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Short-Term Nitrogen Addition Does Not Significantly Alter the Effects of Seasonal Drought on Leaf Functional Traits in *Machilus pauhoi* Kanehira Seedlings

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Received: 21 November 2018; Accepted: 18 January 2019; Published: 22 January 2019



Abstract: Research Highlights: Short-term nitrogen (N) addition did not significantly alter the effects of seasonal drought on the leaf functional traits in *Machilus pauhoi* Kanehira seedlings in N-rich subtropical China. Background and Objectives: Seasonal drought and N deposition are major drivers of global environmental change that affect plant growth and ecosystem function in subtropical China. However, no consensus has been reached on the interactive effects of these two drivers. Materials and Methods: We conducted a full-factorial experiment to analyze the single and combined effects of seasonal drought and short-term N addition on chemical, morphological and physiological traits of *M. pauhoi* seedlings. Results: Seasonal drought (40% of soil field capacity) had significant negative effects on the leaf N concentrations (LNC), phosphorus (P) concentrations (LPC), leaf thickness (LT), net photosynthetic rate (A), transpiration rate (E), stomatal conductance (Gs), and predawn leaf water potential (ψ_{PD}), and significant positive effects on the carbon:N (C:N) ratio and specific leaf area (SLA). Short-term N addition (50 kg N·hm⁻²·year⁻¹ and 100 kg N·hm⁻²·year⁻¹) tended to decrease the C:N ratio and enhance leaf nutrient, growth, and photosynthetic performance because of increased LNC, LPC, LT, leaf area (LA), SLA, A, E, and ψ_{PD} ; however, it only had significant effects on LT and Gs. No significant interactive effects on leaf traits were detected. Seasonal drought, short-term N addition, and their interactions had significant effects on soil properties. The soil total C (STC), nitrate N (NO₃⁻-N) and soil total N (STN) concentrations were the main factors that affected the leaf traits. Conclusions: Seasonal drought had a stronger effect on *M. pauhoi* seedling leaf traits than short-term N deposition, indicating that the interaction between seasonal drought and short-term N deposition may have an additive effect on *M. pauhoi* seedling growth in N-rich subtropical China.

Keywords: leaf functional trait; seasonal drought; short-term N deposition; additive effect; soil properties; *M. pauhoi* Kanehira seedling

1. Introduction

Global environmental change is amongst the most crucial factors that influence the biodiversity, structure and function of forest ecosystems [1–3]. As the most important drivers of environmental change, the climate change (especially the drought stress) and atmospheric nitrogen (N) deposition seriously affect the primary productivity and stability, carbon (C) and water cycles of the forest ecosystems [3,4]. Plants are subject to periodic atmospheric or soil droughts not only in arid areas but also in non-arid and semi-arid areas [5], which causes a decrease in the soil water content and an increase of the plant water deficit. In addition, drought can also decrease the availability of soil nutrients, resulting in plant N limitation, which stunts growth and increases mortality [4,6,7]. As N is a limiting element in terrestrial ecosystems [8], elevated N deposition caused by anthropogenic activities can increase the soil available N, and possibly alleviate N limitation in the ecosystems owing to high N availability [9,10], which can increase plant aboveground productivity and C sequestration capacity [11]. However, N deposition can also alter soil nutrient cycle, and increase soil acidification and cations leaching from the soil [12], resulting in plant nutrient imbalances and further stunting of growth [13,14].

Plant functional traits link the plants to the environment [15]. Leaves have a relatively large contact area to the environment, so are most sensitive to environmental conditions [16]. Leaf functional traits are crucial determinants of plant physiology and biogeochemical cycles [15,17] and are extremely sensitive to environmental change, reflecting the adaption of plants to their environments and their self-regulation in complex habitats [18]. Therefore, the responses and adaptation mechanisms of plants to environmental change are best investigated by using plant leaves as research objects [18]. It is becoming increasingly recognized that leaf chemical, morphological and physiological traits can be altered by drought or N deposition alone, as well as their combination [19–23]. For example, plants usually have a lower specific leaf area (SLA) in response to drought on the global, regional and local scale [24–28] to maximize C absorption and reduce water consumption [25]. However, plants can also increase their SLA by reducing leaf thickness (LT) and/or density rather than by decreasing leaf area (LA) under drought [29]. Therefore, how leaf traits respond to drought and/or N addition is unclear because of environmental change and species-specific characteristics.

Considerable evidence has been accumulated to demonstrate that drought and N deposition might affect ecosystem responses in two ways. Firstly, co-occurring drivers can have additive effects (i.e., no statistically significant effect of the interaction between drought and N deposition is detected) [30], which can be predicted from single-factor studies [31]. Secondly, the combination of drought and N deposition may not be additive, but instead may have non-additive effects on plants (i.e., the interaction is statistically significant), meaning that opposite or synergistic interaction effects may occur through multi-factor approaches [2,3,20,30,32–35]. Many studies have reported that N deposition increases plant sensitivity to drought stress [2,3,32,34,36,37], because high productivity caused by N fertilization may decrease plants' drought resistance and increase mortality [30]. However, N deposition may alleviate the negative effects of drought stress on plants [19,38], but no consistent conclusion on this matter has been summarized yet. The seedling stage is the most vulnerable stage in a plant's life history; and is particularly sensitive to environmental change [20,39,40]. Therefore, it is of great significance to study how the functional traits of seedling leaves respond to the combined effects of drought stress and N deposition.

Extensive evergreen broadleaf forests are widely distributed in southern China [41], where frequent seasonal droughts have occurred in recent decades [42] accompanied by high N deposition. Southern China, generally known as “the oasis of the subtropical zone”, is characterized by a subtropical moist monsoon climate, whereas other subtropical regions have more arid climates. Therefore, it is essential to investigate the ecological effects of atmospheric N deposition and seasonal drought on forest plants in China's subtropical region.

Machilus pauhoi Kanehira is amongst the most dominant evergreen broadleaf tree species in subtropical China. It is distributed in Fujian, Zhejiang, Jiangxi, Anhui, Hunan, Guangdong, Guangxi and other provinces in China [43]. As it is widely used and has high economic value, it has attracted much attention. Subject to changes in precipitation, seasonal droughts often occur in the range of *M. pauhoi* [44], as well as high N deposition. We evaluated how seasonal drought and N addition interactively affected the leaf nutrients, morphology, and photosynthetic physiology of *M. pauhoi* seedlings. A full-factorial experiment of short-term N addition and drought treatment was conducted to explore two key questions: (1) Does short-term seasonal drought have a significant effect on leaf functional traits in *M. pauhoi* seedlings? (2) Does short-term N deposition aggravate the effects of seasonal drought on the leaf functional traits of *M. pauhoi* seedlings in N-rich subtropical China? The results are expected to facilitate a better understanding of the adaptive strategy of *M. pauhoi* to its habitat, and provide a scientific guidance to further investigate plants' response mechanisms to global environmental changes.

