moss ( $\chi^2 = 0.69$ , d.f. = 2,  $P = 0.71$ , RMSEA < 0.001, (a);  $\chi^2 = 0.29$ , d.f. = 1,  $P = 0.59$ , RMSEA < 0.001, (b)), forest floor ( $\chi^2 = 1.35$ , d.f. = 1,  $P = 0.25$ , RMSEA < 0.001, (c);  $\chi^2 = 0.49$ , d.f. = 1,  $P = 0.48$ , RMSEA < 0.001, (d)), and soil ( $\chi^2 = 0.25$ , d.f. = 2,  $P = 0.29$ , RMSEA < 0.001, (e);  $\chi^2 = 0.01$ , d.f. = 1,  $P = 0.98$ , RMSEA < 0.001, (f)). Continuous and dashed arrows represent significant ( $P < 0.05$ ) and non-significant ( $P \geq 0.05$ ) pathways, respectively. Values on the arrows represent standardised path coefficients, proportional to the arrow width. Positive and negative values represent positive and negative relationships, respectively.  $r^2$  indicates the variation of N fixation explained by the models.

changing both the forest floor N and C : N (15%,  $P \leq 0.007$ ; Fig. 4c,d). Forest succession indirectly stimulated soil N fixation by changing the soil C : (N : P) (44%,  $P < 0.001$ ), and forest succession indirectly inhibited the soil N fixation by changing the soil P (40%,  $P \leq 0.009$ ) and by changing both the soil N and C : N (or C : (N : P)) (54–61%,  $P < 0.001$ ; Fig. 4e,f).

#### N fixation and substrate stoichiometry along forest age-gradient

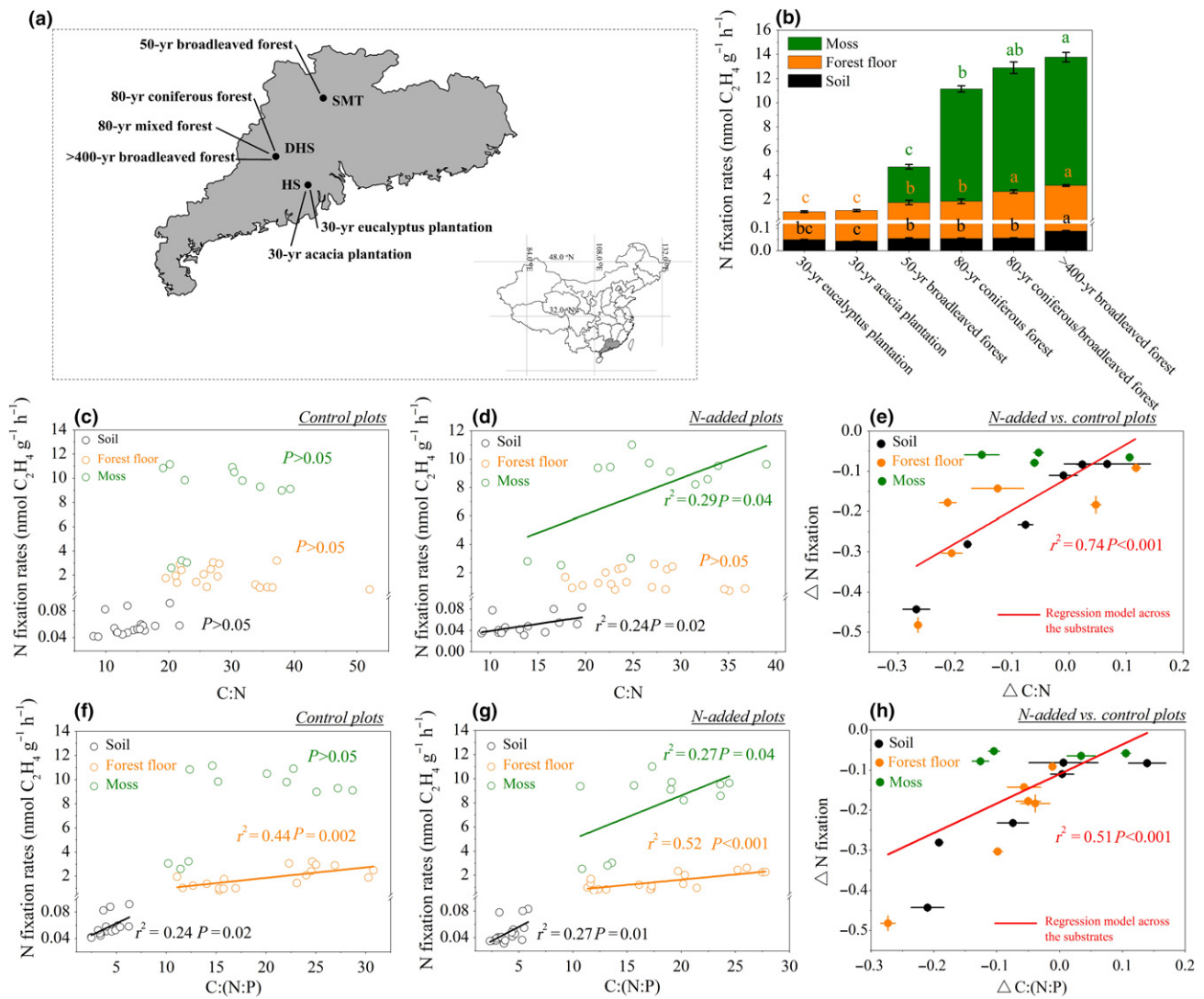
Generally, N fixation rates (per unit mass; hereafter) increased along forest age-gradient (Fig. 5b). Moss N fixation rates were the highest in the >400-year broadleaved forest, followed by the 80-year coniferous/broadleaved forest, 80-year coniferous forest, and 50-year broadleaved forest ( $F_{3,8} = 104.83$ ,  $P < 0.001$ ; ANOVA), and moss was absent in the 30-year acacia plantation and 30-year eucalyptus plantation. Forest floor N fixation rates were the highest in the >400-year broadleaved forest, followed by the 80-year coniferous/broadleaved forest, 80-year coniferous forest, 50-year broadleaved forest, 30-year acacia plantation, and 30-year eucalyptus plantation ( $F_{5,12} = 39.72$ ,  $P < 0.001$ ; ANOVA). Soil N fixation rates were the highest in the >400-year broadleaved forest, followed by the 80-year coniferous/broadleaved forest, 80-year coniferous forest, 50-year broadleaved forest, 30-year eucalyptus plantation, and 30-year acacia plantation ( $F_{5,12} = 48.72$ ,  $P < 0.001$ ; ANOVA). Nitrogen fixation rates per unit area showed a similar pattern with the N fixation rates per unit mass, with the total fixation rates increasing from 1.1 to 2.9 mg N m<sup>-2</sup> day<sup>-1</sup> along forest age-gradient (Fig. S8b).

