



Research paper

Intra-annual dynamics of xylem growth in *Pinus massoniana* submitted to an experimental nitrogen addition in Central China

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In recent decades, anthropogenic activities have increased nitrogen (N) deposition in terrestrial ecosystems. This higher availability of N is expected to impact plant growth. However, the effects of N deposition on tree growth remain inconclusive due to the wide variability of experimental methods used. This study aimed to test the effect of short-term N addition on the intra-annual wood formation of Chinese red pine (*Pinus massoniana* Lamb.) in a warm-temperate forest of Central China. From 2013, solution containing 25 kg N ha⁻¹ year⁻¹ was applied monthly to the understory of experimental plots from April to December to double the current natural N deposition. Each week from March to December in 2014 and 2015, cambial activity and the timings and dynamics of xylem formation were monitored by collecting microcores from stems. Xylem formation lasted from March to November, producing an average of 19 and 33 cells for all studied trees in 2014 and 2015, respectively. No difference in xylem cell production was observed between control and N-treated trees. Moreover, N-treated trees had similar timings, rates and durations of xylem formation as control trees. These findings indicated that short-term N addition was unable to affect timings and dynamics of xylem formation in Chinese red pine of warm-temperate forest.

Keywords: cambium, Chinese red pine, intra-annual wood formation, microcoring, nitrogen.

Introduction

Anthropogenic activities have caused dramatic changes in the active nitrogen (N) content of Earth in recent decades (IPCC 2014). Increasing N deposition has affected the cycle and availability of N for tree growth. In turn, trees may have adjusted their growth to the new levels of N (Galloway et al. 2004, Thomas et al. 2010). Tree growth in most forest ecosystems, including many Chinese forests, is widely considered to be limited by N availability (Aber 2002, Fang et al. 2005, Zhao and Liu 2009). Thus, understanding the effects of increased N deposition on tree growth is critical for improving predictions of tree growth responses to future climate.

Nitrogen is the most important macroelement for plants. Consequently, increasing atmospheric N deposition would influence the dynamics of tree growth, as demonstrated by several studies (Zhao and Liu 2009, Hutchison and Henry 2010, Ma et al. 2011, Gentilesca et al. 2013). More xylem cells in hybrid poplar were produced after N fertilization (Plavcova et al. 2013). An greenhouse experiment conducted on warm temperate forest species showed an early growth and biomass accumulation in *Picea asperata* (Mast.) and *Pinus tabulaeformis* (Carr.) after N addition (Zhao and Liu 2009). However, negative effects of N addition have also been detected. For example, a delayed bud burst that led to a shorter growing season was detected in

mature *Picea mariana* (Mill.) in the boreal forest of Canada (Bigras et al. 1996, De Barba et al. 2016). Nitrogen concentrations exceeding the tolerance of trees can decrease tree growth due to soil acidification and nutrient imbalances in trees (Mohren et al. 1986, Katzensteiner et al. 1992, Bobbink et al. 2010). However, some studies found no significant effects of N deposition on tree growth (Lovett et al. 2013, Dao et al. 2015). Observations in an eastern US forest showed no change in woody biomass increment in five deciduous species after 6 years of N addition (Lovett et al. 2013). Dao et al. (2015) also demonstrated that a 6-year-long N addition did not affect xylem phenology and cell production of mature black spruce in the boreal forest. All these diverging results represent a challenge to the assessment of tree growth responses to the current increase of atmospheric N deposition.

According to the literature, most studies were conducted with unrealistically high amounts of N addition (for example more than 100 kg N year⁻¹ ha⁻¹), which failed to realistically simulate natural N deposition (Hawkins et al. 1995, Gentilesca et al. 2013). Some experiments were conducted on seedlings, which have different sensitivities and responses to environmental signals compared with mature trees (Day et al. 2001, Vieira et al. 2008). Studies have focused on boreal forest and ignored other ecosystems, such as the warm-temperate forest at the transitional zone between the temperate and subtropical climate (Dao et al. 2015, De Barba et al. 2016). Transitional zones are generally considered to be sensitive regions for climate changes and could respond to environmental changes more easily than the colder temperate or boreal forests (Holland and Risser 1991, Loehle 2000, Thuiller et al. 2005).

