



Photosynthesis and growth responses of *Fraxinus mandshurica* Rupr. seedlings to a gradient of simulated nitrogen deposition

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Abstract

• **Key message** During an N-deposition simulation experiment, we showed that low to medium addition of N had beneficial effects on growth and photosynthetic rates of *Fraxinus mandshurica* Rupr. seedlings, while beyond a threshold of 80 kg N ha⁻¹ year⁻¹, performance plateaued and even declined at higher immissions.

• **Context** Temperate forests are shifting from naturally N-limited toward N-saturated status with increasing N deposition. Yet, our knowledge regarding how seedling growth and physiology respond to excessive N input in temperate tree species remains very limited.

• **Aims** The objective of this study was to examine growth and photosynthetic responses of *F. mandshurica* seedlings to a gradient of simulated N deposition.

• **Methods** We conducted a 4-year study to investigate growth and photosynthetic responses of *F. mandshurica* seedlings to a large gradient of simulated N deposition (0, 20, 40, 60, 80, 100, and 120 kg N ha⁻¹ year⁻¹). Biomass accumulation and allocation, photosynthetic gas exchange, expression, and activities of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) in leaves were determined during the fourth growing season. Soil biochemical properties were measured to link them to the alterations in growth and photosynthetic traits across the N addition gradient.

• **Results** Seedling growth and photosynthesis were dependent upon the rates of N deposition. The maximum rate of carboxylation ($V_{c,max}$) and the net photosynthetic rate under saturating light (A_{sat}) reached a maximum under 60 kg N ha⁻¹ year⁻¹. By contrast, high-level N inputs (100 and 120 kg N ha⁻¹ year⁻¹) resulted in suboptimal values in biomass and photosynthetic activity. Nitrogen deposition also modulated the activity and expression of Rubisco in leaves with a maximum around 80–100 kg N ha⁻¹ year⁻¹. Redundancy analysis (RDA) showed that the changes of seedling growth and photosynthesis along the gradient of N deposition were mostly attributed to the variations of soil pH and total N content.

• **Conclusion** Our data suggest that the threshold of N deposition is about 80 kg N ha⁻¹ year⁻¹ for *F. mandshurica* seedlings in this region. Excessive N input decreased performance on the seedling growth and photosynthesis.

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Contribution of the co-authors Miao Wang conceived and designed the experiments.

Miao Wang, Wei-Wei Zhang, Na Li, Yan-Yan Liu, and Xing-Bo Zheng performed the experiments.

Miao Wang analyzed the data, prepared figures, and wrote and revised the manuscript.

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1 Introduction

Elevated nitrogen (N) deposition has become one of the most challenging processes that terrestrial ecosystems are experiencing (Galloway et al. 2008; Hungate et al. 2003). Owing to fossil fuel combustion and agriculture, the annual bulk N deposition in China has increased to 21.1 kg N ha⁻¹ in the 2000s from 13.2 kg N ha⁻¹ in the 1980s (Liu et al. 2013a). At present, China has become by far the largest creator and emitter of N globally (Liu et al. 2013a). In addition to the areas developed by industry and agriculture, enhanced N deposition also has been frequently observed in forest ecosystems (Hyvönen et al. 2007; Fang et al. 2011). In some temperate forests of northeastern

China, the annual deposition rate has reached 27 kg N ha^{-1} , increasing 6–8-fold in the last 30 years (Zhou et al. 2015). With elevated anthropogenic N deposition, the temperate forest ecosystems are shifting toward N-saturated state (Aber et al. 1998; Fenn et al. 1998). Excessive N may reduce tree vitality and cause forest decline in tropical forests (Lu et al. 2010; Liu et al. 2011). Yet, studies on the impact of N immission on temperate forests are relatively scarce, mainly focusing on the coniferous tree species at the high latitude areas in northern Europe and North America (Boxman et al. 1998). In some temperate regions of East Asia, high N load could adversely affect growth, physiological functions, and nutrient status of Japanese forest tree species (Nakaji et al. 2001). Additionally, N deposition has a significant influence on photosynthetic gas exchange and stomatal conductance in temperate trees (Kinose et al. 2017; Tetteh et al. 2015; Azuchi et al. 2014; Kinose et al. 2014). The effects of N deposition on forest ecosystems, even at a similar deposition rate and duration, are probably different for each region, and mainly depend on the dominant species, soil properties, and preexisting climatic conditions (Jiang et al. 2014).

N deposition increases the amount of N available to plants, contributing to the photosynthetic capacity and the growth of forest trees (Latham 1992; Aber et al. 2003; Lu et al. 2010; Wang et al. 2012). Yet, under N saturation, additional N input may have negative effects on the structure and function of the forest ecosystem (Chen et al. 2015; Bobbink et al. 2010; Templer et al. 2012). It is known that photosynthesis activity is associated with leaf N content, which could significantly increase with additional N immission. The photosynthetic rates increase linearly with leaf N concentrations in five species of herbaceous plants (Pons and Poorter 2014) and in northern hardwood trees (Mitchell and Hinckley 1993). Net photosynthetic rate might decline in plants exposed to high N (Shangguan et al. 2000). The adverse effects of high N input on tree growth are likely due to alterations of photosynthetic rate in leaves. However, it is not known how the photosynthetic rate in the seedlings responds to different N input, especially under supra-optimal N. The resulting insights would be of upmost importance to determine the potential threshold of atmospheric N deposition leading to a decrease of the photosynthetic capability in forest tree species.

In leaves, more than half of N is used in the photosynthetic apparatus, and thus photosynthesis is strongly influenced by N availability (Lambers et al. 1998; Manter et al. 2005). As a key enzyme for carbon dioxide (CO_2) assimilation, the activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) could shift following the changes of leaf N content and then affect photosynthetic rates (Cheng and Fuchigami, 2000). In addition, Rubisco could function as a storage protein in leaves, which is an important component of leaf chloroplast (Warren et al. 2003). Our previous study showed that enhancement of photosynthetic capability under N addition is associated with upregulation of leaf Rubisco expression in *F. mandshurica*

seedlings (Wang et al. 2012). However, this report was not sufficient to conclude that photosynthetic rates are influenced by expressional alteration of Rubisco, because of a single-dose N deposition experiment. It is needed to investigate the dynamic of leaf Rubisco activity and expression along a large gradient of N deposition. Development of molecular techniques, such as immunofluorescent staining (Wang et al. 2012), provides a tool to determine the location and expression of Rubisco in leaves.