Based on the result that N fixation was affected by resource stoichiometry (i.e. C : N and C : (N : P)) rather than N or P throughout succession (Fig. 3), this study also found that N

fixation rates were regulated by resource stoichiometry along forest age-gradient (Fig. 5). Linear regression models showed that substrate C : N explained 24–29% ( $P \leq 0.04$ ) of the N fixation variation in N-addition plots (Fig. 5d), and that substrate C : (N : P) explained 24–44% ( $P \leq 0.02$ ) and 27–52% ( $P \leq 0.04$ ) of the N fixation variation in control and N-addition plots, respectively (Fig. 5f,g). Under N-addition treatment, substrate  $\Delta$ C : N and  $\Delta$ C : (N : P) had positive relationships with  $\Delta$ N fixation ( $r^2 = 0.74$ ,  $P < 0.001$  and  $r^2 = 0.51$ ,  $P < 0.001$ , respectively; Fig. 5e,h).

#### DISCUSSION

Contrary to our hypothesis that N fixation might decline throughout succession (H1), we found that N fixation rates in all substrates increased throughout succession with peaks in the late-successional forest (Fig. 1 and Fig. S8), where the soil was already N-saturated (Fang *et al.* 2009). This finding extends a few previous findings that N fixation rates in moss increased with forest rehabilitation after fire disturbance (but the soil remained N limiting, Zackrisson *et al.* 2004; DeLuca *et al.* 2008) and contrasts with many previous findings that N fixation declined or remained constant throughout succession (Nohrstedt 1985; Hope & Li 1997; Crews *et al.* 2000, 2001; Son 2001; Pérez *et al.* 2004). Our findings extend the traditional view and empirical evidence that N fixation peaks in early- or mid-successional ecosystems (Gorham *et al.* 1979; Matzek & Vitousek 2003; Schmidt *et al.* 2008; Menge & Hedin 2009; Taylor *et al.* 2019) and support the prior assumption (Hedin *et al.* 2009) and observations (Reed *et al.* 2008; Menge & Hedin 2009; Zheng *et al.* 2018a) that N fixers remain active in many old-growth N-rich forests, which thereby maintains forest N richness (Menge & Hedin 2009).