To our knowledge, studies that have used concentrations of N addition comparable to those expected in the future are still scarce (Zhao and Liu 2009, Lupi et al. 2012, Dao et al. 2015). Until now, few studies of the effects of N addition on mature trees have been conducted in a warm-temperate forest ecosystem.

Wood formation, a cyclic process of successive xylem cell growth, occurs during the growing season (Rossi et al. 2006b). The dynamics of wood formation have been reported to be sensitive to the surrounding environmental conditions (Li et al. 2013). Although some N addition experiments have been conducted on tree growth (Gentilesca et al. 2013, De Barba et al. 2016), few studies have focused on the intra-annual wood formation (Lupi et al. 2012, Dao et al. 2015). Thus, investigations on intra-annual wood formation under increasing N addition are important to understand whether and to what extent the increases in atmospheric N deposition affect the dynamics of xylem growth.

As a pioneer species, the Chinese red pine (*Pinus massoniana* Lamb.) has a life strategy to exploit resources better than other sympatric species (Cuny et al. 2012). Thus, we expect that this species might exhibit a higher sensitivity to N addition. In addition, Chinese red pine is a key economic species, broadly distributed in Central and Southern China, and widely used in

reforestation. Therefore, the potential changes in wood formation of Chinese red pine following increasing atmospheric N deposition might substantially affect the overall forest productivity of wide regions in Eastern Asia.

This study aimed to test the effects of inorganic N addition on the intra-annual dynamics of wood formation in mature trees of a warm-temperate forest in Central China. Based on previous studies (Zhao and Liu 2009, Hutchison and Henry 2010, Ma et al. 2011, Gentilesca et al. 2013), we raised the hypotheses that N deposition (i) enhances xylem cell production, and consequently (ii) increases the rate or duration of xylem formation. To test these hypotheses, we designed an experiment in the field where twice the current amount of inorganic N deposition was applied to experimental plots since 2013 and the growth of xylem cells was monitored during 2014–15.

Materials and methods

Study site

The study was conducted in a natural mixed deciduous forest of the Jigongshan National Nature Reserve (31°51'N, 114°05'E) of Henan Province, Central China. The site is located in the transitional zone between the subtropical and warm-temperate climate where Chinese red pine (*P. massoniana*) is associated with dominant species such as oaks (*Quercus acutissima* Carruth. and *Quercus variabilis* Bl.) and sweetgum (*Liquidambar formosana* Hance). The forest is 45 years old and grows on soil classified as yellow-brown loam (Zhang et al. 2015).

Based on 60-year climate data (<http://data.cma.cn/site/index.html>), the mean annual temperature is 15.2 °C, with a monthly mean temperature of 27.5 °C and 1.9 °C in July and January, respectively. Total annual precipitation is 1119 mm, of which 80% falls from April to October. Rain provides a natural source of N deposition estimated at an average of 19.6 kg N ha⁻¹ year⁻¹ (Zhang et al. 2015).

Experimental design and tree selection

Four blocks were established at the study site. In each block, two circular plots were randomly assigned. One of the plots was submitted to inorganic N addition, while the other, the control, remained untreated. Plots were separated by at least a 20 m buffer zone and polyvinylchloride boards were installed to avoid soil contamination between adjacent plots. A tree core sampling area was identified at the center of each plot to avoid the edge effect. In each plot, one healthy mature Chinese red pine with an upright stem was selected for sampling. Overall, the analysis involved four N-treated trees (abbreviated as N-trees) and three control trees (C-trees), as one C-tree died during the first experimental year.

Nitrogen solution was sprinkled as homogeneously as possible at 1.5 m aboveground towards the soil and understory by five sprinklers per plot. During the 2013–15 growing seasons

(April–October), the equivalent amount of N in 3 mm of rain was applied once a month to the understory for a total N deposition of 25 kg N ha⁻¹ year⁻¹. The N treatment provided the trees with 21 mm of additional rain per year, representing <1% of the total annual precipitation of the site. Therefore, the confounding effect of water addition by the treatment is considered marginal. No experimental manipulation was done in control plots.