2. Materials and Methods

2.1. Experimental Design

The study was conducted at the Forestry Technology Center of Shunchang County (117°48' E, 26°48' N), Nanping City, Fujian Province, southern China. It is characterized by a subtropical moist monsoon climate, with an annual average temperature of 18.9 °C, a coldest monthly average temperature of 7.9 °C in January, and a hottest monthly average temperature of 28.1 °C in July. The average annual precipitation ranges from 1600 to 1900 mm and is concentrated from February to September. The annual frost-free period is 305 days, with an average sunshine duration of about 1741 hours.

Southern China has experienced frequent seasonal drought in recent decades [42], and the frequency of summer and autumn droughts in Fujian Province is relatively high [44]. Therefore, in order to simulate seasonal drought, a full-factorial experiment including drought and N addition was conducted for three months from August 2014. To eliminate any effects of seedling germplasm resource differences, the study used one-year-old similar-sized cuttings from the same seed tree in Suichun County, Jiangxi Province, with an average tree height of 17.95 ± 0.72 (mean \pm standard deviation) cm and an average basal diameter of 3.12 ± 0.75 (mean \pm standard deviation) mm [45]. Seedlings were transplanted into polyvinyl chloride pots with an inner diameter of 30 cm and height of 30 cm on July 1st, 2014. Pots containing one plant each were placed in a greenhouse covered with a sunshade net (75% shading) for routine water management at 2.5 m above the ground. The experimental pots contained red soil from Shunchang County. The soil total C, N, and phosphorus (P) concentrations were $4.35 \text{ mg}\cdot\text{g}^{-1}$, $1.24 \text{ mg}\cdot\text{g}^{-1}$, and $0.14 \text{ mg}\cdot\text{g}^{-1}$, respectively, and the pH was 4.67.

The N addition and drought treatments were conducted from 1 August 2014 to 31 October 2014. N deposition in Fujian Province in southeastern China has been about $50 \text{ kg}\cdot\text{hm}^{-2}\cdot\text{year}^{-1}$ in recent years, so the seedlings were arranged in three N addition levels: 0 (control, N_0), $50 \text{ kg N}\cdot\text{hm}^{-2}\cdot\text{year}^{-1}$ (low N, N_1), and $100 \text{ kg N}\cdot\text{hm}^{-2}\cdot\text{year}^{-1}$ (high N, N_2) [45]. The drought treatments were set at two levels: 80% of soil field capacity (control with a normal water supply, D_0) and 40% of soil field capacity (drought stress, D) [45]. Therefore, there were six treatments in total: D_0N_0 (control), D_0N_1 (low N addition), D_0N_2 (high N addition), DN_0 (drought treatment, no N), DN_1 (combined treatment of drought and low N), and DN_2 (combined treatment of drought and high N). Three replicates were included for each treatment, with 10 seedlings in each replicate. N was added with ammonium nitrate (NH_4NO_3), and the soil surface area was calculated based on the 30-cm inner diameter of the experimental pot. The amount of N added per pot was calculated using this area (N_1 treatment $1.01 \text{ g NH}_4\text{NO}_3$; N_2 treatment $2.02 \text{ g NH}_4\text{NO}_3$). The total amount of NH_4NO_3 was divided into six equal parts, and each part was added every two weeks for three months. NH_4NO_3 was dissolved in 10 mL of distilled water and uniformly applied with a pipetting gun. An equal amount of distilled water was used in

the control treatment. For the drought treatment, soil moisture was monitored and maintained at the desired soil field capacity, in the way that pots were weighed and watered with distilled water to a given soil water content every two days. Besides, the soil surfaces of the pots were covered with a plastic film to prevent water evaporation.

2.2. Leaf Sampling and Functional Traits Determination

In October 2014, the predawn leaf water potential (ψ_{PD} , Mpa) and midday leaf water potential (ψ_M , Mpa) were determined using a Dewpoint Potential Meter (WP4C, Decagon, Pullman, WA, USA) at 4:00 a.m. and 12:00 p.m., respectively. Photosynthetic parameters were measured using a portable CIRAS-3 photosynthesis system (PP Systems, Amesbury, MA, USA). Measurements were conducted from 08:00 a.m. to 10:30 a.m. to avoid midday stomatal closure. The temperature and relative humidity in the leaf chamber were kept at ambient values. The net photosynthetic rate (A , $\mu\text{mol CO}_2 \cdot \text{m}^{-2} \cdot \text{s}^{-1}$), transpiration rate (E , $\text{mmol H}_2\text{O} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$), stomatal conductance (G_s , $\text{mmol H}_2\text{O} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$), and intercellular carbon dioxide (CO_2) concentrations (C_i , $\mu\text{mol CO}_2 \cdot \text{mol}^{-1}$) of upper, fully expanded, healthy leaves from three seedlings in each treatment were measured. The instantaneous water use efficiency (WUE , $\mu\text{mol CO}_2 \cdot \text{mmol}^{-1} \text{H}_2\text{O}$) was calculated as the ratio of the net photosynthetic rate to the transpiration rate [46].

Sample leaves were collected at the end of the experiment in October 2014. Relatively complete, fully expanded, and well-grown leaves (including petioles) without pests or diseases were selected from the experimental seedlings and taken to the laboratory.

A Vernier caliper with an accuracy of 0.01 mm was used to measure three thicknesses of the leaves (top, middle and bottom) at an intermediate position between the border and the midrib (avoiding important secondary veins) [47], and the average was used as the single LT (mm). A LI-3000C portable leaf-area meter (Li-Cor, Lincoln, NE, USA) was used to measure LA (cm^2) [47]. The leaf fresh weight (FW) was immediately recorded, and the saturated weight (SW) was determined after soaking leaf samples in deionized water for 24 h in the dark [19]. Subsequently, the leaf samples were oven-dried at 65 °C to a constant weight and the dry weight (DW) was determined. Three seedlings with similar average basal diameters and tree heights were selected for each treatment, and the average of three measurements was used to determine leaf morphological traits. The SLA ($\text{cm}^2 \cdot \text{g}^{-1}$) was calculated as the ratio of LA to leaf DW [22,47]. The leaf relative water content (LRWC, %) was calculated in the following manner: $\text{LRWC} = (\text{FW} - \text{DW}) / (\text{SW} - \text{DW}) \times 100\%$ [19,48].

The dried leaf samples were ground into a fine powder using a sample grinder and screened with a 1-mm sieve for chemical analysis. Leaf C and N concentrations per dry mass (LCC and LNC, $\text{mg} \cdot \text{g}^{-1}$) were determined using a CHNS/O Elemental Analyzer (Vario EL III, Elementar, Langenselbold, Germany) [49,50]. Leaf P concentrations per dry mass (LPC, $\text{mg} \cdot \text{g}^{-1}$) were determined using a Continuous Flow Analytical System (SAN ++, Skalar, Breda, Holland) after digestion and boiling in a solution of $\text{H}_2\text{SO}_4\text{-HClO}_4$ [49]. Their stoichiometric ratios were then calculated.