Fraxinus mandshurica Rupr. is among the most dominant tree species in natural forests of northeastern China (Wang et al. 2012). Here we investigated the changes of photosynthetic capacity as well as leaf Rubisco expression and activity in *F. mandshurica* seedlings along a gradient of stimulated N deposition. We had three specific objectives: (i) to determine how excessive N immission, above a N deposition threshold, affects the photosynthesis and growth performance of *F. mandshurica* seedlings; (ii) to investigate changes of leaf Rubisco expression and activity across the N gradient; and (iii) to explore the variation of soil properties and its roles in the shift of plant biomass production and photosynthetic efficiency in response to increasing N input.

2 Materials and methods

2.1 Study site and experimental designs

This study was conducted over a 4-year period (from May 2010 to September 2013) in the Changbai Mountain Forest Ecosystem Research Station (CBFERS), Chinese Academy of Sciences, northeastern China ($42^\circ 24' 09'' \text{ N}$, $128^\circ 05' 45'' \text{ E}$, 738 m a.s.l.). The area has a temperate continental climate, with a mean annual temperature of 3.6° C (monthly range -15.6 to 19.7° C), as well as a mean annual precipitation of 695 mm (Zhang et al. 2005). The ambient N deposition rate in the region was approximately $12 \text{ kg N ha}^{-1} \text{ year}^{-1}$ (Liu et al. 2011). Two-year-old seedlings of *F. mandshurica* with uniform size were obtained from the local tree nursery (Guangming Seedling Nursery Co., Erdao Forest Farm, Jilin Province, China) and planted into plastic pots (one seedling per pot) in early May 2010. The initial height, stem base diameter, and whole dry biomass of seedlings were $18.50 \pm 2.42 \text{ cm}$, $3.64 \pm 0.67 \text{ mm}$, and $2.01 \pm 0.38 \text{ g}$, respectively. Each of the pots (40 cm high; 30 and 28 cm for the upper and lower diameters, respectively) was filled with an aliquot (22 kg) of dark brown forest soils uniformly collected from broad-leaved Korean pine mixed forest. Prior to filling pots, the soils were passed through a 5-mm sieve to reduce heterogeneity. The initial N content of the soils was about $0.30 \pm 0.024\%$ ($3.01 \pm 0.24 \text{ g N kg}^{-1}$). After planting the seedlings, the pots were placed in an open area with full sunlight during the experiment. The potted seedlings were

randomly divided into seven groups for different treatments, i.e., seven different levels of N simulating N deposition rates of 0, 20, 40, 60, 80, 100, and 120 kg N ha⁻¹ year⁻¹. For each treatment, 20 seedlings in individual pots were used. Ammonium nitrate solutions with six different concentrations (0.010, 0.020, 0.030, 0.040, 0.051, and 0.061 mol L⁻¹) were sprayed four times into the pots once a month (125 mL per pot) from June to September each year for four consecutive years, respectively. Seedlings were watered regularly during the growing seasons, and pots were relocated randomly every month to minimize effects of potential differences in environmental conditions among the seven treatments. All seedlings were grown under the same conditions with the exception of variations in soil N levels.

2.2 Photosynthetic gas exchange

Gas exchange was recorded with a LI-6400 portable photosynthesis system equipped with a LED light source (LI-6400-02B, LI-COR Inc., Lincoln, NE) between 9:00 a.m. and 11:30 a.m. on July 20–24, 2013. Six seedlings per N treatment were randomly chosen for the measurements. For each individual, two fully expanded youngest leaves from the top of canopy were measured. The light response curves (A/PPFD) were obtained at different light levels of 1600, 1400, 1200, 1000, 800, 600, 400, 300, 200, 150, 100, 80, 50, 30, and 0 $\mu\text{mol m}^{-2} \text{s}^{-1}$, and the CO₂ concentration within the leaf chamber was maintained at $360 \pm 10 \mu\text{mol m}^{-2} \text{s}^{-1}$ at leaf temperature of 25 °C and relative humidity of 65–75% inside the leaf chamber. The carbon assimilation (A) to intercellular CO₂ (C_i) response (A/C_i) curves were performed subsequently, and the response of A to changing CO₂ was measured at 400 $\mu\text{mol mol}^{-1}$, which was decreased to 300, 200, 150, 100, and 50 $\mu\text{mol mol}^{-1}$, then returned to 400, and subsequently increased to 500, 600, 800, 1000, and 1200 $\mu\text{mol mol}^{-1}$ under saturating irradiance (1300 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD). Curves plotted against the intercellular CO₂ concentration (C_i) were analyzed to estimate the V_{cmax} (maximum carboxylation rate of Rubisco) and J_{max} (RuBP regeneration capacity mediated by maximum electron transport rate). Light-saturated photosynthetic rate (A_{sat}), light saturation point (L_{sp}), and dark respiration (R_{dark}) were determined by analyzing A/PPFD curves (Long and Bernacchi 2003; Sharkey et al. 2007).

2.3 Chemical analysis of leaf carbon and nitrogen

Two or three nonshaded leaves per seedling (six plants per treatment) were randomly harvested on July 10, 2013, for the chemical analysis. The area of the fresh leaves was measured after petiole removal, with an area meter (LI-3000A; LI-COR). The leaves were washed with deionized water, and then dried in an oven at 70 °C for 48 h. Dried leaves were weighed to determine the specific leaf area (SLA). Dried

leaves were ground to fine powder with a vibrating sample mill (MM 400 Retsch, Haan, Germany), and then the concentration of C and N was determined with a C/N analyzer (MT-700, Yanaco, Japan).

2.4 Measurements of Rubisco activity and concentration

Leaves were freshly collected on July 26, 2013, and stored in liquid nitrogen for biochemistry analyses. The activities of leaf Rubisco were assayed according to the procedures we described previously (Wang et al. 2012). In brief, the frozen samples were ground to fine powder in liquid nitrogen, and Rubisco was then extracted with the buffer containing 50 mM HEPES-KOH (pH 8.0), 10 mM MgCl₂, 0.5 mM EDTA, and 1% (w/v) polyvinylpyrrolidone. The extract was centrifuged at 16,000g for 15 min, and the supernatants were used for assessment of Rubisco activity and content. The activity of Rubisco was spectrophotometrically determined by measuring the disappearance rate of NADH. To determine the initial activity of Rubisco, the reaction was started by adding 60 μL of 10 mM RuDP, immediately after combining the desalted sample solution (100 μL) with assay solution (830 μL). Here the assay solution was mainly composed of phosphocreatine (5 mM), phosphoglyceric kinase (10 units/mL), glyceraldehyde-3-phosphate dehydrogenase (10 units/mL), and phosphocreatine kinase (20 units/mL) in a buffered solution (50 mM HEPES-KOH (pH 8.0), 10 mM NaHCO₃, 1 mM EDTA, 20 mM MgCl₂, 1.5 mM NADH, 5 mM ATP, 2.5 mM DTT). The changes in the absorption of the reaction solution at 340 nm over 1 min were measured approximately at 20-s intervals. The activation state of Rubisco was represented by the ratio between initial activities to total activities.