**Figure 5** Nitrogen (N) fixation rates (per unit mass) and their relationships with substrate stoichiometry along forest age-gradient. Location of the six forest sites (a). Stacked column chart of N fixation rates in the soil, forest floor, and moss across the six forests (b). Different lowercase letters (within each substrate) represent significant differences among the forests ( $n = 3$ ,  $P < 0.05$ ). The white line in pane (b) represents the break of y-axis, which makes each column visible. Linear regression models of N fixation rates against C : N and C : (N : P) within each substrate in control plots ( $n = 18$  for the soil and forest floor and  $n = 12$  for the moss because moss was rare in the two HS plantations) (c, f). Linear regression models of N fixation rates against C : N and C : (N : P) within each substrate in N-addition plots ( $n = 18$  for the soil and forest floor and  $n = 12$  for the moss) (d, g). Linear regression models of response ratios of N fixation rates against those of C : N and C : (N : P) following N addition (*note*: the six solid points (with error bars) within each substrate represent the six forests (except for moss, which only occurred in four forests)) (e, h). Each solid point and error bar represents mean response ratio and 95% confidence interval following N addition, respectively. HS, Heshan research station; SMT, Shimentai natural reserve; DHS, Dinghushan biosphere reserve.

Consistent with prior theories (Walker & Syers 1976; Menge & Crews 2016) and observations (Vitousek & Farrington 1997; Crews *et al.* 2001), we found that substrate N and N : P increased throughout succession (Fig. 2). This finding, combined with our previous findings of net ecosystem N losses (Fang *et al.* 2009) and distinct microbial P limitation (Liu *et al.* 2012) in the studied late-successional forest, indicates that forest succession results in soil N richness and P limitation. However, our regression models showed that neither substrate N nor P could explain the N fixation variation (Fig. 3), which did not support our hypothesis (H2). Even under N-addition treatment, we found

no evidence that the N fixation variation was caused by N or P variation within each substrate (soil, forest floor, and moss) (Fig. 3). These results agree with the previous finding of Menge & Hedin (2009) that N fixation in litter, lichens, and bryophytes was not affected by soil N richness along the soil chronosequence. Our findings contrast with previous views and findings that high N or low P availability constrains N fixation during forest succession (Crews *et al.* 2000; Barron *et al.* 2011; Yelenik *et al.* 2013) and indicate that N fixation is not controlled by substrate N (*N richness hypothesis*) or P (*P limitation hypothesis*) throughout succession dynamics.



We propose two reasons to explain why our results contradict prior knowledge. First, the traditional view and assumed mechanisms of declining N fixation throughout succession are derived from and applied to symbiotic N fixation in plant root nodules. Theoretically, N-fixing plants (e.g. legumes) reduce their energy costs of nodule production and N fixation when soil N is sufficient (Gutschick 1981), which allows them to invest more energy toward acquiring limiting nutrients (e.g. P; Batterman *et al.* 2013b; Nasto *et al.* 2014). Thus, soil N richness and P limitation often result in declines in nodule N fixation (e.g. Pearson & Vitousek 2001; Batterman *et al.* 2013a; Menge & Crews 2016). Unlike nodule N fixation, which is energetically controlled by N-fixing plants, N fixation performed by autotrophic N-fixing microbes (e.g. cyanobacteria) is energetically dependent on photosynthesis of cyanobacteria or the C pools of host plants (e.g. moss; Rai *et al.* 2002) while N fixation performed by heterotrophic N-fixing microbes is energetically dependent on organic matter from the environment (e.g. soil and litter layers; Reed *et al.* 2011). Thus, N-fixing microbes can sustain high rates of N fixation in substrates with high C quantity and quality (Vitousek & Hobbie 2000).

Second, the growth and functions of N-fixing microbes are regulated not only by N and P but also by C availability (stoichiometry principle; Reed *et al.* 2011), such that resource stoichiometry may predict N fixation rates better than N or P alone. Empirical evidence has been found in several mature forests where substrates (e.g. leaf litter, foliage, and/or epiphytes) with high labile C but low total N contents (i.e. high C : N stoichiometry) exhibited high N fixation rates despite soil N richness and P limitation (Vitousek & Hobbie 2000; Pérez *et al.* 2010; Zheng *et al.* 2018a). Our study found increases in labile substrate C (e.g. ROC, MBC, and DOC) concentrations throughout succession (Fig. S3), similar to the pattern of increasing N fixation rates (Fig. 1). This evidence together suggests that the traditional view and hypotheses regarding N fixation in succession theory may have neglected the importance of substrate C availability.

Because substrates with high C (e.g. labile C), high P, and low N concentrations favor N fixation (Reed *et al.* 2011), we hypothesised that substrate C : N or C : (N : P) might explain the N fixation variation throughout succession better than substrate N or P (H3). This hypothesis was supported by our results. First, we found that labile C concentrations increased proportionally with total C concentrations throughout succession (Figs S3 and S6) and the response ratios of increased labile C were higher than those of increased N : P ratios driven by accumulation of N (Fig. S4). Second, our regression models showed that substrate N and P had no relationship with N fixation rates, whereas substrate C : N and C : (N : P) explained 12–66% of the N fixation variation (Fig. 3). Third, our structural equation models revealed that forest succession stimulated N fixation primarily via increases in substrate C : N ( $r^2 = 20\text{--}43\%$ ) and C : (N : P) ( $r^2 = 19\text{--}50\%$ ) (Fig. 4). Fourth, we found that forest succession changed all of the resource (particularly C) concentrations simultaneously rather than N or P concentrations alone (Table S4). These findings indicate that forest succession increases N fixation rates via changes in resource stoichiometry rather than

single resources, which provides new insight into succession theory and advances our understanding that N fixation is co-regulated by substrate C, N, and P during succession.

