horticulturae

Article

Effect of Simulated Organic–Inorganic N Deposition on Leaf Stoichiometry, Chlorophyll Content, and Chlorophyll Fluorescence in *Torreya grandis*

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Abstract: Atmospheric nitrogen (N) deposition is coupled with organic nitrogen (ON) and inorganic nitrogen (IN); however, little is known about plant growth and the balance of elements in *Torreya grandis* growing under different ON/IN ratios. Here, we investigated the effects of ON/IN ratios (1/9, 3/7, 7/3, and 9/1) on leaf stoichiometry (LF), chlorophyll content, and chlorophyll fluorescence of *T. grandis*. We used ammonium nitrate as the IN source and an equal proportion of urea and glycine as the ON source. The different ON/IN ratios altered the stoichiometry and photochemical efficiency in *T. grandis*. Although the leaf P content increased significantly after treatment, leaf N and N:P maintained a certain homeostasis. *Torreya grandis* plants performed best at an ON/IN ratio of 3/7, with the highest values of chlorophyll-a, total chlorophyll, maximum photochemical efficiency, and photosynthetic performance index. Thus, both ON and IN types should be considered when assessing the responses of plant growth to increasing N deposition in the future. Our results also indicated that the leaf P concentration was positively correlated with Chl, Fv/Fm, and PIabs. This result further indicates the importance of the P element for plant growth against the background of nitrogen deposition. Overall, these results indicate that *T. grandis* might cope with changes in the environment by maintaining the homeostasis of element stoichiometry and the plasticity of PSII activity.

Keywords: *Torreya grandis*; N deposition; foliar nitrogen/phosphorus; leaf P concentration; photosystem II

Citation: Yuan, Y.; Zhang, H.; Shi, X.; Han, Y.; Liu, Y.; Jin, S. Effect of Simulated Organic–Inorganic N Deposition on Leaf Stoichiometry, Chlorophyll Content, and Chlorophyll Fluorescence in *Torreya grandis*. *Horticulturae* **2023**, *9*, 1042. https://doi.org/10.3390/horticulturae9091042

Academic Editor: Lucia Guidi

Received: 23 August 2023
Revised: 11 September 2023
Accepted: 13 September 2023
Published: 16 September 2023

1. Introduction

Atmospheric nitrogen (N) deposition, mainly arising from agricultural N fertilization, fossil fuel consumption, and emissions from cowsheds and stables, is currently impacting forests, grasslands, and heathlands [1,2]. This phenomenon has strongly influenced subtropical China, which has incurred a maximum N deposition rate of 63.53 kg N $ha^{-1}\cdot yr^{-1}$, and is predicted to become the region with the highest N deposition in the world by 2030 [3]. Such a change significantly affects the balance of various ecosystems in the global N cycle or global environment [4]. The existing studies have shown that appropriate IN deposition can promote plant growth, but it will have a negative impact when N is sufficient; however, this is mainly determined by tree species [5,6]. The few existing studies about ON-IN N deposition showed that ON and IN N deposition had different effects on soil nutrient cycling and nitrogen nutrition of plants [7,8]. However, these studies did not explore the effects of ON-IN N deposition on leaf stoichiometry and plant growth.

Foliar nitrogen/phosphorus (N/P) ratios are widely considered an efficient indicator of spatiotemporal variations between plant physiological and ecosystem biogeochemical functions, including N and or P limitation or co-limitation [9], which can be described by the growth rate hypothesis, which states that fast-growing plants require rapid protein synthesis, requiring large amounts of P-rich RNA [10]. Therefore, a higher N:P ratio is often associated with a lower growth rate [10–12]. In general, the correlation between increasing N deposition and foliar N concentration is positive. Furthermore, the decline in soil pH is higher with IN addition than with ON addition [13–16], resulting in a decrease in P availability in the soil [17] and changing the nutrient content, including P, in plants. Especially, the aggravated P limitation on tree growth and the variation in P content in plants under N addition has attracted the attention of researchers.