2.5 Immunolocalization and expression of Rubisco in the leaves

For each treatment, six leaves were randomly selected for immunofluorescent assay from the nitrogen canister. The samples were cut into small pieces (2 × 2 mm² approximately) and then embedded in optimal cutting temperature embedding medium (Tissue-Tek O.C.T. Compound, Sakura Finetek, CA). Sections of 5 μm were cut and were attached to poly-lysine-coated slides. Slides were extensively washed with phosphate-buffered saline (PBS) and blocked with 1% bovine serum albumin in PBS for 20 min at room temperature. The polyclonal primary rabbit anti-Rubisco antibody diluted by 1:2000 was added into the slides and incubated overnight at 4 °C in a humidity chamber. After washings with PBS, slides were incubated in Alexa Fluor 488-conjugated secondary antibody (1:500) (Molecular Probes, Eugene, OR, USA) for 30 min. Nuclei were stained with DAPI (4',6'-diamidino-2-phenylindole) (Molecular Probes, Eugene, OR, USA).

Sections were visualized with a Leica TCS SP2 confocal scanning microscope (Heidelberg GmbH, Mannheim, Germany).

2.6 Biomass accumulation and allocation

During mid-September 2013, a total of 42 seedlings (6 replicates of each treatment) were randomly harvested to determine the growth response to N additions. Roots were collected by sieving the seedling at the root collar, and were then washed carefully to remove soil. The shoots were separated into two main parts: the leaves and stem. The collected samples were dried at least 72 h in an oven at 80 °C and then weighed.

2.7 Soil biochemical properties

Soil samples of 10-cm depth from a seedling collar were taken in late September 2013, and transported back to the laboratory in an ice box and analyzed within 24 h for soil water and inorganic N content. Soil samples were extracted in 2 mol L⁻¹ KCl and were analyzed for NO₃⁻-N and NH₄⁺-N by flow injection analysis (FIA) (Lachat Instruments, Mequon, Wisconsin, USA). Subsamples were dried at 105 °C for 48 h for water content, and parallel samples were air-dried at room temperature and soil pH was measured with a glass electrode in a 1:2.5 ratio (soil/solution) (H₂O) (Thermo Orion T20, Waltham, MA). The other samples were passed through a 0.5-mm sieve for soil C and N content. Total organic carbon (TOC) and total nitrogen (TN) in soils were measured using an Element Analyzer (Analyzer vario MICRO cube, Elementar, Germany).

2.8 Statistical analyses

Quantitative data are presented as means ± standard deviation (SD). One-way analysis of variance (ANOVA) was used to test the effects of different N treatments on the physiological characteristic and growth of the seedlings. LSD multiple range test was conducted to determine whether differences between treatments existed. A *P* value of less than 0.05 was considered significant. Statistical analyses were performed with the SPSS software package (SPSS Inc., Chicago, IL). Redundancy analysis (RDA) was conducted to estimate the potential links of the growth and photosynthetic traits of the seedlings with soil biochemical properties. The growth and photosynthetic parameters as well as the activities of Rubisco in leaves were viewed as response traits, and soil nutrients and pH values as environmental factors for this analysis. The significance and proportions of the total variance in the seedling growth explained by environmental variables were detected with the Monte Carlo permutation test using

CANOCO 4.5 software package (Microcomputer Power, Ithaca, NY).

3 Results

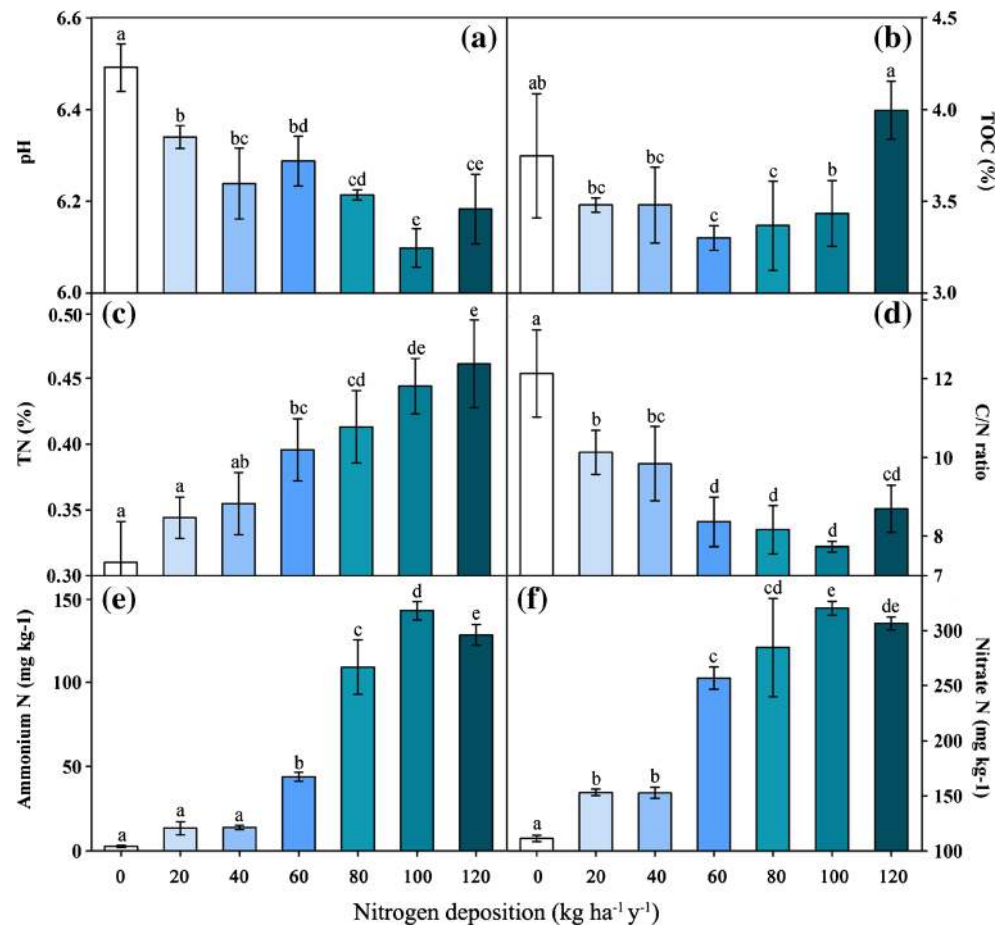
3.1 Changes of soil physicochemical properties following different N depositions