Soil collection and measurement

The soil chemical content was analyzed and published by Shi et al. (2016). Soil samples were collected during the summer of the second monitoring year, August 2014. Each plot was divided into four parts; two soil samples (0–10 cm depth) were randomly collected from each part, and pooled together for chemical analysis. Shi et al. (2016) showed that the available N content, pH and the percentage of cation exchange capacity (CEC) for Mg, Na, Fe, K and Mn cations did not change between treatments. A significant increase and decrease in the percentage of CEC for Al and Ca cations were observed, respectively (Zhang et al. 2015, Shi et al. 2016).

Sample collection and preparation

Each week from March to December during 2014–15, wood microcores were collected. Samples were taken following a spiral trajectory up the stem with a Trephor (Rossi et al. 2006a). Wood microcores were taken at least 5 cm apart from each other to avoid disturbance by previous samplings. The microcores contained at least three recent tree rings and the developing annual layer with cambial zone and adjacent phloem tissues. Microcores were placed in Eppendorf micro-tubes containing 50% aqueous ethanol solution and stored at 4 °C.

A total of 476 microcores were dehydrated successively in ethanol and D-limonene, and embedded in paraffin (Rossi et al. 2006a). Transverse sections of 8–10 µm were cut from the samples with a rotary microtome, stained with cresyl violet acetate (0.05% in water) and observed with visible and polarized light at magnifications of 400× to differentiate the cambium and developing xylem (including radial enlarging, wall thickening and mature cells) (Rossi et al. 2006a). The cambial and radial enlarging cells showed only a primary wall, which, unlike the secondary wall, did not shine under polarized light (Abe et al. 1997). In cross section, cambial cells were characterized by thin cell walls and small radial diameter (Rossi et al. 2006b), while enlarging cells were normally defined by a standard of at least twice the radial width diameter of the cambial cells. During maturation, cells in wall thickening show a coloration varying from light to deep violet. When the violet was completely replaced by blue, xylem cells were considered mature (Rossi et al. 2006b, 2014). The number of cambium, radial enlarging, wall-thickening and mature cells were counted along three radial files. Total xylem

cells were calculated as the sum of enlarging, wall-thickening and mature cells.

During January to February 2016, 5–10 additional microcores per tree were collected at regular intervals around the stems and prepared using the abovementioned procedures. The number of xylem cells produced during 2011–15 was counted along three radial rows and averaged.

Curve fitting and statistics

The number of cambium and xylem cells was used in repeated measures analysis of variance (ANOVA) to compare treatments including the day of the year (DOY) as a repeated factor.

The variations in total xylem cells during 2014 and 2015 were fitted with Gompertz functions according to Rossi et al. (2009). The function is defined as:

$$y = A \exp[-e^{-\kappa(t-T_i)}]$$

where y represents the number of total xylem cell and t the day of year. The three parameters are the upper asymptote A , the rate of change of the shape κ and the x -axis placement of the inflection point T_i . The residuals were regressed onto the partial derivatives with respect to the parameters until the estimates converged. Several possible starting values were specified for each parameter, so that the procedure evaluated each combination of initial values using the interactions producing the smallest residual sums of squares. Evaluation of the nonlinear regressions was based on statistics for goodness of fit, fitting behavior and examination of the residuals.

Comparisons of the estimated parameters were performed with bootstrapped t -tests calculated as:

$$t = \frac{E_1 - E_2}{\sqrt{s_1^2 + s_2^2}}$$

where E_i and s_i represent the estimated parameters (A , β or T_i) and their standard error, respectively (Rossi et al. 2010). t -Statistic was repetitively calculated by randomly resampling the original dataset and estimating 5% and 95% confidence intervals of the distribution (Efron 1979). A 10,000-times-bootstrapping was performed in order to improve the robustness of results and differences were considered significant when both confidence intervals were either higher or lower than zero. Statistics were performed using SAS 9.4 (SAS Institute Inc., Cary, NC, USA).