Chlorophyll (Chl) is an important pigment for photosynthesis in photosystems [18]. Chl content plays an indispensable role in photosynthesis machinery by harvesting photons, transferring excitation energy to reaction centers, and supplying electrons to the electron transport chain [19,20]. Significant relationships between N addition and Chl content have been reported due to the underlying investment of nitrogen in Chl molecules [21]. For example, Hua et al. found that alpine grassland had an improved leaf Chl content and capacity for photosynthesis with increasing N addition [22]. Chlorophyll fluorescence (ChlF) parameters are important tools for studying the effects of different environmental stresses on photosynthesis in photosystem II [23]. The sensitive parameters of the maximal photochemical efficiency of photosystem II (Fv/Fm) and the photosynthetic performance index (PIabs) reflect the change in the plant photosynthetic apparatus [24]. The measurement of ChlF has been widely used to observe changes in the leaves of plants under N addition, such as moso bamboo [25] and conifer forests [26].

Torreya grandis is an old relict species within the Taxaceae family, which is endemic in China, and is often referred to as Chinese Torreya. It has been an excellent economic tree used to produce fruit, oil, medicine, wood of ornamental purposes, and medical function in southeastern China [27]. Currently, the distribution of *T. grandis* is limited by increasing N deposition, and in Zhejiang province, the main growing region of *T. grandis*, it is subjected to high N deposition with the rate of 30.9 kg N ha^{-1} yr^{-1} [24,28]. According to research by Zhang et al. [29], IN addition can slow the growth of Torreya seedlings and increase leaf N:P. Our previous research showed that changes in the ratio of organic and inorganic nitrogen deposition could affect both primary growth and secondary metabolism of deciduous species such as *Cyclocarya paliurus* [30]. This study was innovative for studying the effects of the mixed addition of ON and IN on the physiology of *T. grandis* seedlings. To date, no information on the effects of ON versus IN N deposition on leaf stoichiometry (LF) and plant growth of conifer species under increasing N deposition is available.

For this study, two working hypotheses are defined: (1) treatments with ON/IN addition will change the LF and growth of *T. grandis*; (2) moderate ON/IN ratios will promote the growth of *T. grandis*, and there will be a correlation between LF, chlorophyll content, and chlorophyll fluorescence parameters. Testing these hypotheses will provide novel insight into understanding the response strategies of plants under the background of increasing N deposition. Overall, our aims were to examine the effect of simulated organic–inorganic N deposition on LF, chlorophyll content, and chlorophyll fluorescence of *T. grandis*.

2. Materials and Methods

2.1. Plant Materials

This study was conducted at the origination locale of *T. grandis*—Zhuji (29.80° N, 120.12° E), Zhejiang Province, China. The background soil type was acidic and characterized as the Hapludult soil type with respect to Chinese Soil Taxonomy, with the following physicochemical properties (0–20 cm, $n = 3$): 16.06 g/kg organic carbon (OC); 1.67 g/kg total N (TN); 1.56 g/kg P; 139.25 mg/kg available P (AP); and pH 6.39. Grafted *T. grandis* seedlings were planted with a distribution density of 12 trees per treatment (3 × 4) in a 5 m × 5 m plot. The average tree height was 85 ± 5 cm (mean ± SD), and the diameter at breast height (DBH) was 2.2 ± 0.34 cm.

2.2. ON and IN Addition Experiment

In the experiment, an equal mixture of urea and glycine (1:1) was used as the ON addition, and ammonium nitrate (NH_4NO_3) was used as the IN addition [8]. The five treatments were as follows: control (CK) and 10%/90%, 30%/70%, 70%/30%, and 90%/10% of ON/IN additions (N1, N2, N3, and N4, respectively). According to the change trend and related data of N deposition components [29], the N deposition rate was maintained at 30 kg N ha^{-1} $year^{-1}$. Here, we used ammonium nitrate as the IN source and an equal proportion of urea and glycine as the ON source. Fresh batches of fertilizer were dissolved in water for treatments N1–N4 each month from the beginning to the end of the trial (about two years from June 2020 to October 2022). The control group was sprayed with the same amount of water to reduce the influence caused by water addition between treatments. Weeding was carried out once a month without any fertilizer to avoid the influence of other sources of N.