2.3. Soil Sampling and Physicochemical Properties Determination

After leaf sampling, soil from the pots where leaves were sampled was sampled using a steel shovel. The soil samples were passed through a 2-mm sieve and divided into two parts. One part of the fresh soil was used to colorimetrically determine nitrate N ($\text{NO}_3^- \text{-N}$, $\text{mg} \cdot \text{kg}^{-1}$) and ammonium N ($\text{NH}_4^+ \text{-N}$, $\text{mg} \cdot \text{kg}^{-1}$) concentrations using an autoanalyser (SEAL-AA3, Hamburg, Germany) after extraction with a 2 M KCl solution [50]. The other part was air-dried to measure the pH and total C and N concentrations. Soil pH was determined using a pH meter, with a soil:water ratio of 1:2.5 [49]. The soil total C and N concentrations (STC and STN, respectively, $\text{mg} \cdot \text{g}^{-1}$) were determined using a CHNS/O Elemental Analyzer (Vario EL III, Elementar, Langenselbold, Germany) [49,50].

2.4. Data Analysis

The leaf functional traits' responses to N addition and drought were measured as the magnitude of treatment effect (*MTE*). The *MTE* was calculated as $MTE = (X_t - X_c)/(X_t + X_c)$, where X_t is the leaf trait value (except for ψ_{PD} with a negative numerical value) for the N addition or (and) drought treatment, and X_c is the leaf trait value for the control treatment [2]. The *MTE* of ψ_{PD} was calculated as $MTE = (X_c - X_t)/(X_c + X_t)$. The numerical value ranges from -1 to 1 [2].

A Principal Component Analysis (PCA) was conducted to identify important traits using Canoco 5.0 software (Microcomputer Power, Ithaca, NY, USA). Before conducting an analysis of variance (ANOVA), the variance of the data was tested by Levene's homogeneity test. If the variance was homogeneous, one-way ANOVA with Least Significant Difference (LSD) post-hoc tests were performed. If the variance was heterogeneous, a nonparametric Kruskal-Wallis test was performed [51]. The C:N ratio did not meet the assumption of variance homogeneity, so a nonparametric Kruskal-Wallis test was adopted. A two-way ANOVA was used to analyze drought treatment, N addition and their interactive effects. Based on stepwise linear regression analysis between soil variables and leaf traits, the multi-collinearity among soil variables was detected in the case that variables with the square root of variance inflation factor (\sqrt{VIF}) higher than 2 were excluded [52]. Path analysis was then used to analyze the direct and indirect effects from independent variables (soil variables) to dependent variables (leaf traits) [53]. The ANOVA analysis and path analysis were both conducted using SPSS 24.0 software (SPSS Inc., Chicago, IL, USA). Origin 9.0 software (Origin Lab Corp., Northampton, MA, USA) was used to create the figures. Differences at $p < 0.05$ were considered significant. The data are presented as means \pm standard deviations in the figures.

3. Results

3.1. Effects of Drought Treatment, N Addition and their Interaction on Leaf Functional Traits

For leaf chemical traits, the first PCA axis accounted for 67.80% of the total variation, with strong loadings of LNC, C:N ratio and LPC (in descending order) (Table S1, Figure 1). The second PCA axis accounted for 22.62% of the total variation, with strong loadings of the C:P ratio, N:P ratio, LPC, and LCC (in descending order) (Table S1, Figure 1). For leaf physiological traits, the first PCA axis accounted for 59.07% of the total variation, with strong loadings of E , A , G_s , and ψ_{PD} (in descending order) (Table S1, Figure 1). The second PCA axis accounted for 17.89% of the total variation, with strong loadings of C_i and WUE (in descending order) (Table S1, Figure 1). Therefore, LNC, C:N ratio, and LPC were selected as chemical traits, and E , A , G_s , and ψ_{PD} were selected as physiological traits.

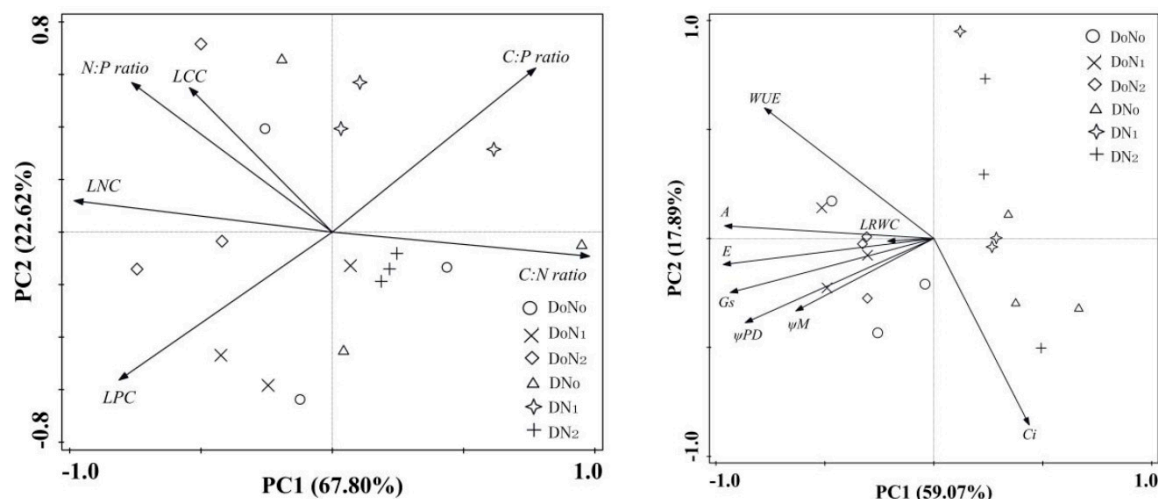


Figure 1. Principal component analysis of leaf chemical and physiological traits in *Machilus pauhoi* seedlings. Leaf C concentrations (LCC); Leaf N concentrations (LNC); Leaf P concentrations (LPC); Leaf C:N ratio (C:N ratio); Leaf C:P ratio (C:P ratio); Leaf N:P ratio (N:P ratio); Net photosynthetic rate (A); Transpiration rate (E); Stomatal conductance (Gs); Predawn leaf water potential (ψ_{PD}); Water use efficiency (WUE); Midday leaf water potential (ψ_M); Interacellular CO_2 concentrations (Ci); Leaf relative water content (LRWC). Each point indicates one replicate.

Drought treatments decreased leaf trait values in comparison with the non-drought treatments, except for the C:N ratio and SLA (Table S2, Figure 2). N addition increased LNC, LPC, LT, SLA, A, E, and ψ_{PD} in comparison with non-N addition but decreased the C:N ratio and Gs (Table S2, Figure 2). No significant differences in LNC, LPC, C:N ratio, or LA were found among the treatments, but there were significant differences in LT, SLA, A, E, Gs, and ψ_{PD} among them (Table S2, Figure 2). The highest LT value was recorded in the D_0N_2 treatment (0.59 ± 0.05 mm) and the lowest in the DN_0 treatment (0.39 ± 0.05 mm) (Table S2, Figure 2). The highest SLA value was in the DN_0 treatment (186.99 ± 6.04 cm²·g^{−1}), and the lowest in the D_0N_0 treatment (142.91 ± 3.07 cm²·g^{−1}) (Table S2, Figure 2). The highest A was in the D_0N_1 treatment (14.85 ± 3.24 μmol CO₂·m^{−2}·s^{−1}), and the lowest in the DN_2 treatment (1.38 ± 1.28 μmol CO₂·m^{−2}·s^{−1}) (Table S2, Figure 2). The highest E, Gs, and ψ_{PD} values were in the D_0N_1 treatment (3.90 ± 0.48 mmol H₂O·m^{−2}·s^{−1}, 363.83 ± 45.32 mmol H₂O·m^{−2}·s^{−1}, -0.04 ± 0.02 Mpa, respectively), and the lowest in the DN_2 treatment (0.83 ± 0.35 mmol H₂O·m^{−2}·s^{−1}, 39.67 ± 21.83 mmol H₂O·m^{−2}·s^{−1}, -0.85 ± 0.08 Mpa, respectively) (Table S2, Figure 2).

