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Impact of Insect Foliar Herbivory on Soil N_2O Emission and Nitrogen Dynamics in Subtropical Tree Species

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Abstract: Insect foliar herbivory is ubiquitous in terrestrial ecosystems, yet its impacts on soil nitrogen cycling processes remain not yet well known. To examine the impacts of insect foliar herbivory on soil N_2O emission flux and available nitrogen (N), we conducted a pot experiment to measure soil available N content and soil N_2O emission flux among three treatments (i.e., leaf herbivory, artificial defoliation, and control,) in two broad-leaved trees (*Cinnamomum camphora* and *Liquidambar formosana*) and two conifer trees (*Pinus massonianna* and *Cryptomeria fortunei*). Our results showed that insect foliar herbivory significantly increased soil inorganic N (i.e., NH_4^+–N and NO_3^-–N), dissolved organic nitrogen (DON) and microbial biomass nitrogen (MBN) contents, and urease activity compared to control treatment. However, there were no differences in soil available N contents and urease activity between artificial defoliation and control treatments, implying that insect foliar herbivory had greater impacts on soil available N contents compared to physical damage of leaves. Moreover, soil N_2O emission fluxes were increased by insect foliar herbivory in *Cinnamomum camphora* and *Pinus massonianna*, but not for the other two tree species, indicating various effect of insect foliar herbivory on soil N_2O emission among tree species. Furthermore, our results showed the positive correlations between soil N_2O emission flux and soil NO_3^-–N, DON, MBN, and acid protease activity, and soil inorganic N, pH, and MBN mainly explained soil N_2O emission. Our results implied that insect foliar herbivory can speed up soil nitrogen availability in subtropical forests, but the impacts on soil N_2O emission are related to tree species.

Keywords: insect foliar herbivory; nitrous oxide flux; soil available nitrogen; subtropical forest; tree species

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1. Introduction

Soil nitrogen not only plays essential roles in improving soil quality, sustaining plant growth, and biogeochemical cycling [1] but also influences the emission of nitrous oxide (N_2O) in forest ecosystems [2]. However, soil nitrogen has been demonstrated to be affected by many factors in subtropical forests, such as temperature [3], humidity [4], and insect herbivory disturbance [5]. Insect foliar herbivory is one of the most common phenomena and ecological processes in forest ecosystems, and the frequencies of insect pests are increasing in the context of global climate change [6]. Insect foliar herbivory can significantly affect plant growth [7], photosynthetic rate [8], above-/below-ground biomass partition [9], and root exudates [10]. Meanwhile, the changes in plant photosynthetic carbon input and water use efficiency caused by foliar herbivory can lead to changes in soil

environmental factors such as soil acidity, temperature, and humidity [11], which further affect soil microbial activity, soil available nitrogen content, and nitrous oxide emission [12].

Soil available nitrogen is a more accurate indicator of nitrogen supplement for plants [13]. Soil available nitrogen mainly includes ammonium (NH_4^+–N), nitrate (NO_3^-–N), dissolved organic nitrogen (DON), and soil microbial biomass nitrogen (MBN) [14]. Soil NH_4^+–N and NO_3^-–N are sensitive to insect foliar herbivory because they are generally correlated with soil physicochemical and biological properties [15]. Soil dissolved organic nitrogen is not only easily mineralized but also can be directly utilized by plant roots [4]. Soil nitrogen mineralization is mediated by soil microorganisms, and thereby MBN can directly indicate soil N nutrient availability [16]. Currently, there are contrasting responses of soil available nitrogen to insect foliar herbivory. For example, one of the previous studies found that foliar herbivory increased soil inorganic nitrogen content due to accelerating fine root turnover, enhancing root carbon allocation, and increasing microbial activity, which differed among tree species, with more changes in soil inorganic nitrogen in *Abies alba* than in *Fagus sylvatica* [17]. In addition, Nitschke et al. reported that feeding on poplar leaves by the dancing moth reduced soil nitrogen mineralization rate because leaf insect herbivory attenuated plant transpiration and shading, resulting in the increase in soil temperature and humidity and increased soil pH [18]. In addition, Buchkowski et al. found that insect foliar herbivory had less effect on soil available nitrogen [19]. Therefore, more studies are still needed to investigate the effect of foliar herbivory on soil available nitrogen in forests.

Nitrous oxide, as a greenhouse gas, plays an important role in global climate change, with a global warming potential about 150 times as high as that of CO_2 [20]. Soil nitrification and denitrification produce N_2O [21]. Therefore, soil N_2O emissions are closely related to soil available nitrogen content and soil microbial biomass and activity [22]. In recent years, much attention has been paid to how global climate change and anthropogenic practices affect soil available nitrogen and N_2O emission in forests [23]. However, less study focuses on the effect of insect foliar herbivory on soil N_2O emission and its mechanisms, especially for the different responses of tree species.

China has the largest area of subtropical forest in the world [24], storing a vital nitrogen pool in soils [25]. Subtropical forests are only about 8% of global forests, whereas they account for about 14% of the N_2O emissions of global soils and induce important impacts on global climate change [26]. It has been suggested that about 10% of leaf productivity in subtropical forests can be annually consumed by insect herbivory [27]. Thus, it is necessary to know the effect of insect foliar herbivory on soil available nitrogen content and N_2O emission in subtropical forests. In this study, we compared the impacts of insect foliar herbivory on soil available nitrogen (including NO_3^-–N, NH_4^+–N, and DON), soil MBN and N-converting enzyme (i.e., acidic protease and urease) activities, and soil N_2O emission in two broad-leaved trees (*Cinnamomum camphora* and *Liquidambar formosana*) and two conifer trees (*Pinus massonianna* and *Cryptomeria fortunei*) in subtropical China. Furthermore, we tested the relationships between soil N_2O emission and soil available nitrogen, soil microbial biomass N, and enzyme activities. We proposed two hypotheses: (1) Insect foliar herbivory can increase soil available nitrogen content (e.g., NO_3^-–N, NH_4^+–N), which further promotes soil N_2O emission. (2) The impacts of insect foliar herbivory on soil available nitrogen content and N_2O emission vary among tree species, with greater effects on broad-leaved trees.