N additions led to a significant reduction in soil pH, and this effect was strikingly enhanced with continuous increases in the rate of N deposition (Fig. 1a). In contrast, total N (TN) in soils was pronouncedly elevated with increasing N deposition (Fig. 1c). Total organic carbon (TOC) in soils showed a marked decrease under medium-level N additions (60 and 80 kg N ha⁻¹ year⁻¹), while it increased at high N deposition (120 kg N ha⁻¹ year⁻¹) (Fig. 1b). Soil C/N ratios were reduced with increasing N deposition (Fig. 1d). The concentrations of nitrate and ammonium N in soils displayed continuous enhancement with increasing N deposition (Fig. 1e, f). No difference in the soil water contents was observed among different N deposition rates (data not shown).

3.2 Growth response of the seedlings to stimulated N deposition

Compared to those without N input (0 kg N ha⁻¹ year⁻¹), the plant height markedly raised in all of the seedlings receiving stimulated N deposition manipulations (*P* < 0.05) (Fig. 2a). Seedling height reached a maximum value under medium-level N supplies (60 and 80 kg N ha⁻¹ year⁻¹), and declined with increasing N deposition, especially at 120 kg N ha⁻¹ year⁻¹. Similar to the plant height alterations, the dry biomass of leaves, stems, and whole plants increased almost linearly along the gradient N addition at first (*P* < 0.05), reaching a maximum at 60 kg N ha⁻¹ year⁻¹, and subsequently declined at higher N deposition rates (*P* < 0.05, vs. 60 kg N ha⁻¹ year⁻¹) (Fig. 2b–e). In general, root biomass was enhanced following N additions (excluding 120 kg N ha⁻¹ year⁻¹) with respect to controls, but it did not change with N deposition rate (Fig. 2d). Surprisingly, different N deposition intensities also had significant effects on biomass allocation (Fig. 2f, g). The fraction of the aboveground biomass was substantially increased following N additions from 20 to 60 kg N ha⁻¹ year⁻¹ (Fig. 2f). The shoot-root ratio (S/R) markedly increased by N additions, reaching a maximum at 60 kg N ha⁻¹ year⁻¹ (*P* < 0.05) (Fig. 2g). Although it decreased profoundly with further increase of N deposition, S/R remained higher than without N addition. Following N additions, SLA gradually decreased from 20 to 60 kg N ha⁻¹ year⁻¹ and increased and reached a maximum under 120 kg N ha⁻¹ year⁻¹ (Fig. 2h).

Fig. 1 Changes of soil physiochemical properties following different level N deposition rate. **a** Soil pH values. **b** Total organic carbon (TOC). **c** Total nitrogen (TN). **d** Ratios of soil C and N contents. **e** Contents of soil nitrate N. **f** Levels of soil ammonium N



3.3 Leaf photosynthetic capacity across the gradient of N deposition

Leaf photosynthesis and related traits were affected by N deposition (Fig. 3). High N deposition (100 and 120 kg N ha⁻¹ year⁻¹) resulted in a significant decline in A_{sat} ($P < 0.05$), while A_{sat} remained stable at all other deposition rates (Fig. 3a). $V_{c,max}$, J_{max} , and L_{sp} were profoundly increased under low and medium-level N deposition ($P < 0.05$, vs. 0 kg N ha⁻¹ year⁻¹), and markedly lowered under high N deposition rates ($P < 0.05$, vs. 60 kg N ha⁻¹ year⁻¹) (Fig. 3b–d). Leaf dark respiration (R_{dark}) was strikingly elevated under low and medium-level N deposition rates ($P < 0.05$, vs. 0 kg N ha⁻¹ year⁻¹), whereas it was further increased under high N addition ($P < 0.05$, vs. N3) (Fig. 3e). By contrast, leaf stomatal conductance (g_s) was reduced following N additions, reaching the valley value in the seedlings receiving N addition of 60 kg N ha⁻¹ year⁻¹ (Fig. 3f).

3.4 Activity and content of leaf Rubisco under different-level N depositions

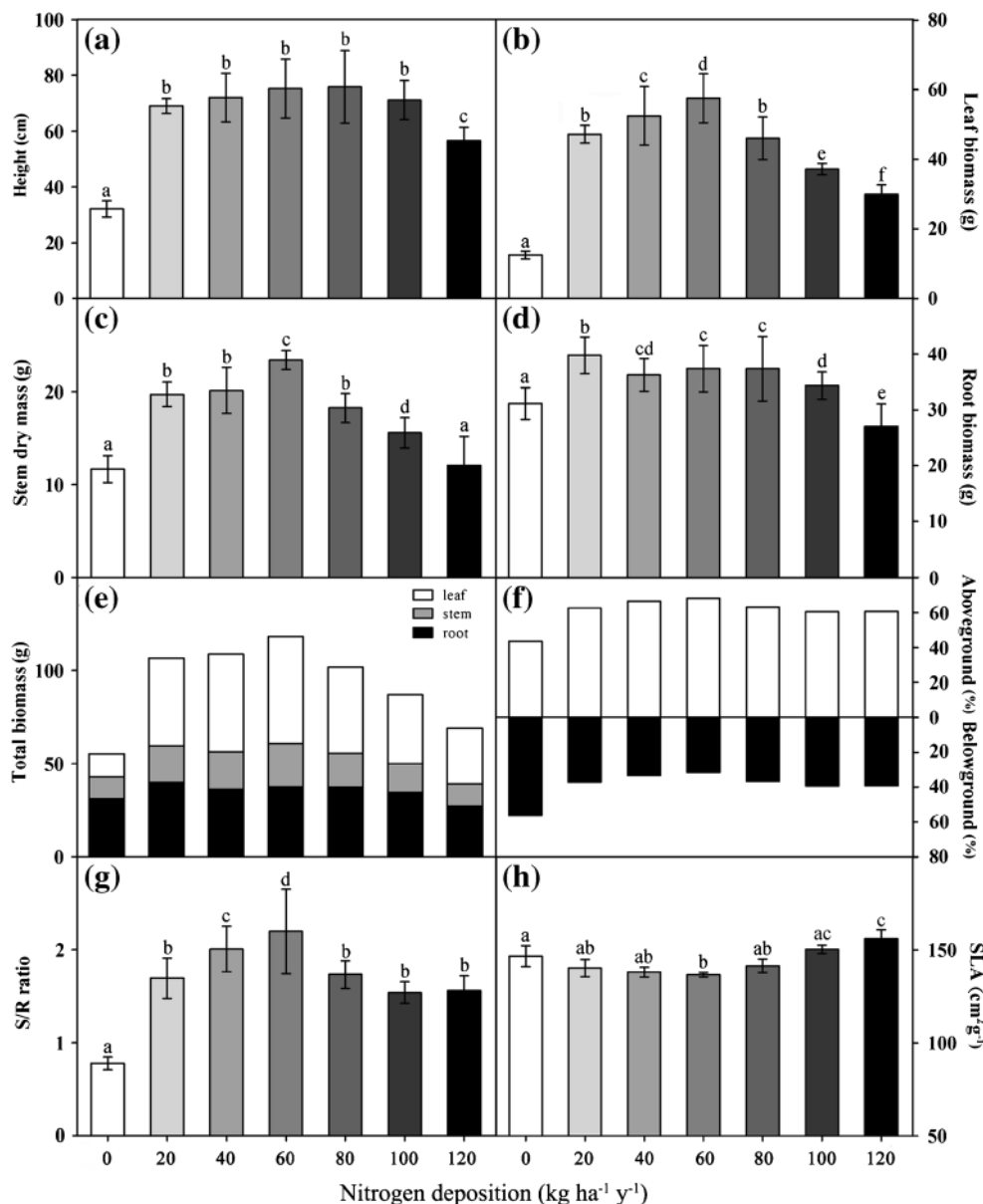
The activity and content of Rubisco in leaves were markedly varied in response to different N deposition rates (Fig. 4). The