2.3. Leaf Stoichiometry Measurement

Three intact seedlings in each treatment were randomly selected and harvested in mid-October 2022 for leaf stoichiometry and chlorophyll measurement. In each treatment, we collected the healthy and mature leaves from the mid-upper for leaf stoichiometry analysis. After that, *T. grandis* leaves were cleaned and collected into plastic bags and immediately transported to the laboratory for laboratory measurements. The *T. grandis* leaves were dried and then sieved before measurement of leaf stoichiometry. The N concentration of *T. grandis* leaves was measured following the method of Wu et al. [31] (n = 3). The P concentration of *T. grandis* leaves was detected according to the method of the General Administration of Quality Supervision in China (Reference Code: GBW08513) (n = 3).

2.4. Chlorophyll Measurement

Briefly, 0.2 g of *T. grandis* fresh leaves was finely cut and extracted with 10 mL of 90% alcohol. The extraction was conducted in the dark for 24 h and shaken about two or three times until the samples were blanched. The optical densities of Chla and Chlb were measured using a spectrophotometer (Shimadzu UV-2550, Kyoto, Japan) at 649 and 665 nm [32]. Thereafter, the Chl concentrations were determined according to [33].

2.5. Chlorophyll Fluorescence Measurement

In the present experiment, ChlF parameters in the leaves were evaluated with sunny weather in mid-August 2022 using a Handy PEA fluorometer (Hansatech Instruments, Norfolk, UK). Before measurement, the *T. grandis* plants were dark-adapted for 30 min. The experiment was repeated three times. The parameters were calculated according to the method of [34].

2.6. Statistical Analyses

The statistical significance of the foliar stoichiometry and photosynthetic parameters in *T. grandis* leaves between different ON/IN treatments were analyzed using a one-way analysis of variance (ANOVA), followed by Tukey's test ($p < 0.05$). Pearson's correlation analysis was performed to detect the relationships among the different indices. The data in the figures and tables present the average values ± standard deviation (SD).

3. Results

3.1. Foliar Stoichiometry Characteristics

Compared with the control, ON/IN addition increased the foliar P concentration (Figure 1) in the leaves in all four sampling groups. However, there were no significant differences in leaf N and N:P among the five treatments. For instance, N2 most significantly increased the P concentration, resulting in a decrease in N:P in the leaves (12.7% and 6.3%, respectively), whereas the concentration of N increased by 8.1%, 5.5%, 6.1%, and 2.1%.

