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.

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 (Cerrin 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 CO2 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 N2 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 (Zackrissone 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 (Dyński & Houlton 2018; Zheng et al. 2019) (N richness hypothesis). Because fixing atmospheric N₂ 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 N₂ 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 & Rue 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 (Alberty 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 (Alberty 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⁻¹ year⁻¹) 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

<table>
<thead>
<tr>
<th>Site</th>
<th>Experiment type</th>
<th>Forest type (Location)</th>
<th>Plots/Replicates/Established</th>
<th>Main tree species</th>
<th>Mean DBH of main tree species (cm)</th>
<th>Soil N (mg g(^{-1}))</th>
<th>Soil C (mg g(^{-1}))</th>
<th>Soil P (mg kg(^{-1}))</th>
<th>Soil MBC (mg kg(^{-1}))</th>
<th>MAT (°C)</th>
<th>MAP (mm)</th>
</tr>
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<tbody>
<tr>
<td>DHS1</td>
<td>Forest succession</td>
<td>Coniferous forest (Early succession)</td>
<td>112°10’ E, 23°10’ N (Control, +N150/n = 5/2007)</td>
<td>Pinus massoniana</td>
<td>19.3§</td>
<td>1.5</td>
<td>26.8</td>
<td>0.26</td>
<td>449§</td>
<td>21†</td>
<td>1927†</td>
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<tr>
<td></td>
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<td>Mixed coniferous and broadleaved forest (Middle succession)</td>
<td>112°10’ E, 23°10’ N (Control, +N150/n = 5/2007)</td>
<td>Pinus massoniana Schima superba Castanopsis chinensis</td>
<td>20.5§</td>
<td>2.1</td>
<td>34.7</td>
<td>0.29</td>
<td>327§</td>
<td>21†</td>
<td>1927†</td>
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<tr>
<td></td>
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<td>Broadleaved forest (Late succession)</td>
<td>112°10’ E, 23°10’ N (Control, +N150/n = 5/2007)</td>
<td>Schima superba Castanopsis chinensis Machilus chinensis Cryptocarya chinensis Syzygium rehderianum</td>
<td>26.0§</td>
<td>2.8</td>
<td>41.2</td>
<td>0.38</td>
<td>564§</td>
<td>21†</td>
<td>1927†</td>
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<tr>
<td>HSSMTDHS2</td>
<td>Forest age-gradient</td>
<td>Eucalypt plantation (30 years)</td>
<td>112°50’ E, 23°34’ N (Control, +N50/n = 3/2010)</td>
<td>Eucalyptus urophylla</td>
<td>11.1*</td>
<td>1.5*</td>
<td>15.5*</td>
<td>0.27</td>
<td>288*</td>
<td>22.5*</td>
<td>1534*</td>
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<tr>
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<td>Acacia plantation (30 years)</td>
<td>112°50’ E, 23°34’ N (Control, +N50/n = 3/2010)</td>
<td>Acacia auricaliformis</td>
<td>15.0*</td>
<td>1.6*</td>
<td>22.1*</td>
<td>0.29</td>
<td>254*</td>
<td>22.5*</td>
<td>1534*</td>
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<td>Broadleaved forest (50 years)</td>
<td>113°05’-113°31’ E 24° 22°-24° 31° N (Control, +N50/n = 3/2013)</td>
<td>Schima superba Castanopsis chinensis Machilus chinensis</td>
<td>18.6g (means of all species available)</td>
<td>1.9</td>
<td>25.4</td>
<td>0.35</td>
<td>334</td>
<td>20.8</td>
<td>2364</td>
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<td>Coniferous forest (80 years)</td>
<td>112°10’ E, 23°10’ N (Control, +N50/n = 3/2003)</td>
<td>Pinus massoniana</td>
<td>17.5#</td>
<td>1.4†</td>
<td>23.3†</td>
<td>0.30†</td>
<td>410†</td>
<td>21†</td>
<td>1927†</td>
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<td></td>
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<td>Mixed coniferous and broadleaved forest (80 years)</td>
<td>112°10’ E, 23°10’ N (Control, +N50/n = 3/2003)</td>
<td>Pinus massoniana Schima superba Castanopsis chinensis</td>
<td>22.0#</td>
<td>2.0†</td>
<td>32.1†</td>
<td>0.25†</td>
<td>450†</td>
<td>21†</td>
<td>1927†</td>
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<td></td>
<td>Broadleaved forest (&gt;400 years)</td>
<td>112°10’ E, 23°10’ N (Control, +N50/n = 3/2003)</td>
<td>Schima superba Castanopsis chinensis Machilus chinensis Cryptocarya chinensis Syzygium rehderianum</td>
<td>23.5#</td>
<td>2.9†</td>
<td>40.0†</td>
<td>0.34†</td>
<td>590†</td>
<td>21†</td>
<td>1927†</td>
</tr>
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</table>

Notes: Data with the label of *, †, ‡, §, ‖, ††, †‡, †§, †‖ 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: Dingushan biosphere reserve; HS: Heshan research station; SMT: Shimentai nature reserve. DHS1 and DHS2 represent different study sites in the Dingushan biosphere reserve. ‘+N50’ and ‘+N150’ represents N addition at a rate of 50 and 150 kg ha\(^{-1}\) year\(^{-1}\), 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⁻¹ year⁻¹. Each 5 × 5 m² plot was surrounded by a 5-m wide buffer strip, and all plots were randomly laid out within each forest. Solutions of NH₄NO₃ 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⁻¹ year⁻¹. Each square plot (10 × 10 m) and 10 × 20 mat the HS and DHS2 sites, respectively) or circular plot (17 m in semidiameter with an area of 907 m² at the SMT site) was surrounded by a more than 10-m wide buffer strip. At the SMT site, NH₄NO₃ 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 (NH₄NO₃) 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 cm²). 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 cm² 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 C₂H₂ (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 Exeter® (Labco, High Wycombe, UK) and returned to the laboratory for analysis. The C₂H₄ concentrations of each gas sample were measured using a gas chromatograph (Shimadzu GC14, Tokyo, Japan). The background C₂H₄ concentrations in the pure C₂H₄ gas and the C₂H₄ concentrations naturally produced by the samples were measured and subtracted. Nitrogen fixation rates per unit mass were expressed as the C₂H₄ production rates (nmol C₂H₄ g⁻¹ dry weight h⁻¹; Table S1), and N fixation rates per unit area were scaled up using the C₂H₄ 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 Fe²⁺ 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.
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. 1a). The late-successional forest had the higher total N fixation rates ($2.9 \pm 0.2 \text{ mg N m}^{-2} \text{ day}^{-1}$) than the early-successional ($2.2 \pm 0.1 \text{ mg N m}^{-2} \text{ day}^{-1}$) and mid-successional ($2.3 \pm 0.1 \text{ mg N m}^{-2} \text{ day}^{-1}$) 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 $F_{2,42} = 10.88 \ P < 0.001$, 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 : 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 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 and C : (N : P) (19–20%, $P \leq 0.002$), and forest succession indirectly inhibited moss N fixation by changing both the moss C : N and 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
and negative relationships, respectively. $r^2$ indicates the variation of N fixation explained by the models.

 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 ($F_{3,12} = 39.72, P < 0.001$; 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 ($F_{3,12} = 48.72, P < 0.001$; 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$^{-2}$ day$^{-1}$ 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 ΔC : N and ΔC : (N : P) had positive relationships with ΔN 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; Matzke & 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).
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 nodules 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 (r² = 20–43%) and C : (N : P) (r² = 19–50%) (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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