Drought treatment had significant effects on the leaf traits, except LA (Table S3, Figure 2), whereas N addition only significantly affected LT and Gs (Table S3, Figure 2). The interaction between drought treatment and N addition had no significant effect on the leaf traits (Table S3, Figure 2).

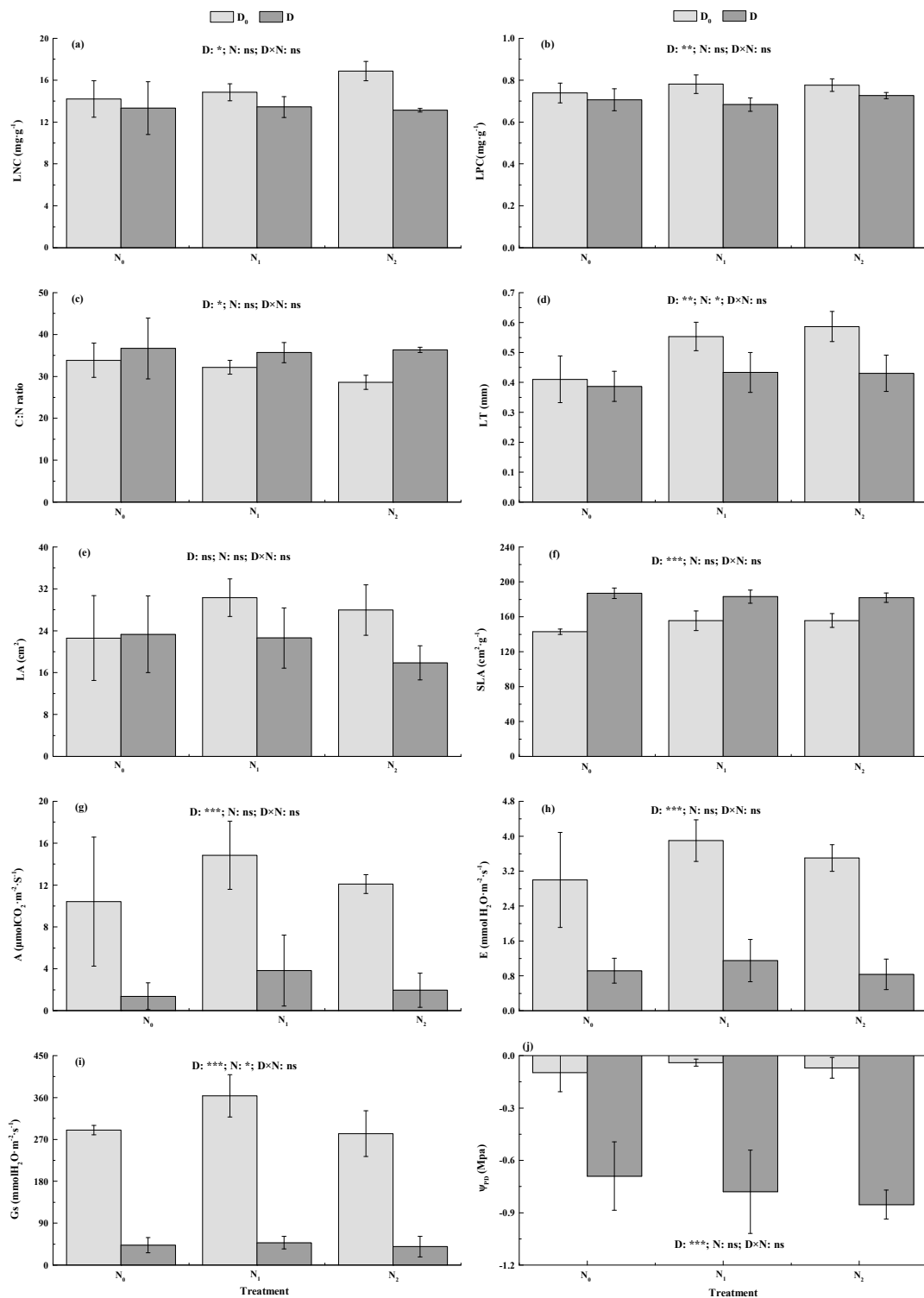


Figure 2. Differences in leaf functional traits of *M. pauhoi* seedlings among different treatments (mean ± standard deviation). (a) Leaf N concentrations; (b) Leaf P concentrations; (c) Leaf C:N ratio; (d) Leaf thickness; (e) Leaf area; (f) Specific leaf area; (g) Net photosynthetic rate; (h) Transpiration rate; (i) Stomatal conductance; (j) Predawn leaf water potential. Normal water supply (D₀); Drought treatment (D); No N addition (N₀); Low N addition (N₁); High N addition (N₂). Note: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, ns $p > 0.05$.

3.2. Treatment Effects of Drought, N Addition, and their Interaction on Leaf Functional Traits

The responses of leaf functional traits to drought treatment and N addition were further analyzed by measuring their *MTE*. Short-term low and high N addition had positive effects on LNC, LPC, LT, LA, SLA, A, E, and ψ_{PD} (Figure 3a–c). In comparison with the control treatment, low N addition increased these parameters by 2.22%, 2.77%, 14.88%, 7.83%, 4.22%, 17.55%, 13.04%, and 41.46%, respectively, and high N addition by 8.55%, 2.45%, 17.73%, 3.78%, 4.31%, 7.48%, 7.69%, and 16.00%, respectively (Figure 3a–c). Short-term low N and high N addition had negative effects on the C:N ratio by decreasing it by 2.16% and 8.11%, respectively, in comparison with the control treatment (Figure 3a). A positive effect of low N addition on G_s was found, which increased by 11.29% in comparison with the control treatment (Figure 3c), whereas high N addition had a negative effect on G_s and decreased it by 1.31% in comparison with the control treatment (Figure 3c). Short-term drought stress had negative effects on LNC, LPC, LT, LA, A, E, G_s , and ψ_{PD} , the values of which were 3.22%, 2.25%, 2.93%, 5.25%, 76.55%, 53.19%, 74.26%, and 75.42% lower, respectively, than those of the control treatment, and positive effects on the C:N ratio and SLA, with 13.36% and 3.24% higher values, respectively, than the control treatment (Figure 3a–c). There were positive interactions between drought and low N addition and between drought and high N addition on the C:N ratio, LT, and SLA. The interaction between drought and low N addition increased the C:N ratio, LT, and SLA by 2.96%, 2.77%, and 12.31%, respectively, in comparison with the control treatment, while the interaction between drought and high N addition increased them by 4.00%, 2.38% and 11.96%, respectively (Figure 3a,b). However, the effects on LNC, LPC, LA, A, E, G_s , and ψ_{PD} were negative. The interaction between drought and low N addition decreased LNC, LPC, LA, A, E, G_s , and ψ_{PD} by 2.78%, 3.91%, 6.79%, 46.20%, 44.58%, 71.60% and 77.95%, respectively, in comparison with the control treatment, while the interaction between drought and high N addition decreased them by 3.91%, 0.77%, 18.33%, 68.24%, 56.52%, 75.94%, and 79.65%, respectively (Figure 3a–c).