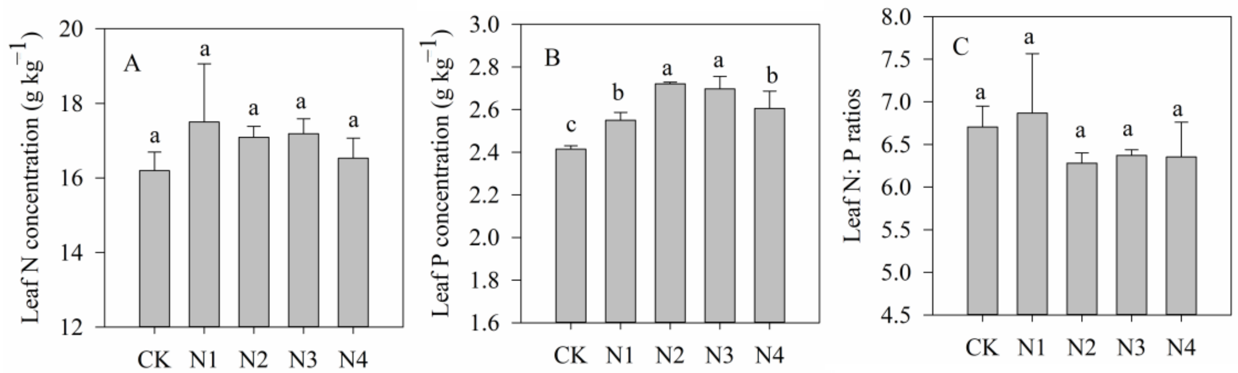

Figure 1. Responses of N (**A**), P (**B**) concentrations and N:P (**C**) in the leaves of five groups of seedlings to the change in organic nitrogen (ON)/ inorganic nitrogen (IN). Bars within groups with different letters indicate significant differences ($p < 0.05$). Treatments: control (CK) and 10%/90%, 30%/70%, 70%/30%, and 90%/10% of ON/IN additions (N1, N2, N3, and N4, respectively).

3.2. Chlorophyll Characteristics

ON/IN addition has a significant effect on the Chl content of the *T. grandis* leaves. Compared with CK, the contents of Chla and Total Chlorophyll were significantly increased in *T. grandis* leaves from the four ON/IN treatments. The Chla and Total Chlorophyll showed a trend of first increasing (Figure 2A,C) and then decreasing. Compared with CK, the Chlb content significantly decreased in the *T. grandis* leaves treated with N1–N3.

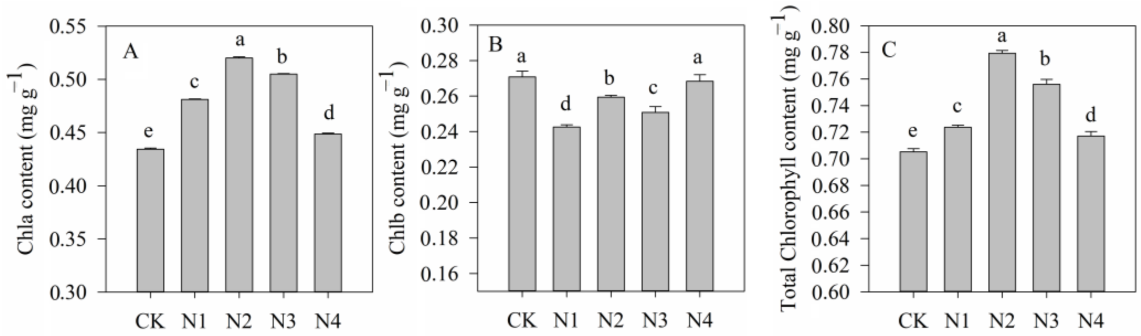

Figure 2. Variation in plant photosynthetic pigment parameters (Chla (**A**), Chlb (**B**), and total chlorophyll (**C**)) of *Torreya grandis* leaves with the change in the ON/IN ratio. Bars within groups with dissimilar letters denote significant differences. Treatments: control (CK) and 10%/90%, 30%/70%, 70%/30%, and 90%/10% of ON/IN additions (N1, N2, N3, and N4, respectively).

3.3. Chlorophyll Fluorescence Characteristics

The increase in leaf fluorescence transients in *T. grandis* plants under different N treatments showed a typical OJIP shape (Figure 3). The Fv/Fm and PIabs of the *T. grandis* leaves in the N-treatment groups were significantly higher than those of CK (Table 1). Compared with CK, Fv/Fm and PIabs of the four N-treatment groups increased by 2.1%, 9.8%, 7.7%, and 1.0% and by 16.1%, 163.5%, 59.0%, and 14.1% (Table 1). After N treatment, light energy absorption (ABS/RC), heat dissipation (DIo/RC), capture (TRo/RC), and non-photochemical quenching per cross-section (DIo/CSm) generally showed a decreasing trend. In general, when the ON/IN ratio was 3/7, the fluorescence parameters of the leaves performed best.