Furthermore, we propose a conceptual framework to explain our findings of increasing N fixation rates driven by substrate stoichiometry throughout succession (Fig. S7). In early-successional forests, although substrates with low N and high P availability favor N fixers, substrates with low C (e.g. labile C) availability inhibit N fixation (Vitousek & Hobbie 2000; Zheng *et al.* 2017). Thus, low substrate C : N or C : (N : P) stoichiometry limits N fixation during early succession. As N accumulates (via N deposition and fixation; Crews *et al.* 2001; Menge & Hedin 2009) and P is depleted (via biological uptakes and leaching losses; Walker & Syers 1976; Hedin *et al.* 2003), plant species and biomass increase in mid-successional forests, leading to increases in substrate total C (including labile C) and thereby N fixation rates. Thus, increases in substrate C : N or C : (N : P) stoichiometry stimulates N fixation at the mid-successional stage. In late-successional forests, although soil N richness or P limitation does not favor N fixers, N fixation in the litter and canopy layers is decoupled from and less controlled by soil N pool (Hedin *et al.* 2009; Menge & Hedin 2009), and high substrate C (e.g. ROC, MBC, and DOC; Fig. S3) availability becomes a key driver of N fixation. Thus, high substrate C : N or C : (N : P) stoichiometry supports N fixation at the late-successional stage (Fig. 3).

The findings and conceptual framework above were confirmed in our age-gradient forests. We found that N fixation rates (per unit mass and per unit area) increased along forest age-gradient (from young to old-growth forests; Fig. 5b, Fig. S8) due to increases in substrate C : N and C : (N : P) (explaining 24–74% of the N fixation variation; Fig. 5, Figs S9 and S10). This evidence supports our findings that forest succession dynamics drive N fixation via changes in substrate stoichiometry, and highlights that N fixation is co-regulated by C, N, and P during succession. Yet, plantations may not behave similarly to natural forests during succession. Our age-gradient forests that included young plantations only represent forests with a difference in stand ages rather than in naturally successional gradient, and more relevant studies are needed to verify these findings in the future. Together with previous findings that asymbiotic N fixers retain high fixation rates in old-growth forests (Reed *et al.* 2008; Menge & Hedin 2009; Zheng *et al.* 2018a), we conclude that N fixation plays an important role in maintaining soil N richness in late-successional forests, which challenges the traditional view (i.e. N fixation peaks in early- or mid-successional ecosystems where soils are limited by N) and offers new insight into succession theory.

## CONCLUSIONS

This study found that forest succession stimulated N fixation via changes in resource stoichiometry rather than in single resources, which has three implications for theoretical and applied ecology. First, our findings offer an important line of evidence that N fixation rates increase in all tested substrates throughout forest succession and along forest age-gradient

regardless of soil N richness and P limitation. These results renew our previous understanding of the highest N fixation rates occurring in early- or mid-successional forests (where soils are N-limited), which is predicted by succession theory and supported by many empirical studies. Second, we found no evidence that N fixation was controlled by N or P in any of the substrates throughout succession dynamics, which extends previous assumptions and modelling results that N fixers in the canopy and litter layers are decoupled from and less controlled by soil N richness (Menge & Hedin 2009; Hedin *et al.* 2009; Menge & Levin 2017). Third, our study uncovered a key mechanism driving N fixation throughout succession and stand age that labile substrate C increased rapidly compared to substrate N (increased C : N stoichiometry) and N : P (increased C : (N : P) stoichiometry), which favors N fixers. This stoichiometric mechanism indicates that N fixation rates are co-controlled by substrate C, N, and P simultaneously throughout forest dynamics, which supports a previous view that substrate stoichiometry may predict N fixation rates better than single nutrients (Reed *et al.* 2011) and helps understand the long-standing N paradoxical phenomenon that tropical forests rich in soil N sustain up-regulation of N cycling (Hedin *et al.* 2009). Overall, this study highlights the utility of ecological stoichiometry in illuminating the mechanisms that couple forest succession and N cycling.

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#### AUTHORS' CONTRIBUTIONS

MHZ and JMM designed the study. MHZ performed the research and analysed the data. All authors wrote the paper.

#### DATA AVAILABILITY STATEMENT

Data supporting the results are available from the Dryad Digital Repository: <https://doi.org/10.5061/dryad.5dv41ns2r>.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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