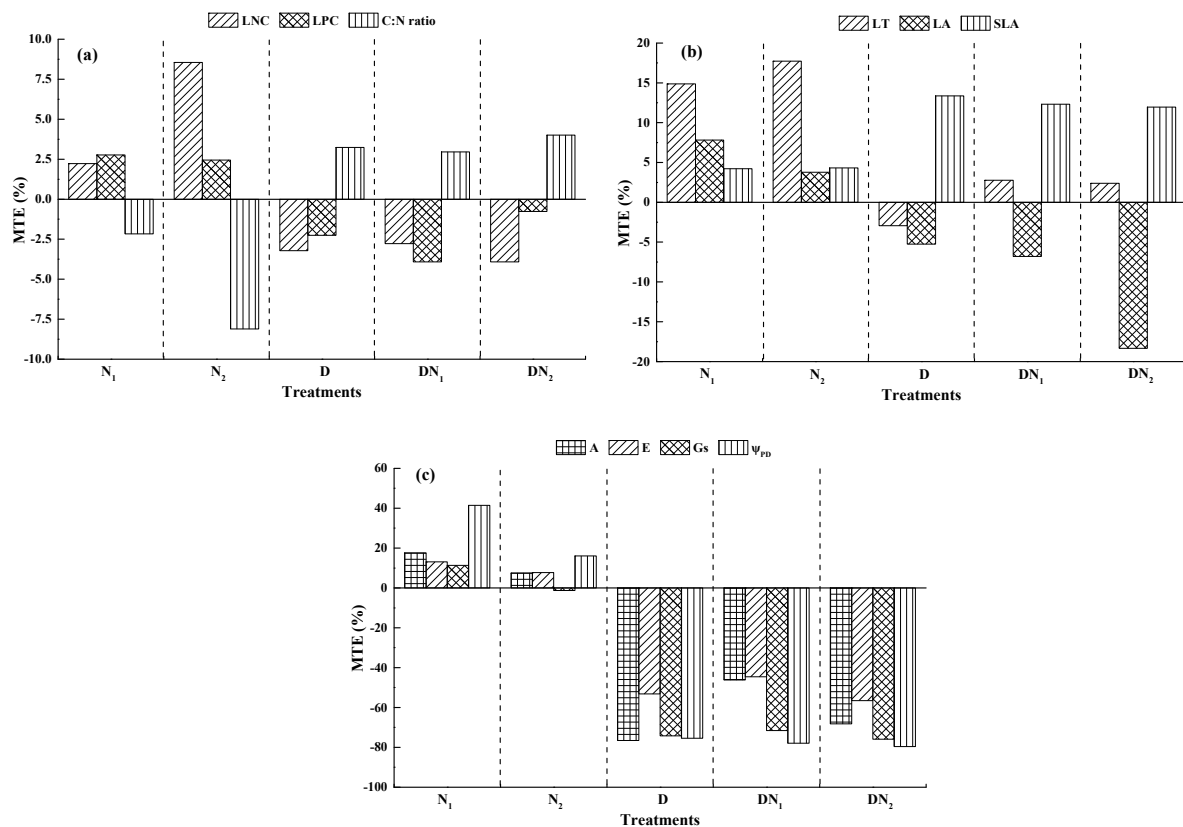


Figure 3. Magnitude of treatment effect of leaf functional traits of *M. pauhoi* seedlings in different treatment groups. (a) Leaf chemical traits; (b) Leaf morphological traits; (c) Leaf photosynthetic traits. Low N addition (N₁); High N addition (N₂); Drought treatment (D); Combined treatment of drought and low N (DN₁); Combined treatment of drought and high N (DN₂).

3.3. Effect of Drought Treatment, N Addition and their Interaction on Soil Physicochemical Properties

Soil properties significantly differed among the six treatments (Table S4, Figure 4). Soil pH, STN and STC in the D₀N₀ treatment, the soil C:N ratio (SCN) in the DN₂ treatment, and NO₃⁻-N and NH₄⁺-N in the D₀N₁ treatment had the lowest values (Table S4, Figure 4). The pH in the D₀N₂ treatment, STN in the DN₂ treatment, STC in the DN₁ treatment, SCN in the D₀N₂ treatment, and NO₃⁻-N and NH₄⁺-N in the DN₁ treatment had the highest values (Table S4, Figure 4). Significant effects of N addition on pH, STN, SCN, NO₃⁻-N and NH₄⁺-N were detected, but not on STC (Table S5, Figure 4). Drought treatment as well as the interaction between drought treatment and N addition had significant effects on all of the soil properties (Table S5, Figure 4).