initial and total activity of Rubisco in leaves was significantly increased in the seedlings receiving N additions from 20 to 60 kg N ha⁻¹ year⁻¹ ($P < 0.05$, vs. 0 kg N ha⁻¹ year⁻¹) (Fig. 4a, b). The activity of the leaf Rubisco was then declined with further increase of the N deposition rate, which was even lower than in the seedlings without N input ($P < 0.05$). Yet, no significant change in the activation state of Rubisco in leaves was observed following N deposition, except at the N deposition rate of 40 kg N ha⁻¹ year⁻¹ ($P < 0.05$, vs. 0 kg N ha⁻¹ year⁻¹) (Fig. 4c). The content of Rubisco in leaves enhanced following increase of N deposition from 40 to 80 kg ha⁻¹ year⁻¹. The Rubisco content was subsequently reduced in which the N deposition rate was above 80 kg N ha⁻¹ year⁻¹, but it remained much higher than in the baseline (0 kg ha⁻¹ year⁻¹) (Fig. 4d).

3.5 Expressional variations of leaf Rubisco along N deposition gradient

The Rubisco proteins were labeled by the green fluorescence in leaves of the seedlings, and the expression of Rubisco was pronouncedly shifted across the gradient of N deposition (Figs. 4b and 5a). The N deposition rate from 40 to 80 kg N ha⁻¹ year⁻¹ induced significant increases in the expression

Fig. 2 Biomass of *F. mandschurica* seedlings with increasing N deposition rates. Seedling height (a), leaf biomass (b), stem biomass (c), root biomass (d), and total biomass (e) under different N deposition rates. (f) Distribution of the aboveground and belowground biomass. Variations in the ratio of the shoot and root biomass (S/R) (g) and the specific leaf area (SLA) (h) along the N-deposition gradient. Means with different letters are significantly different ($P < 0.05$)



of leaf Rubisco ($P < 0.05$, vs. 0 kg ha⁻¹ year⁻¹) (Fig. 5b). In contrast, the expression of Rubisco markedly declined with increasing N deposition rate, which was close to the baseline level at 120 kg N ha⁻¹ year⁻¹.

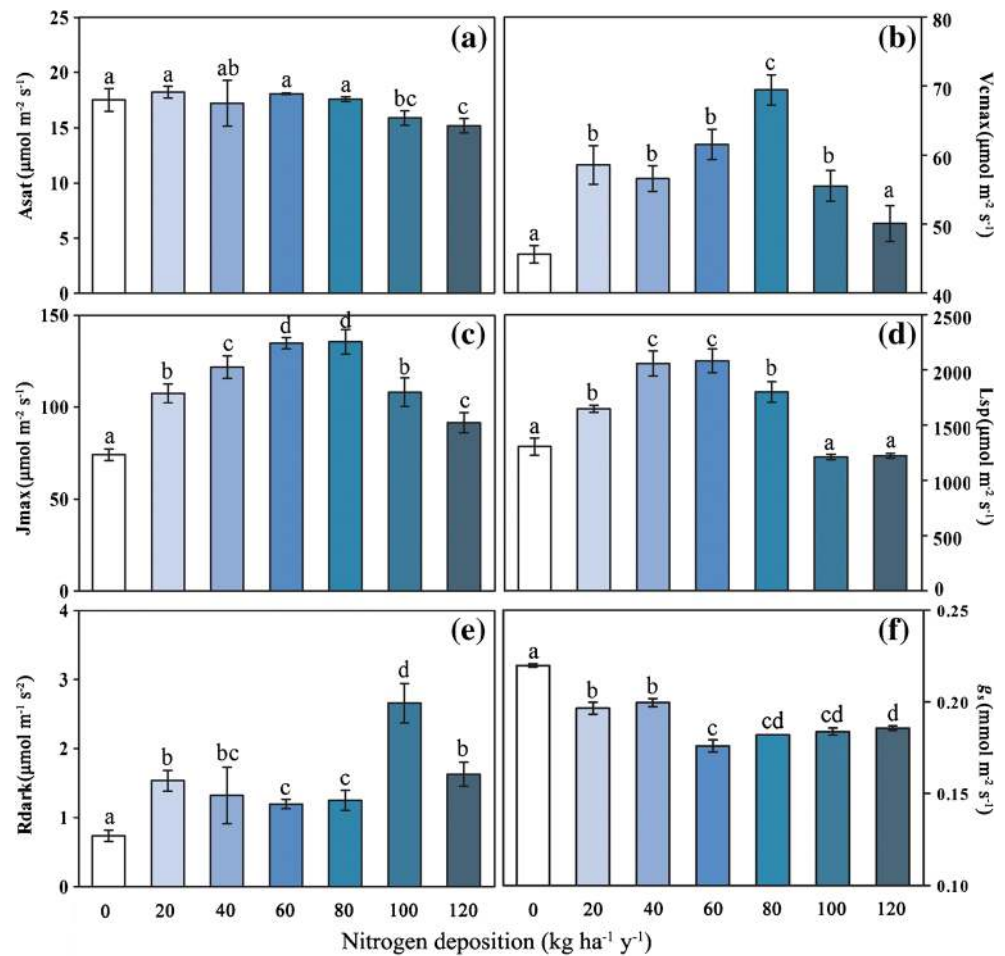
3.6 Changes of leaf nutrients in response to N deposition