Results

Intra-annual wood formation

In 2014 and 2015, the number of cambial cells varied between five and eight without a clear annual pattern (Figure 1). The dynamics of enlarging and wall-thickening cells were characterized by two peaks in spring and autumn, although this pattern

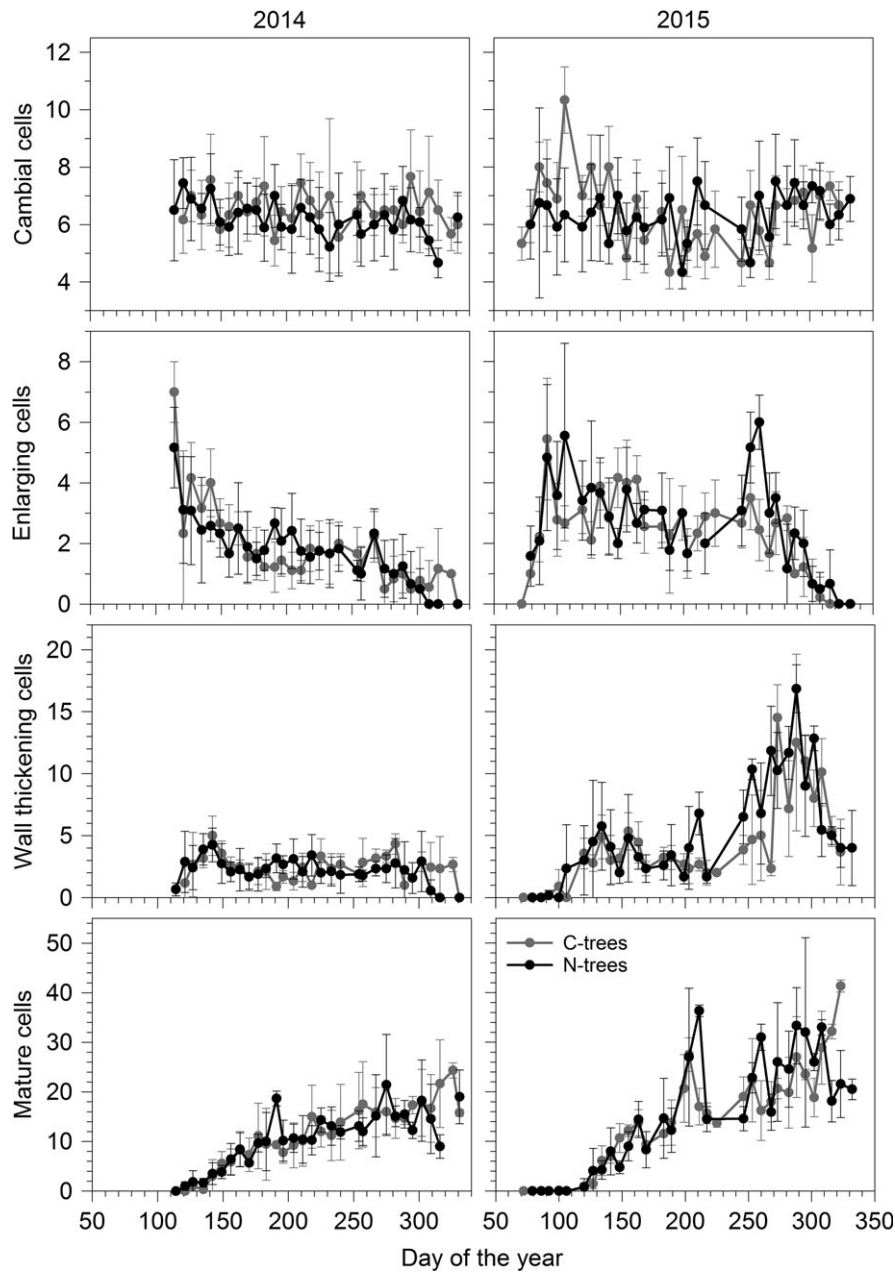


Figure 1. Number of cambial and differentiating xylem cells of Chinese red pine in 2014 and 2015 in Central China. Bars represent the standard deviations among trees.

was less evident in 2014 because sampling began after the reactivation of xylem differentiation. In 2015, the first enlarging and wall-thickening cells were observed on DOY 72 and 104, respectively. The end of enlargement and wall-thickening occurred in similar periods, in mid-November, between DOY 310 and 320. The first mature cells were observed at the end of April (DOY 120–121) in both years.