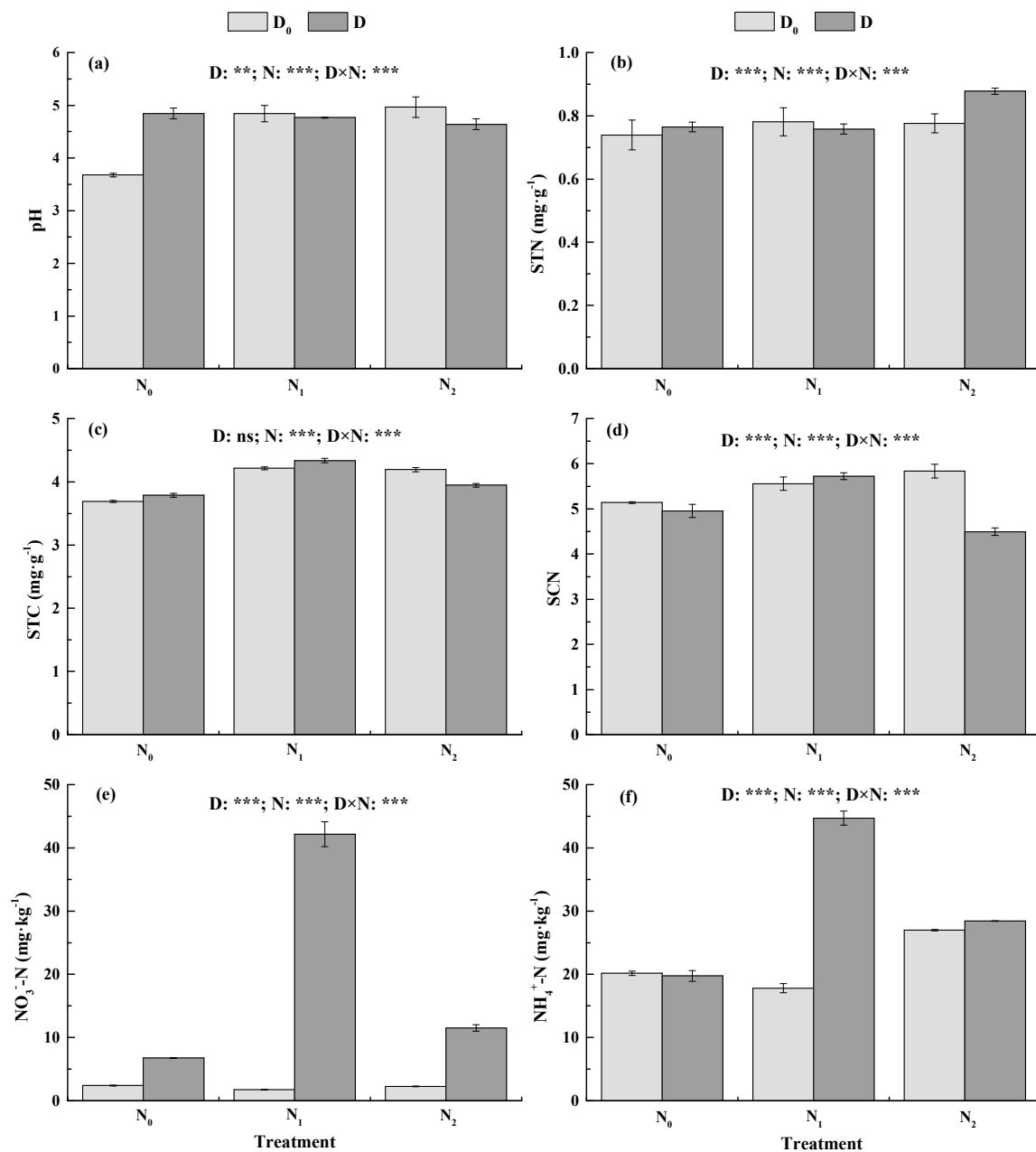


Figure 4. Differences of soil properties among different treatments (mean \pm standard deviation). (a) Soil pH; (b) Soil total N concentrations; (c) Soil total C concentrations; (d) Soil C:N ratio; (e) Nitrate N concentrations; (f) Ammonium N concentrations. Normal water supply (D₀); Drought treatment (D); No N addition (N₀); Low N addition (N₁); High N addition (N₂). Note: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

3.4. Main Soil Factors Affecting Leaf Functional Traits

A path analysis was conducted in order to ascertain which soil factors mainly affected leaf traits. The correlation between pH and STC was the highest, with the regression coefficient (R) of 0.662; followed by STC and NO₃⁻-N, with R of 0.508 (Figure 5). With regard to the correlation between leaf traits and soil properties, for LPC, LT, and A, high correlation was detected in NO₃⁻-N, STC, and STN, respectively (Figure 5). For SLA, E, Gs, and ψ_{PD} , high correlation was all detected in STN and NO₃⁻-N (Figure 5). For LPC, NO₃⁻-N had the greatest direct effect, and STC had the greatest indirect effect through NO₃⁻-N (Table 1). For LT, STC had the greatest direct effect, and NO₃⁻-N had the greatest indirect effect through STC. For SLA, the direct effect of STN was slightly greater than

that of NO_3^- -N, and the indirect effect of NO_3^- -N through STN was slightly greater than that of STN. For A, STN had a direct effect, but no indirect effect was detected. For E and Gs, STN had the greatest direct effect, and STN had the greatest indirect effect through pH, STN, and NO_3^- -N. For ψ_{PD} , NO_3^- -N had the greatest direct effect, and STN had the greatest indirect effect through pH, STN, and NO_3^- -N. For LPC, E, Gs, and ψ_{PD} , the contribution on NO_3^- -N to the R^2 was the highest (Table 1). For LT, the contribution on STN to the R^2 was the highest (Table 2). For SLA, the contribution on STN to the R^2 was slightly higher than that of NO_3^- -N (Table 2). Therefore, STN, NO_3^- -N, and STN were the main factors that affected the leaf traits of *M. pauhoi* seedlings.

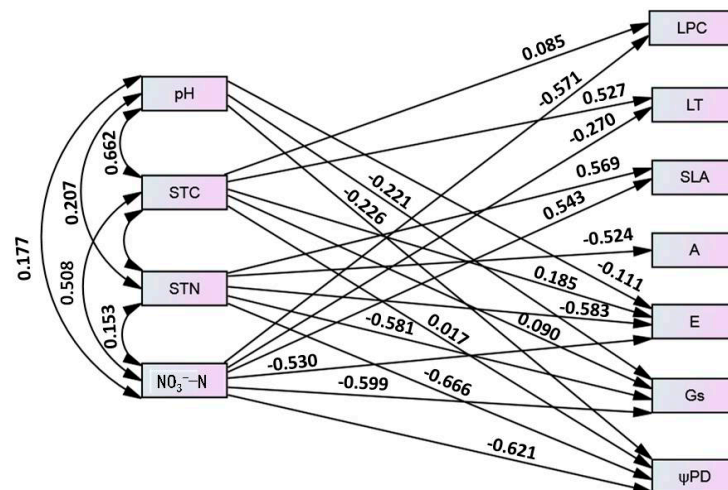


Figure 5. Correlations between *M. pauhoi* seedling leaf traits and soil properties. Soil pH (pH); Soil total C concentrations (STC); Soil total N concentrations (STN); Nitrate N concentrations (NO_3^- -N); Leaf P concentrations (LPC); Leaf thickness (LT); Specific leaf area (SLA); Net photosynthetic rate (A); Transpiration rate (E); Stomatal conductance (Gs); Predawn leaf water potential (ψ_{PD}). Values above arrows indicate correlation coefficients between soil properties and leaf traits.

Table 1. Path analysis of *Machilus pauhoi* seedling leaf traits and soil properties.