Foliar N was lower under low-level N deposition (20 and 40 kg N ha⁻¹ year⁻¹) compared to the baseline value ($P < 0.05$). Subsequently, it reached a peak at 120 kg N ha⁻¹ year⁻¹ ($P < 0.01$, about 2.2-fold, vs. 0 kg N ha⁻¹ year⁻¹) (Fig. 6a). By contrast, leaf C peaked at 60 kg N ha⁻¹ year⁻¹ (Fig. 6b). The leaf C/N ratios were also altered significantly following N deposition (Fig. 6c).

3.7 Relationship between the seedling growth dynamic and soil nutritional status

The divergence in the growth and photosynthesis of the seedlings was clearly observed across N-deposition gradient, as characterized by the significant distribution differences of the sampling dots in the ordination diagram (Fig. 7). For the RDA, the first and second axes explained 54.3 and 17.4% of the variance, respectively. Results from the Monte Carlo test indicated that the correlations between response variables and environmental factors were of special significance. Soil TN contents were positively related to seedling growth and photosynthesis under low and medium-level N deposition, while adversely associated with them under high N deposition

Fig. 3 Response of photosynthetic activity in the seedlings to different N deposition rate. **a** A_{sat} : light-saturated net assimilation rates. **(b)** $V_{\text{c,max}}$: maximum rate of carboxylation. **c** J_{max} : maximum rate of electron transport. **d** L_{sp} : light saturation point. **e** R_{dark} : dark respiration. **f** g_s : stomatal conductance. The leaf samples were collected, and the photosynthetic performance was assessed in July 2013. Means with different letters are significantly different ($P < 0.05$)



rate. Soil nitrate and ammonium N also presented similar patterns with TN to be correlated to the growth of the seedling. By contrast, soil pH and C/N ratio displayed

weak association with seedling growth and photosynthesis under low and medium N deposition rates, but strongly associated with them under high N.

Fig. 4 Alterations of Rubisco activity and content under different level N deposition rate. Changes in the initial activities **(a)**, the total activities **(b)**, activation state **(c)**, and contents **(d)** of leaf Rubisco following different-level N deposition rates. Leaf samples were collected, and enzyme activities and content were assessed in July 2013. Means with different letters are significantly different ($P < 0.05$)

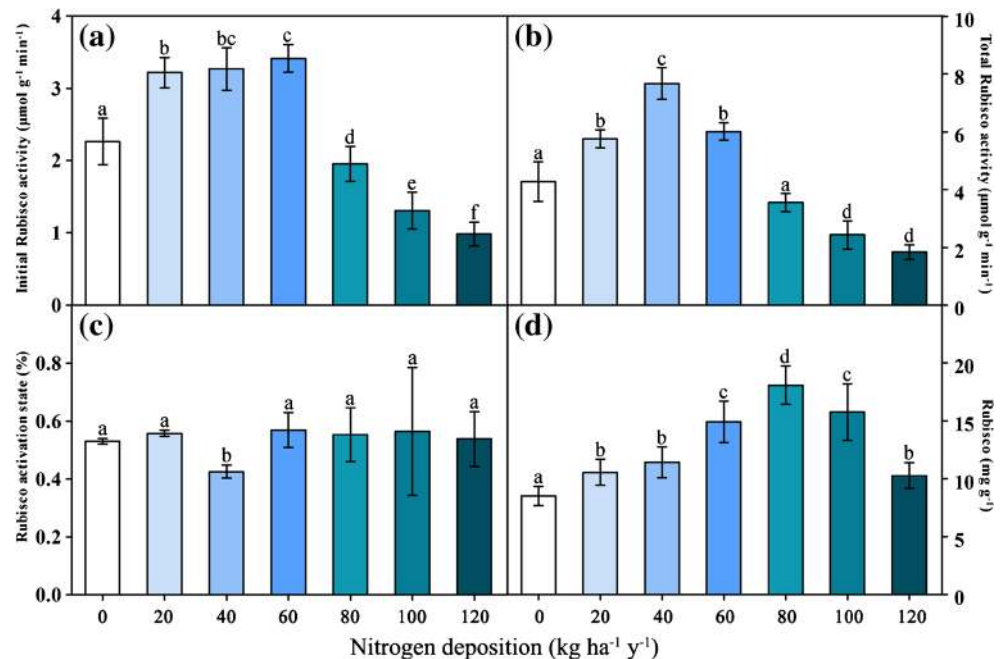
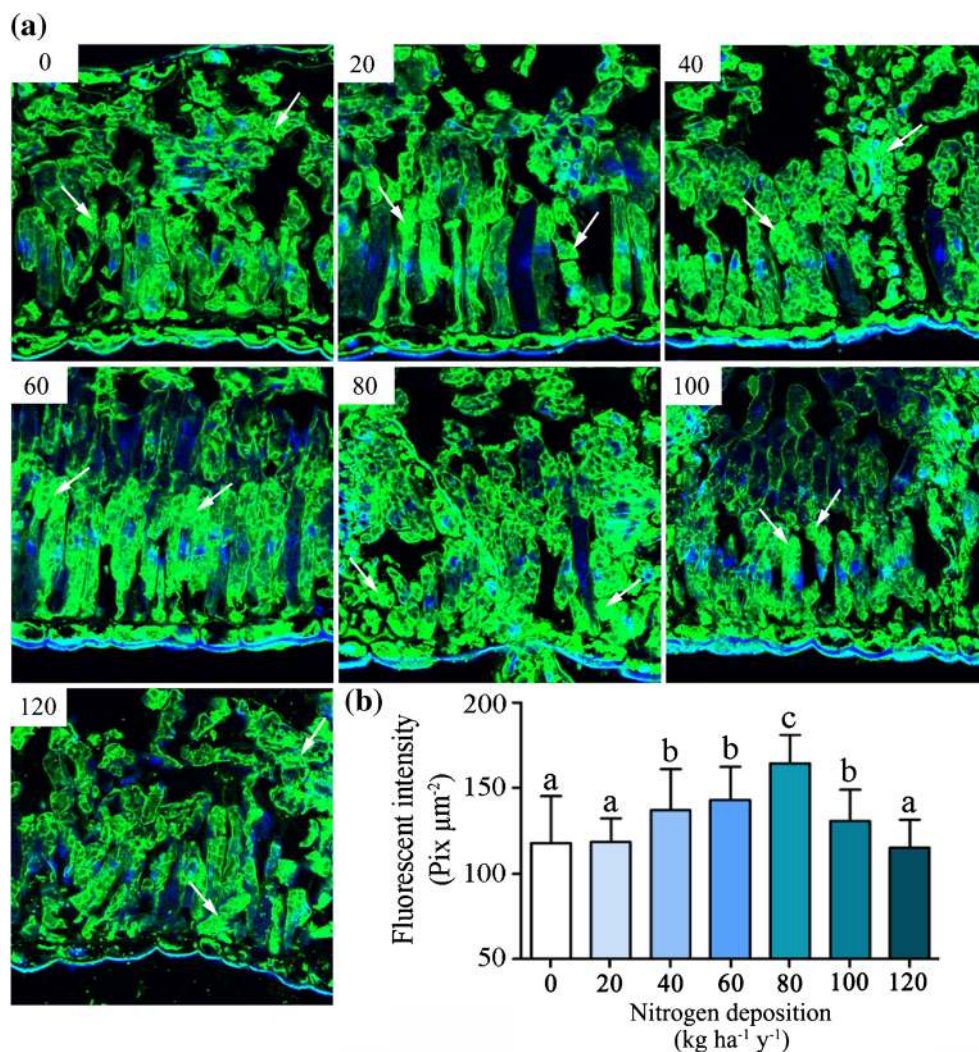