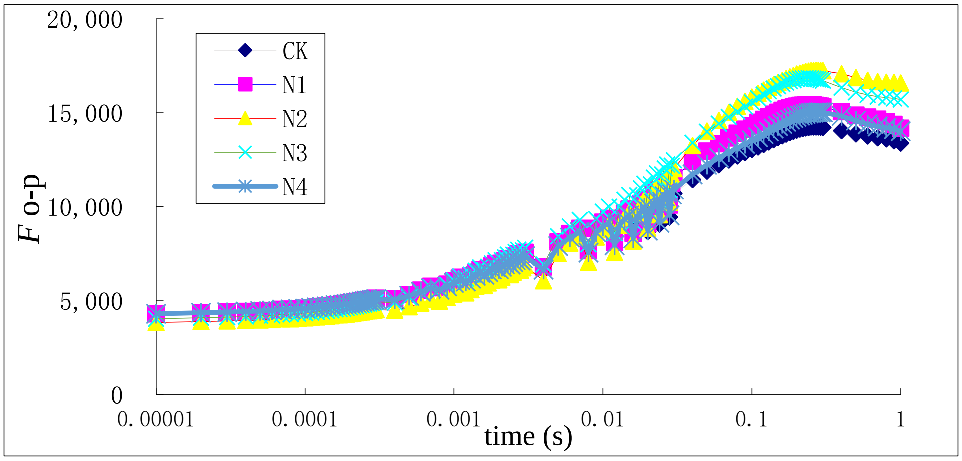

Figure 3. Chlorophyll a fluorescence transient of dark-adapted *Torreya grandis* leaves exposed to different ON/IN ratios. Treatments: control (CK) and 10%/90%, 30%/70%, 70%/30%, and 90%/10% of ON/IN additions (N1, N2, N3, and N4, respectively).

Table 1. Chlorophyll fluorescence characteristics of *Torreya grandis* leaves under different organic nitrogen (ON)–inorganic nitrogen (IN) deposition treatments. CK (control, distilled water), N1 (90% IN and 10% ON), N2 (70% IN and 30% ON), N3 (30% IN and 70% ON), and N4 (10% IN and 90% ON). Significant differences ($p < 0.05$) between N treatments were indicated by different lowercase letters.

Treatment	Fv/Fm	PIabs	ABS/RC	DIo/RC	TRo/RC	DIo/CSm
CK	0.71 ± 0.00 b	5.19 ± 1.00 b	1.56 ± 0.10 a	0.46 ± 0.02 a	1.10 ± 0.08 a	4388.67 ± 549.19 a
N1	0.72 ± 0.00 b	5.95 ± 1.34 b	1.49 ± 0.08 ab	0.42 ± 0.02 a	1.08 ± 0.06 ab	4306.33 ± 611.09 a
N2	0.76 ± 0.02 a	13.51 ± 3.02 a	1.28 ± 0.05 c	0.29 ± 0.03 b	0.99 ± 0.02 b	3851.33 ± 572.06 a
N3	0.76 ± 0.01 a	8.15 ± 2.45 b	1.35 ± 0.09 bc	0.32 ± 0.02 b	1.03 ± 0.07 ab	4035.00 ± 354.27 a
N4	0.71 ± 0.00 b	5.85 ± 1.18 b	1.53 ± 0.10 a	0.44 ± 0.03 a	1.09 ± 0.07 ab	4317.00 ± 362.30 a

3.4. Correlation Analysis

The results showed that there were no significant correlations between leaf N concentration, leaf N:P and chlorophyll fluorescence parameters; however, the leaf P concentration was significantly positively correlated with Chl, Fv/Fm, and PIabs (Figure 4) ($p < 0.05$). Meanwhile, significant negative correlations were observed between the leaf P concentration and ABS/RC and between DIo/RC and TRo/RC ($p < 0.05$).