Leaf Traits	Variables	Correlation Coefficients	Direct Effect	Indirect Effect					
				Total Indirect Effect	pH	STN	STC	NO ₃ [−] -N	NH ₄ ⁺ -N
Nutrient	LPC	STC	0.085	0.505	−0.420	—	—	—	−0.420
		NO ₃ [−] -N	−0.571	−0.827	0.257	—	—	0.257	—
Morphology	LT	STC	0.527	0.896	−0.368	—	—	—	−0.368
		NO ₃ [−] -N	−0.270	−0.725	0.455	—	—	0.455	—
	SLA	STN	0.569	0.498	0.071	—	—	—	0.071
		NO ₃ [−] -N	0.543	0.466	0.076	—	0.076	—	—
Photosynthetic physiology	A	STN	−0.524	−0.524	—	—	—	—	—
		pH	−0.111	−0.546	0.436	—	−0.058	0.651	−0.158
	E	STN	−0.583	−0.280	−0.302	−0.113	—	−0.053	−0.136
		STC	0.185	0.984	−0.798	−0.361	0.015	—	−0.452
		NO ₃ [−] -N	−0.530	−0.890	0.360	−0.097	−0.043	0.500	—
		pH	−0.221	−0.662	0.442	—	−0.051	0.661	−0.168
	Gs	STN	−0.581	−0.244	−0.336	−0.137	—	−0.054	−0.146
		STC	0.090	0.998	−0.908	−0.438	0.013	—	−0.483
		NO ₃ [−] -N	−0.599	−0.951	0.352	−0.117	−0.037	0.507	—
		pH	−0.226	−0.491	0.265	—	−0.082	0.497	−0.150
	ψ _{PD}	STN	−0.666	−0.395	−0.271	−0.102	—	−0.041	−0.129
		STC	0.017	0.750	−0.733	−0.325	0.021	—	−0.429
		NO ₃ [−] -N	−0.621	−0.845	0.234	−0.087	−0.060	0.381	—

Note: Path coefficients of direct effect are simple, standardized regression coefficients. Results of stepwise linear regression and multi-collinearity are in Table S6 in supplementary materials. NH₄⁺-N with \sqrt{VIF} higher than 2 was excluded from Gs path analysis.

Table 2. Determination coefficients (R^2) between *M. pauhoi* seedling leaf traits and soil properties and the contribution of each soil variable to the R^2 .

Leaf Traits		Variables	Determination Coefficient (R^2)				Contribution to R^2
			pH	STN	STC	NO ₃ [−] -N	
Nutrient	LPC	STC			0.255	−0.424	0.043
		NO ₃ [−] -N				0.684	0.472
		Error term <i>e</i>					0.485
Morphology	LT	STC			0.803	−0.660	0.472
		NO ₃ [−] -N				0.526	0.196
		Error term <i>e</i>					0.332
	SLA	STN		0.248		0.071	0.283
		NO ₃ [−] -N				0.217	0.253
		Error term <i>e</i>					0.464
Photosynthetic physiology	A	STN		0.275			0.275
		Error term <i>e</i>					0.725
		pH	0.298	0.063	−0.711	0.172	0.061
	E	STN		0.078	0.030	0.076	0.163
		STC			0.968	−0.890	0.182
		NO ₃ [−] -N				0.792	0.472
		Error term <i>e</i>					0.122
		pH	0.438	0.067	−0.875	0.223	0.146
		STN		0.060	0.026	0.071	0.142
	Gs	STC			0.996	−0.964	0.090
		NO ₃ [−] -N				0.904	0.570
		Error term <i>e</i>					0.052
	ψ _{PD}	pH	0.241	0.080	−0.488	0.147	0.111
		STN		0.156	0.032	0.102	0.263
		STC			0.563	−0.644	0.013
		NO ₃ [−] -N				0.714	0.525
		Error term <i>e</i>					0.088

4. Discussion

4.1. Response to Drought Stress

Tree species growing in humid habitats are generally considered sensitive to drought stress [54]. Drought treatment had a negative but not significant effect on the LA of *M. pauhoi* seedlings, which corroborates previous findings, demonstrating that the *M. pauhoi* seedlings decreased LA and transpiration to prevent cell turgor and ψ_{PD} reduction in response to drought stress. LT is associated with resource acquisition, water conservation and CO₂ assimilation [22]. Plants usually increase LT to cope with drought [22], which is inconsistent with our results, as we found a significant negative effect of drought stress on LT. It is possible that drought stress affected the seedlings' water metabolism and decreased their ψ_{PD} . Leaf water deficit hindered the growth and division of mesophyll cells and reduced the gaps between cells and compacted them, indicating that *M. pauhoi* leaves preserve and make full use of the limited water available under drought conditions by reducing E. This may be a typical economic adaptation.

Considerable evidence has been accumulated to demonstrate that plants increase leaf mass per area (LMA) (or decrease SLA) in response to drought on the global scale [25], reflecting the adaptive strategies employed by plants to maximize C absorption and reduce water consumption [25]. The relatively consistent results also apply on regional and local scales [26–28]. However, our results were contrary to previous findings, but agreed with the findings reported by [20,29]. *M. pauhoi* seedlings significantly increased SLA by reducing LT and/or density more than decreasing LA under drought, as previously suggested [29], indicating a tradeoff between leaf investment and survival under limited photosynthetic conditions. Elevated SLA is beneficial to *M. pauhoi* seedlings because it improves leaf structure efficiency in response to drought stress, so compensates for leaf biomass allocation reduction [55]. In addition, a larger SLA shortens the distance from stomatal pores to carboxylation sites, thus counteracting the resistance of mesophyll and stomata [56]. *M. pauhoi* seedlings decreased LT under drought stress. SLA is negatively correlated with LT [22], which corresponds well in our observations for *M. pauhoi* seedlings ($R^2 = -0.474$, $p < 0.01$). Accordingly, it was confirmed that the *M. pauhoi* seedlings increased SLA in response to drought stress. SLA and LNC are two vital traits correlated closely with C gain and the maximum assimilation rate [15], but our results do not support a positive relationship between them [22]. Drought treatment significantly decreased LNC, which decreased LPC, because N is tightly coupled with P [57–59].

Drought treatment significantly decreased A, E, Gs and ψ_{PD} , mainly due to the fact that drought-associated soil water shortage leads to CO₂ limitation through stomata closure (a decrease in Gs) and metabolic constraints [35,60]. Specifically, soil water shortage attributed to drought has a negative effect on N metabolism by regulating the activities of crucial enzymes that participate in N assimilation and catabolism, which results in damage to cell membranes and further decreases photosynthetic capacity [35,61].

4.2. Response to N Addition

Our results indicated N addition (50 kg N·hm⁻²·year⁻¹ or 100 kg N·hm⁻²·year⁻¹) increased LNC, LPC, LT, LA, SLA, A, E, and ψ_{PD} in *M. pauhoi* seedlings, indicating that appropriate N addition promotes their growth, but no significant effects were detected except for LT and Gs. Previous studies showed that N addition of 100 kg N·hm⁻²·year⁻¹ increases the LA of *Acer truncatum* Bunge seedlings, but when N addition exceeds 200 kg N·hm⁻²·year⁻¹, LA decreases [62]. Appropriate N input (20, 40, or 60 kg N·hm⁻²·year⁻¹) improves the growth of *Fraxinus mandshurica* Ruprecht seedlings, but when N addition is above 80 kg N·hm⁻²·year⁻¹, growth is inhibited [63]. The threshold was considered as “N critical load”, that was, the ecosystems can tolerate N addition without any obviously adverse effects [64]. The critical N capacity varies with ecosystem type, study area, and forest type. Therefore, it is reasonable to infer that N input of 100 kg N·hm⁻²·year⁻¹ may not reach the N critical load.

Therefore, more attention should be paid to atmospheric N deposition in the range of *M. pauhoi* in order to evaluate any negative effects caused by N deposition.