Similar intra-annual dynamics in cambium and xylem cells were observed between treatments (Figure 1), and no significant difference in cambium and differentiating cells were observed by repeated measures ANOVA (Table 1, $P > 0.05$). Except for

cambium and enlargement cells in 2014, the factor DOY was always significant (Table 1, $P < 0.05$), indicating that a different number of cells was counted throughout the year. The interaction treatment \times DOY was significant only for mature cells in 2015, which indicated that the variation in the number of mature cells during the growing season diverged between treatments.

Wood formation dynamics

Nonlinear regressions explained a proportion of variation between 0.35 and 0.73, with the lowest R^2 estimated for N-trees and the year 2014, which was attributed to the higher

variability in the data and the lack of observations at the growth resumption in 2014 (Table 2). The absence of patterns in the distributions of the residuals confirmed the model quality and goodness of fit for all three variables. On average, trees produced 16.2–35.3 xylem cells per year, as indicated by the asymptote A (Table 2). Xylem cell production rates culminated between DOY 119 and 143 indicated by T_i (Table 2). The rate of change of the growth curves varied between 0.013 and 0.028 (κ parameters in Table 2) during 2014–15. The bootstrapped t -tests showed no statistical difference for the three parameters (Figure 2), which indicated that C-trees and N-trees had similar xylem production and growth dynamics.

Xylem cell production rates peaked between DOY 131 and 133 for C-trees, and between DOY 119 and 143 for N-trees (T_i parameter in Table 2 and Figure 2). The patterns of xylem cell production rate were similar between treatments in both years. During DOY 100–168, differences in cell production rate appeared, the N-trees at the time of culmination being 38% higher than C-trees in 2014. However, in 2015, during DOY 100–153, values of cell production rate in N-trees were lower with the peaked rate 5% lower in N-trees compared with C-trees.

Comparison between years and treatments

Xylem cell production differed between years (Table 3, $P < 0.0001$), with the number of cells ranging from 23 in 2011 to 38 in 2015. N-trees produced between 23 and 34 xylem cells,

in 2011 and 2015, respectively, which was less than C-trees (25–38) (Figure 3). No significant effect of treatments or interaction of treatment \times year was detected by repeated measures ANOVA (Table 3, $P > 0.05$). When comparing Figures 2 and 3, the results of cell production diverged between C-trees and N-trees, with Gompertz functions estimating a higher number of xylem cells for N-trees in 2015. As data used for the two figures were collected on different samples, and the differences between treatments were not significant, we concluded that the variations observed in the averages were related to sampling variability.

Discussion

In this study, the effect of increased N deposition on xylem wood formation in mature Chinese red pine in a natural warm-temperate forest of Central China was tested. In our experiment, since 2013, we sprinkled a water solution (25 kg N ha⁻¹ year⁻¹) on to the understory (at the height of 1.5 m above the ground) monthly from April to October. A relatively low N concentration was used to simulate the conditions of N deposition expected for the near future. The observations on growth and dynamics of xylem formation showed no significant effect of the treatment in 2014 and 2015. Therefore, the hypotheses that N addition increases xylem cell production and enhances the rate or duration of tree growth were rejected.

Table 1. Comparison of cambial and differentiating cells between treatments using repeated measures ANOVA in Chinese red pine in Central China in 2014 and 2015.

	Source of variation	Year 2014		Year 2015	
		F-value	P	F-value	P
Cambium	Treatment	0.49	0.52	0.23	0.65
	DOY	0.91	0.60	1.68	0.03
	Treatment \times DOY	1.02	0.45	1.11	0.35
Enlarging cells	Treatment	0.76	0.42	1.56	0.27
	DOY	0.95	0.55	4.62	<0.0001
	Treatment \times DOY	0.99	0.49	1.17	0.28
Wall thickening cells	Treatment	1.97	0.22	6.56	0.05
	DOY	2.04	<0.01	10.55	<0.0001
	Treatment \times DOY	1.51	0.07	1.43	0.09
Mature cells	Treatment	0.02	0.90	0.68	0.45
	DOY	3.41	<0.0001	13.25	<0.0001
	Treatment \times DOY	0.83	0.72	1.72	0.02

Table 2. Parameters and R^2 of the Gompertz functions fitted on the number of xylem cells in C-trees and N-trees.