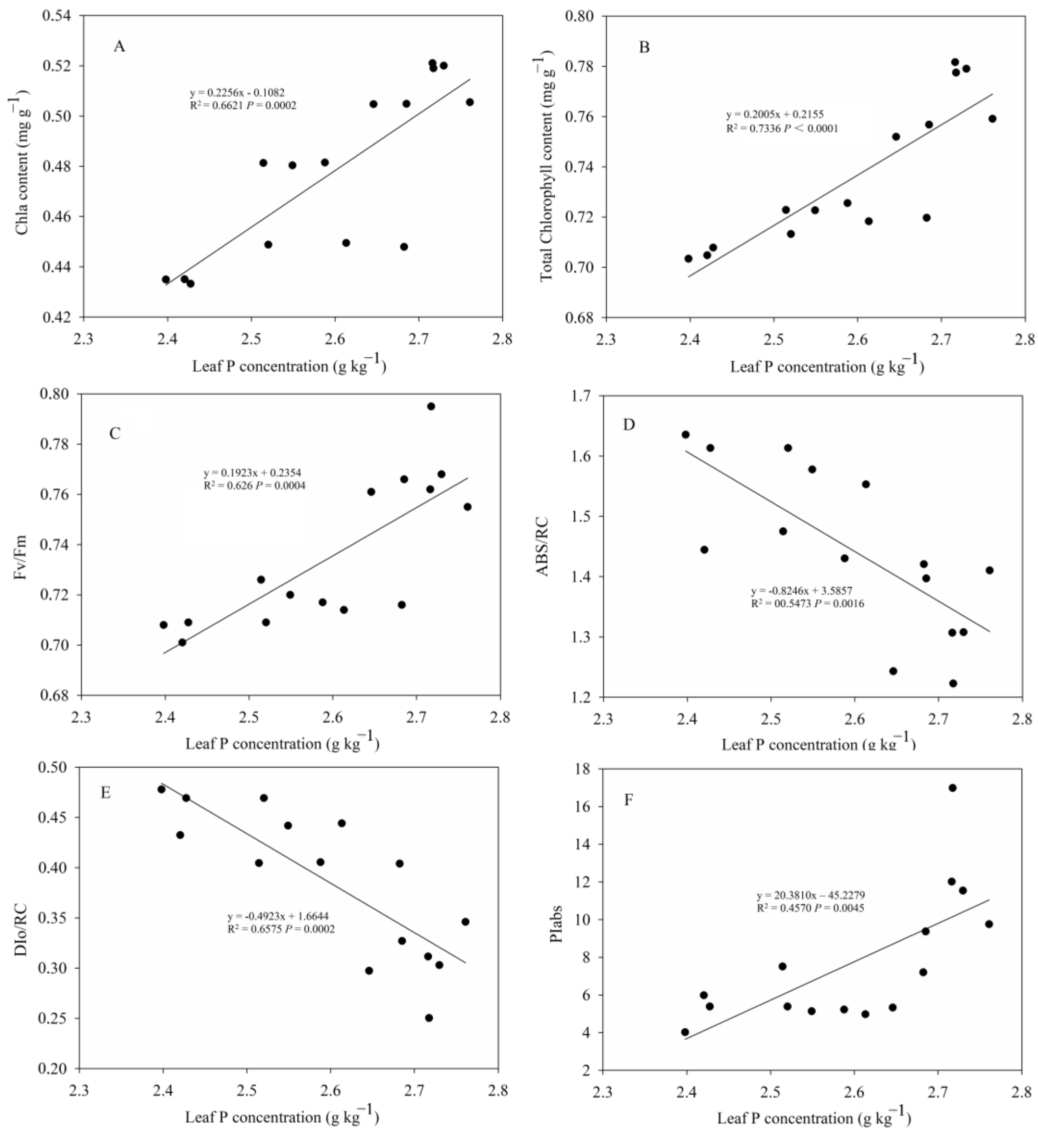

Figure 4. Relationship between leaf P concentration, photosynthetic pigment parameters and chlorophyll fluorescence characteristics (**A**–**F**) of *Torreya grandis* leaves.