Fig. 5 Localization and expression of Rubisco in the leaves of the seedlings of *F. mandshurica*. **a** Representative images indicating the variations of the Rubisco localization and expression in response to N deposition. The arrows indicate the fluorescent staining (green) of Rubisco protein. Numbers presenting in the images represent the rates of N deposition ($\text{kg N ha}^{-1} \text{ year}^{-1}$). **b** Quantitative comparisons of the Rubisco expression under different N deposition rate. The leaf samples were collected in July 2013 and were stored in liquid nitrogen until analysis. Means with different letters are significantly different ($P < 0.05$)



4 Discussions

Here we present a 4-year experiment to evaluate the effects of N deposition on photosynthetic and growth performance of *F. mandshurica* seedlings. Low to medium-level N deposition rate induced positive effects on photosynthesis and growth of the seedlings, but the performance plateaued and even declined when the deposition rate was above $80 \text{ kg N ha}^{-1} \text{ year}^{-1}$. Further, shifts of leaf Rubisco expression and activity may be involved in the differential effect of N deposition on seedling growth. In addition, soil acidification and nutrient imbalance are likely considered as another important factor influencing seedling growth under excessive N input.

Temperate forest ecosystems are often N-limited, and substantial increase of N deposition could exceed the capacity for N uptake by plants, leading to N saturation (Aber et al. 1998; BassiriRad 2015). In the present study, the growth responses to increasing N were nonlinear and the peak values were observed at medium-level N addition (60 and $80 \text{ kg N ha}^{-1} \text{ year}^{-1}$), suggesting the beneficial effects of N

deposition (Brown et al. 1996; Li et al. 2004; Liao et al. 2010). Compared to medium-level N deposition, however, the whole-plant biomass significantly declined under high-level N deposition (100 and $120 \text{ kg N ha}^{-1} \text{ year}^{-1}$), which is similar to previous findings in three bog shrubs (Bubier et al. 2011) and *Laelia speciosa* Shltr. (Diaz-Alvarez et al. 2015). It seems that an additional N input beyond 80 kg N ha^{-1} per year may exceed the biotic N command and induce N saturation for the tree species.

The difference in the effects of N inputs on seedling growth is likely caused by alteration of C assimilation and leaf area. Plant biomass allocation reflects an integration of plant growth and environmental adaptability, which varies in response to environmental disturbance (Hättenschwiler and Körner 1998). The findings presented here showed that the biomass distributions shifted following different N deposition rates. A substantial increase in aboveground biomass, especially leaf biomass, was observed under medium-level N deposition, indicating that adequate N could stimulate seedlings to allocate more nutrients to

leaves, and then promote the development of leaf photosyn-

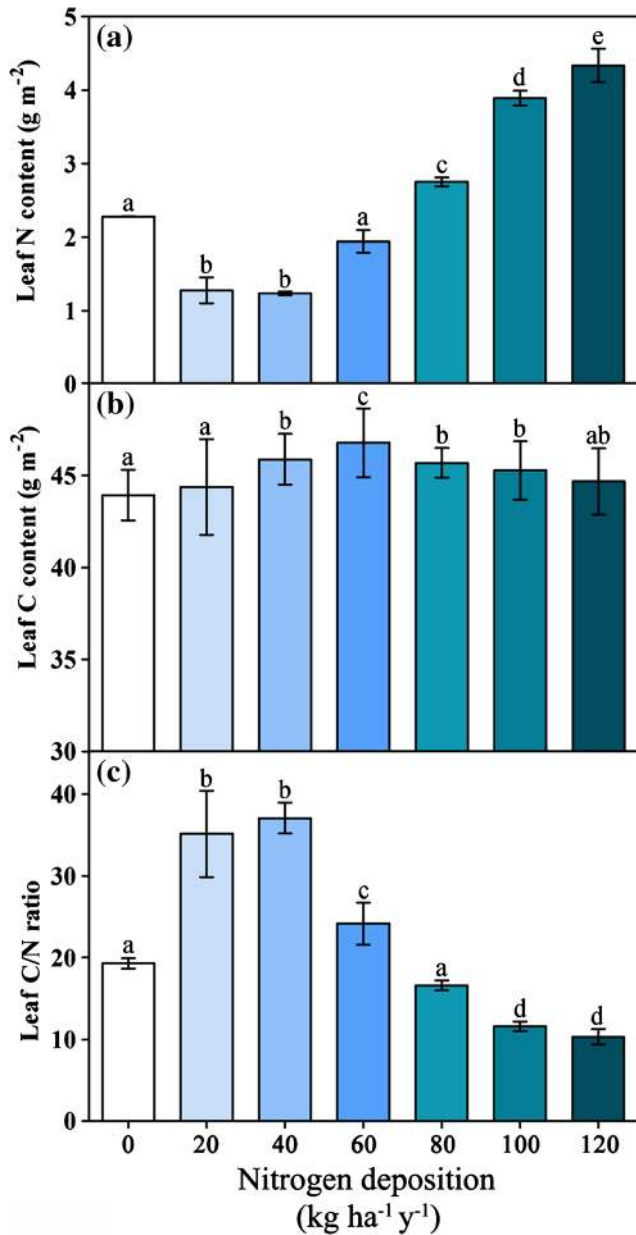


Fig. 6 Leaf nutrients (N and C) under different N deposition rates. Leaf samples were collected and analyzed in July 2013. Means with different letters are significantly different ($P < 0.05$)