N addition increased LT, LA and SLA, which is consistent with numerous previous findings [26,65,66]. SLA (the reciprocal of LMA) indicates captured light resources on LA per unit leaf dry matter investment, which is closely related to plants' light interception efficiency [15]. N addition increased SLA, as documented previously [63,65], possibly because it promoted growth and thus increased leaf photosynthesis [21], accelerated the resource cycle to increase resource capture efficiency [67], or reduced the availability of canopy light to increase the SLA [68]. Elevated LT, LA, and SLA caused by N fertilization suggests that *M. pauhoi* leaves have a higher resource acquisition ability and relative growth rate (characterized by having a reduced dry matter investment per unit LA) under exogenous N input.

N addition increased the LNC of *M. pauhoi* seedlings by increasing the soil available N (the sum of NO_3^- -N and NH_4^+ -N) [21,69], as identified previously [27,50,59,65,70,71]. We also found that N addition increased the LPC, as reported by [72], but contrary to other results [27,73]. There are two possible explanations for the increase in LPC caused by N addition. Firstly, N is tightly coupled with P from cell to ecosystem [57–59], so the absorption of one affects the availability of the other [74]. Specifically, N addition increased the LPC on account of the close coupling between leaf N and P [59], leading to increased soil available P concentrations. Secondly, Fujita et al. (2010) suggested that elevated N input could increase soil P absorption by increasing root phosphatase activity [75]. Plants can use excess N to assemble more phosphatase under N addition and thus promote the absorption of soil P, leading to an increase in the LPC [76,77]. Elevated LNC and a relatively stable C level resulted in a decrease in the C:N ratio under N addition.

N addition increased the photosynthetic performance of the *M. pauhoi* seedlings by increasing A and the photosynthetic N use efficiency (PNUE), as previously reported [20,23,78]. Elevated A can be explained in two ways. Firstly, it may have increased the leaf chlorophyll content [35,63]. Secondly, it may have increased the LNC possibly fixed in photosynthetic pigments and enzymes, resulting in a higher A [63]. PNUE that reflects nutrition-related physiological traits of leaves [79], can be used to evaluate plants' photosynthetic capacity [78,80].

4.3. Additive or Non-Additive Effects?

Water and N are the main limiting factors for plant growth [81]. No significant interactive effects of drought stress and N addition on the leaf functional traits of *M. pauhoi* seedlings were detected, possibly owing to a tradeoff between water and N input. Under a combination of drought stress and short-term N addition, LNC, LPC, LA, A, E, Gs, and ψ_{PD} decreased, while the SLA and C:N ratio increased. It reflects that the effect of short-term N deposition on plants is relatively weaker than that of drought stress, and drought is the main limiting factor [56] that affects the full utilization of additional N [82]. It is possible that short-term addition of NH_4NO_3 solution is easily immobilized and incorporated by C molecules into relatively stable soil organic storage, so is difficult for plants to absorb by plants [83]. Additionally, the NH_4NO_3 solution was directly added to the soil in our experiment, and it takes some time for roots to uptake nutrients from the soil that are then transported to the leaves. Drought, N addition and their interaction all had significant effects on soil properties, but N addition and the interaction between N addition and drought had no significant effects on leaf traits. The response of leaves to N input may indicate a hysteresis effect, which is not immediately apparent. Therefore, it is reasonable to conclude that short-term N addition does not significantly alter the effects of seasonal drought on *M. pauhoi* seedling leaf traits, and the interaction between short-term drought and N deposition has an additive effect on *M. pauhoi* seedling leaf traits.

However, because of the different N addition levels, fertilization forms, and drought intensity and duration, the adaptive strategies of *M. pauhoi* under different N addition and drought levels differ, which should be investigated in future studies.

5. Conclusions

A full-factorial experiment was conducted to analyze the single and combined effects of seasonal drought and short-term N addition on the chemical, morphological and physiological traits of *Machilus pauhoi* seedlings. Seasonal drought (40% of soil field capacity) had significant negative effects on LNC, LPC, LT, A, E, Gs, and ψ_{PD} , and significant positive effects on the C:N ratio and SLA. Short-term N addition (50 kg N·hm⁻²·year⁻¹ and 100 kg N·hm⁻²·year⁻¹) increased LNC, LPC, LT, LA, SLA, A, E, and ψ_{PD} , and decreased the C:N ratio; however, it only had significant effects on LT and Gs. It was concluded that seasonal drought had a stronger effect on *M. pauhoi* seedling leaf traits than short-term N deposition. The interaction between drought and N deposition was additive. Drought, N addition, and their interaction all had significant effects on soil properties. Path analysis indicated that the STC, NO₃⁻-N, and STN were the main factors that affected the leaf traits. In summary, short-term N addition does not significantly alter the effects of seasonal drought on *M. pauhoi* seedling leaf traits in N-rich subtropical China. However, the effects of N fertilization on *M. pauhoi* seedlings may not be fully taken into account because of the short experimental duration. Therefore, long-term N fertilization combined with seasonal drought experiments should be conducted in the future. Our results provide deeper insights into the adaptive responses of *M. pauhoi* to its habitat, and lay the groundwork for future studies that investigate the response mechanisms of plants to global environmental change.

Supplementary Materials: The following are available online at <http://www.mdpi.com/1999-4907/10/2/78/s1>. Table S1: Loading scores of six leaf chemical and physiological traits in the principal component analysis, Table S2: Differences of leaf functional traits of *Machilus pauhoi* seedlings among different treatments (mean ± standard deviation), Table S3: Effect of drought treatment, N addition and their interactions on leaf functional traits of *M. pauhoi* seedlings, Table S4: Differences of soil properties among different treatments (mean ± standard deviation), Table S5: Results of a two-way ANOVA of drought treatment, N addition, and their interactions on soil properties; Table S6: Results of stepwise linear regression analysis of leaf functional traits of *M. pauhoi* seedlings and soil properties.

Author Contributions: Conceptualization, H.Y. and Q.Z.; methodology, H.Y. and B.L.; software, H.Y. and Z.Z.; validation, H.Y. and C.X.; formal analysis, H.Y.; investigation, C.X., Z.Z. and Y.Z.; resources, H.Y.; data curation, H.Y.; writing—original draft preparation, H.Y.; writing—review and editing, H.Y., Q.Z. and D.C.; supervision, B.L.; funding acquisition, H.Y., Q.Z. and D.C.

Funding: This research was funded by the National Natural Science Foundation of China (31170596, 31370589, 31470501), the National Key Research and Development Plan of China (2017YFC0505400), the Seed Industry Innovation Project of Fujian Province of China (2014S1477-4), the Major S&T Project of Fujian Province of China (2014N5008), the Natural Science Foundation of Fujian Province of China (2018J01479), and the Educational and Scientific Research Project of Fujian Provincial Education Department of China (JAT170443).

Acknowledgments: We would like to thank Yonghong Wu, Yuxing Zou and Shaofei Jin for their assistance in collecting plant and soil materials used for experiments. We are sincerely grateful to the anonymous reviewers for their valuable comments to improve the manuscript.

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

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