4. Discussion

N deposition changes the soil nutrient content directly and then modulates the stoichiometric characteristics of plants [8,35]. Güsewell et al. [36] and Greenwood et al. [37] reported that ratios of N:P on a mass basis of <10 indicate limited N, and ratios >20 indicate limited P. Therefore, in N-limited forest ecosystems, N addition increases soil IN content and soil N availability, resulting in a corresponding increase in the N concentration in all plant organs [38]. In support of our first hypothesis, foliar stoichiometry showed significant differences among the simulated ON/IN ratios. This result is inconsistent with Zhou et al. [39], who reported no detectable differences among ammonium nitrate, urea, and glycine addition on LF. Therefore, it seems reasonable to investigate the effect of N deposition on LF under ON/IN addition in *T. grandis*. The effects of IN are higher than those of ON because IN can be utilized directly by plants [40]. However, IN is more easily dispersed in the soil than ON due to leaching and volatilization [41,42]. Previous studies of IN N deposition have shown that IN could inhibit plant growth due to the soil properties negatively affected by IN N deposition [7,43]. For example, Zhang et al. [43] showed that IN N deposition decreased soil pH, available N, P and K in *Torreya grandis* plantations. However, compared with CK, treatments with a higher ON:IN ratio significantly promoted leaf P content and photosynthetic performance of *T. grandis*. These results indicate that exogenous N input changes the phosphorus concentration of *T. sinensis*, and the primary reason might be the change in soil available phosphorus content, and the second reason might be the change in plant phosphorus absorption capacity and phosphorus use efficiency [13]. A previous study also found that a substitution ratio of 25–75% of chemical fertilizers under ON/IN fertilizers could ensure high and stable plant nutrient uptake [44]. This result may be related to species differences and further explains the necessity of studying the effects of N deposition components on foliar stoichiometry and plant growth. ON/IN addition may also affect plant growth by altering soil microbial activity, which is correlated to the C:N ratio, and there is evidence that nitrogen addition can affect the soil C:N ratios [8,45]. However, further experiments are needed to verify soil-related indexes in the future.

Exploring the underlying reasons for the effects of IN and ON on foliar N/P in *T. grandis*, we compared LF between CK and N4 and found that higher ON/IN ratios increased foliar N and P concentrations, resulting in a decreased foliar N:P ratio in *T. grandis* seedlings. This may correspond to the suggestion that plants assimilate more P to maintain N:P homeostasis when N concentrations increase [46]. According to the growth rate hypothesis, fast-growing organisms often need to invest many resources in phosphorus-rich RNA to support rapid protein synthesis [10]. Collectively, the studies mentioned above are consistent with our results for the five N treatments. As N deposition levels continue to accelerate, N-limitation is gradually alleviated, which is consistent with the conclusion that in subtropical areas of China, there is no longer any N limitation, but the growth limitation caused by low P availability has been aggravated due to increased foliar N:P [37]. However, ON/IN treatment may enhance the P efficiency of the soil and plants to improve P uptake in several ways. First, P availability through the regulation of soil acidity improves more with IN addition than with ON addition [13,14] and may increase P mobility in the soil [47]. Second, based on arbuscular mycorrhizal symbioses, the hyphae of arbuscular mycorrhizal (AM) fungal hyphae extend further from roots than root hairs [48]. Hence, a substantial number of mechanisms may lead to ON/IN addition enhancing P uptake by plants, which is consistent with our results. However, further trials are still needed to confirm this. Our results also indicated that the leaf P concentration was positively correlated with Chl, Fv/Fm, and PIabs (Figure 3) ($p < 0.05$). This result further indicates the importance of the P element for plant growth against the background of nitrogen deposition [37]. These results not only support our first hypothesis stating that ON/IN addition changes the foliar N:P ratio but are also in agreement with our studies demonstrating that plant growth may be enhanced with ON addition than with IN addition (especially at an ON/IN ratio equal to 3/7).