Year	Treatment	A	κ	T_i	R^2
2014	C-trees	21.45 \pm 3.48	0.013 \pm 0.067	133.24 \pm 13.85	0.37
	N-trees	16.27 \pm 0.87	0.028 \pm 0.009	119.99 \pm 7.63	0.35
2015	C-trees	30.54 \pm 2.04	0.019 \pm 0.004	131.04 \pm 6.88	0.73
	N-trees	35.34 \pm 3.44	0.016 \pm 0.004	143.30 \pm 9.94	0.64

Note: Values represent the estimated parameters and their standard error.

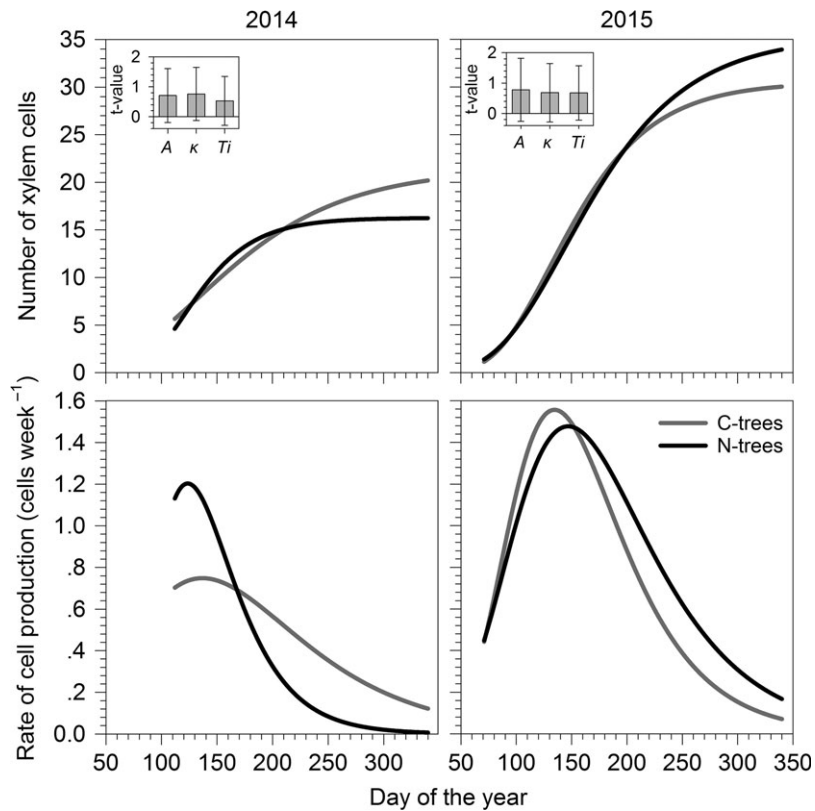


Figure 2. Sigmoid functions representing xylem cell production of Chinese red pine in 2014 and 2015 in Central China. The enclosed vertical bars represent average and 5% and 95% confidence intervals of the bootstrapped t -statistics calculated by 10,000 replications. t -Test is considered significant when both confidence intervals are either higher or lower than zero. Since we did not observe the whole growing season in 2014 and 2015, the sigmoid function illustrated in the figure is based on the observed data.

Table 3. Comparison of xylem cells between treatments using repeated measures ANOVA in Chinese red pine in Central China during 2011–2015.