thetic organelles (Hättenschwiler and Körner 1998). Similarly, SLA also declined under medium-level N deposition of 60 kg N ha⁻¹ year⁻¹. The increase in leaf thickness would provide more photosynthetic tissues and cell/intercellular air space contact for CO₂, which is beneficial for photosynthetic C assimilation (Hikosaka 2004; Shipley et al. 2006). Adequate N input could drive biomass allocation to leaves, which may in turn enhance photosynthetic capacity and promote the biomass production of the seedling. By contrast, SLA strikingly increased under high N deposition rate, suggesting that excessive

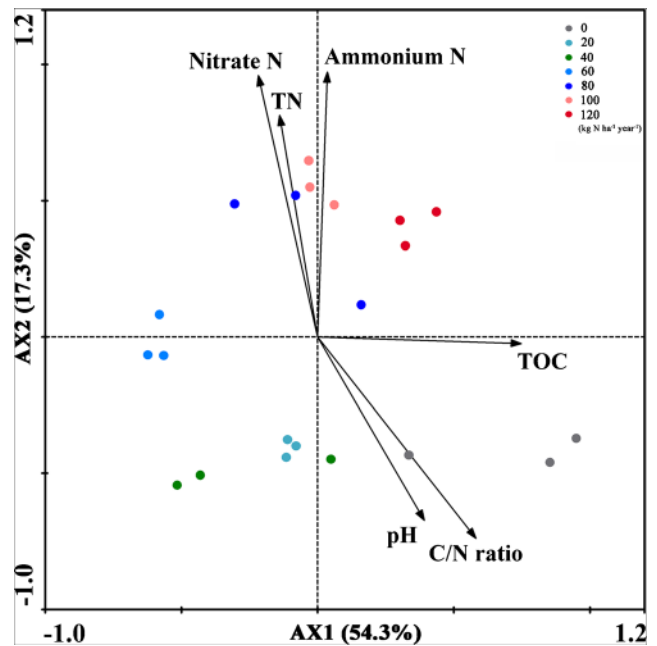


Fig. 7 Relationships between the seedling growth dynamic and soil nutritional status. Redundancy analysis displaying the relationship between the seedling growth and soil properties including soil pH, total N content, organic C content, nitrate N, and ammonium N

N input might induce a reduction of biomass allocation to leaves. Generally, the total C assimilation of seedlings depends on photosynthetic capacity and total leaf area. Therefore, besides the changes in photosynthetic efficiency, altered leaf area might be another factor causing a reduction of leaf biomass under high N deposition rate.

The correlation of V_{cmax} and J_{max} with N availability showed bell-shaped curves, reaching peak values under medium N deposition rate. Enhanced photosynthetic efficiency appears to be related to N availability, which is consistent with previous findings in *Robinia pseudoacacia* L. seedlings (Liu et al. 2013b). On the contrary, photosynthetic efficiency reduced with further increase of N deposition. The findings are similar to the previous reports (Wortman et al. 2012; Braun et al. 2010), providing a potential explanation for the decline of biomass production under extra N input. The growth responses of the seedlings along the N gradient are also accompanied by the dynamic changes of whole-plant hydraulic conductance (Wang et al. 2016). Thereby, we can conclude that shifts of leaf water-use efficiencies, owing to hydraulic architectural alterations, might represent another mechanism underlying the different responses of seedling growth to N depositions.

Alteration of Rubisco activity displayed a similar pattern with seedling biomass and photosynthetic rate, suggesting that the N-saturated threshold for the Rubisco activity is likely consistent with that of seedling growth. Given that the initial and total activities of the Rubisco were decreased under high N deposition, it is possible that the reduction of leaf

photosynthetic efficiency under excessive N was partly due to changes of the enzymatic activities (Manter et al. 2005; Ethier et al. 2006). However, the enzymatic activities might not explain completely the variations of the seedling growth, especially under high-level N deposition rate. In fact, whole plant biomass of the seedlings under high N deposition rate remained higher than that of the seedlings without N input, but the activities of Rubisco were reduced in the former. In contrast, the content and expression of Rubisco under high N deposition rate are close to the baseline, implying that excessive N input might induce more Rubisco protein into an inactive state leading to a decrease in the enzymatic activities. These findings might imply that the inactive Rubisco protein could function as an N store to play a role in maintenance of the seedling biomass under high N deposition (Stitt and Schulze 1994).

Changes in soil nutritional status have previously been reported to impact seedling growth. Soil acidification has been considered as a key factor for inducing the loss of the seedling biomass in high N input (Carroll et al. 2003; Högberg et al. 2006). Excessive N input into soils could induce overproduction of nitrate and ammonium ions, leading to decreases in buffering capacity and pH (Mohren and Ilvesniemi 1995; Blancojuan et al. 2012). Thereby, the plateauing or declining response of seedling growth to extra N is partly caused by soil acidification and decreased N availability. In addition, soil C/N ratios are adversely correlated with seedling growth and photosynthesis (Fig. 7), implying that the imbalance of soil C and N is likely another factor causing the loss of biomass and photosynthetic capacity under high N deposition rate (Galloway et al. 2003).

Based on differential responses across the N-deposition gradient, we demonstrate that the biomass growth and photosynthetic efficiency of the *F. mandshurica* seedlings is dependent upon N availability. Low and medium N deposition could promote plant growth, but excessive input on top of the threshold ($80 \text{ kg N ha}^{-1} \text{ year}^{-1}$) is less optimum for the seedling growth. The reduced plant growth caused by extra N might arise from alterations of leaf photosynthetic efficiency and soil nutrient imbalance. Additionally, changes of the Rubisco expression and activity with increasing N availability should be taken into account as another factor causing the suboptimal effects of extra N on seedling growth and photosynthesis. This study may represent a first step for estimating the influences of continuously increasing N deposition rate on the biomass production of *F. mandshurica* in the future.

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Compliance with ethical standards

Competing interests The authors declare that they have no competing interests.

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