Plants can properly alter the content of photosynthetic pigments according to their environment [49]. Our research found that different ratios of ON/IN had a certain positive impact on the Chl content of *T. grandis* leaves, while at ratios of 3/7, the Chla and total chlorophyll contents were significantly higher in all N treatment groups (N2 > N3 > N1 > N4 > CK). By contrast, the Chlb concentration has a downward trend, which may reflect the range in the antennae size and efficiency of light harvesting, which were lower than those in CK [50]. Fv/Fm reflects the light energy conversion rate in PSII and is considered an imperative indicator of photoinhibition [51]. Compared with the control, ON/IN addition significantly increased the Fv/Fm values in all experimental N groups. This indicates that ON/IN treatment may promote photosynthesis in *T. grandis*, especially at a ratio of 3/7. In this study, PIabs of *T. grandis* leaves showed an upward trend with different ratios of ON/IN, not only demonstrating that the light energy absorption, light energy conversion rate, and electron conversion capacity were enhanced but also indicating that the overall photosynthetic activity was elevated after ON/IN N treatment. ABS/RC and DIo/RC are influenced by the number of active/inactive RCs, and ABS/RC can also be a measure of relative antennae size [52]. In the present study, the ratios of ABS/RC and TRo/RC decreased with N treatment compared to CK, which indicated that effective antennae size decreased because of inactive reaction centers under ON/IN treatment. The results of this study infer that some ON/IN supplements (especially N2) could promote the photosynthesis capacity in PSII, which might result in better growth in *T. grandis* compared with CK.

5. Conclusions

Atmospheric N deposition is coupled with ON and IN; however, little is known about plant growth and the balance of elements in *T. grandis* growing under different ON/IN ratios. Here, we investigated the effects of ON/IN ratios (1/9, 3/7, 7/3, and 9/1) on leaf stoichiometry (LF), chlorophyll content, and chlorophyll fluorescence of *T. grandis*. Leaf stoichiometry, chlorophyll content, and chlorophyll fluorescence of *T. grandis* were significantly affected by different forms of N addition in our experiment. Although the leaf P content increased significantly after treatment, leaf N and N:P maintained a certain homeostasis. *T. grandis* plants performed best at an ON/IN ratio of 3/7, with the highest values of Chla, total chlorophyll, maximum photochemical efficiency, and photosynthetic performance index. The variation in P content in plants under N addition has attracted the attention of researchers. Our results indicated that the leaf P concentration was positively correlated with Chl, Fv/Fm, and PIabs ($p < 0.05$). This result further indicates the importance of the P element for plant growth against the background of nitrogen deposition. These results indicate that *T. grandis* copes with changes in the environment by maintaining the homeostasis of element stoichiometry and the plasticity of PSII activity. Furthermore, both ON and IN types should be considered when assessing the responses of photosynthesis and plant growth to globally increasing N deposition from a long-term perspective.

Author Contributions: Resources, Y.L.; data curation, writing—original draft preparation, Y.Y. and H.Z.; literature search, formal analysis, X.S. and Y.H.; writing—review and editing, Y.L. and S.J.; supervision, S.J., Y.Y. and H.Z. contributed equally. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the National Natural Science Foundation of China (No. 32001305, 31971641), the National Key Research and Development Project of China (2019YFE0118900), "Leading Talents" R & D Program in Universities of Zhejiang, China (2022), and Jiyang College of Zhejiang A & F University under Grant, China (No. RQ2020B14).

Data Availability Statement: The data presented are available on request from the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest.

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