Source of variation	F-value	P
Treatment	0.44	0.5387
Year	15.55	<0.0001
Treatment \times year	0.11	0.9778

Nitrogen addition and xylem phenology

Contrary to our hypothesis, N addition neither increased xylem cell production nor affected the intra-annual xylem formation. Similarly, no effect was observed when concentrations of N similar to our study were applied to the canopy of mature balsam fir in the boreal forest of Quebec, Canada (D'Orangeville et al. 2013). Also, in a 6-year N fertilization experiment on mature deciduous tree species, no significant changes in the woody biomass or aboveground net primary production were observed (Lovett et al. 2013). These results might be attributed to the lower sensitivity of trees to N compared with other non-woody plants (Nasholm et al. 2009). Wang et al. (2006) demonstrated that trees differ from herbaceous plants in their physiological traits during nutrient foraging. With respect to longer N

fertilizations, our experiment has been unable to affect growth because of the short period of the treatment (Hogberg et al. 2006). For instance, Magill et al. (2000) found that pines suffering from chronic increases in N deposition tend to decrease wood production, while no effect was detected in deciduous forest species after a short-term N fertilization (Lovett et al. 2013). Therefore, after short-term N fertilization, trees will probably not show significant growth changes compared with other plants. However, we can not exclude the possibility that the cumulative long-term effect of increased N deposition on tree growth and wood formation could appear in the longer term (Jarvis and Linder 2000, Yarie and Van Cleve 2010).

Nitrogen addition and growth dynamics of xylem

In our experiment, the dynamics of xylem formation did not change after 3 years of N addition. Photosynthesis and carbohydrate production takes place in leaves, giving them an important role in tree growth. Zhang et al. (2015) demonstrated that leaf area and leaf N content in our experimental plots were not affected by the N addition treatment, which probably can explain the absence of N effects on xylem growth. Furthermore, the N addition did not significantly modify the inorganic N content of the soil, possibly because of the short time period of this

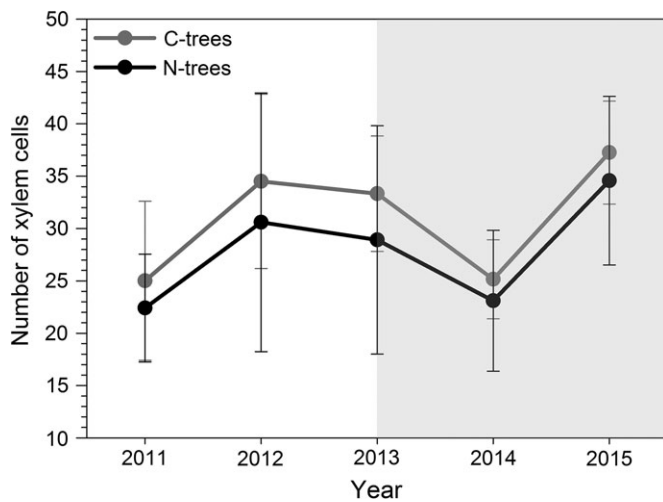


Figure 3. Xylem cell production of Chinese red pine during 2011–15 in Central China. Bars represent the standard deviations among trees, gray background represents the N treatment years.

experiment (Zhang et al. 2015, Shi et al. 2016). Thus, there is no evidence that an addition of $25 \text{ kg N ha}^{-1} \text{ year}^{-1}$ for 3 years was able to substantially modify the growth conditions, and consequently the growth, of Chinese red pines.

Unlike our experiment, many previous studies of N addition were conducted on young trees or used unrealistically high amounts of N addition (Zhao and Liu 2009, Gentilesca et al. 2013). Many studies admitted that the responses of radial growth to environmental conditions are age-dependent as tree physiology changes with age (Day et al. 2001, Vieira et al. 2008). Therefore, it is inappropriate to predict the response of mature trees to N addition based on experiments performed on young trees. The failure of growth stimulation in our 3-year study, which applied realistic increased concentrations of inorganic N to mature trees, suggests that effects of increased N deposition on tree growth, if any, would be expected in Chinese pine of warm-temperate forests only in a longer term.

Conclusion

In this study, we applied $25 \text{ kg N ha}^{-1} \text{ year}^{-1}$ for 3 years to mature Chinese red pines growing in a natural forest of Central China to simulate realistic future changes in N deposition in warm-temperate ecosystems. Contrary to our hypotheses, no effect of N addition was observed on the growth and dynamics of xylem cell production. The lack of effect on tree growth was probably due to the short duration of the experiment or the lower sensitivity of mature trees to environmental changes. Results based on fertilization studies using unrealistically high amounts of N addition or performed on seedlings may have overestimated the impact of short-term N depositions on adult tree growth.

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Conflict of interest

None declared.

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