2. Materials and Methods

2.1. Study Site

Our experiment was conducted in Sanming City, Fujian Province (26°6′–26°40′ N, 117°32′–118°6′ E), in southeastern China (Figure 1). The region has a typical subtropical monsoon climate with a mean annual temperature of 19.6 °C, mean annual precipitation of

1754 mm, and annual sunshine hours of 1878 h and a frost-free period of 303 days. The soil is classified as Utisol developed from granite stone.

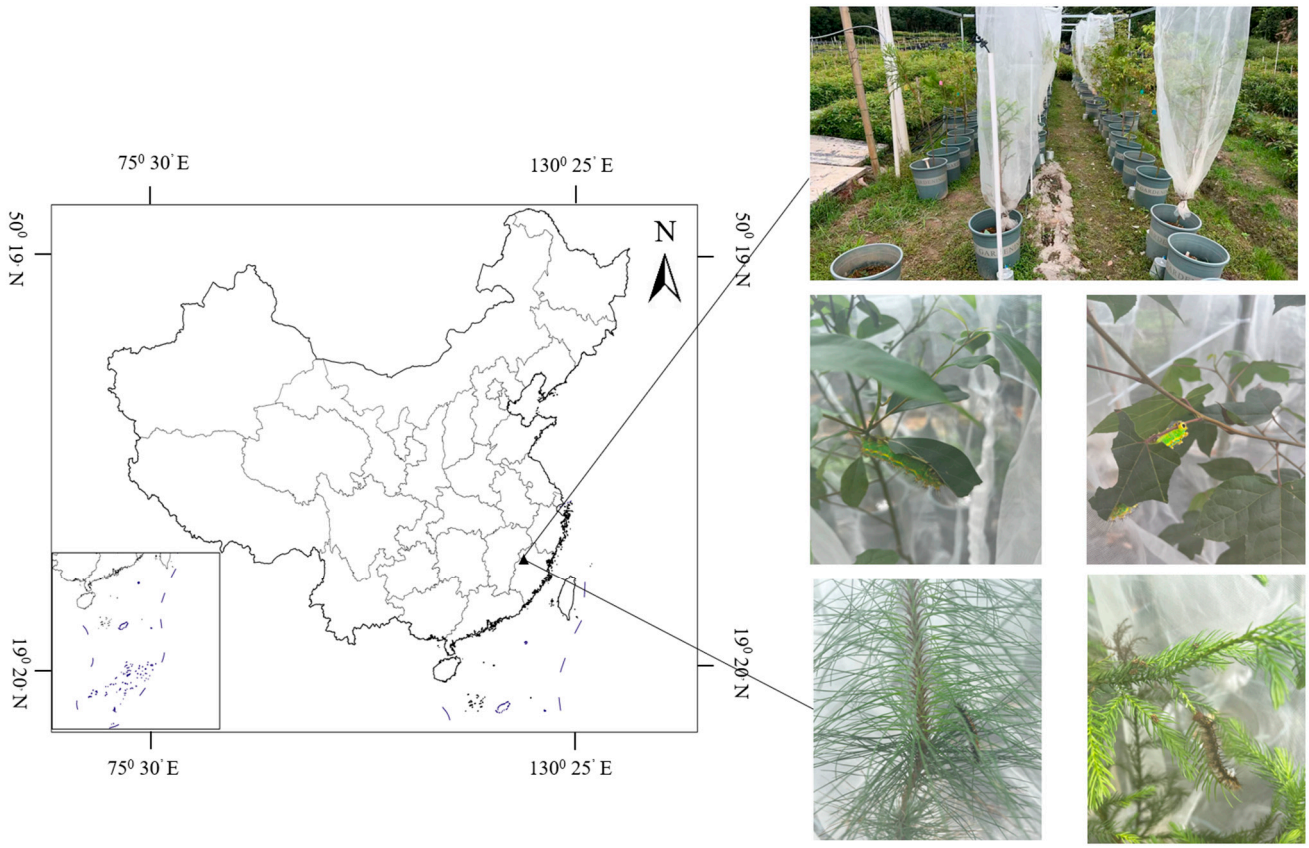

Figure 1. Location map of the study site.

2.2. Experiment Design

In this study, we used a factorial experiment of 4 tree species × 3 herbivory treatments. The 4 tree species were *Cinnamomum camphora*, *Liquidambar formosana*, *Pinus massonianna* and *Cryptomeria fortunei*, respectively. The 3 herbivory treatments included the control of non-herbivory (CK), leaf herbivory (LH), and artificial defoliation (LD). A total of 12 treatments were set up, with 6 replications of each treatment.

In December 2022, the 2-year-old tree seedlings of four species were transplanted into plastic pots. Before transplantation, 0–20 cm soil was collected in one *Cunninghamia lanceolata* plantation forest. The soil was mixed thoroughly without sieving and sterilization, and then field soil (approximately 20 kg dry weight) was used to fill one pot (37.5 cm × 29.5 cm × 40 cm). After that, one seedling was planted in each pot and grown for 5 months in field conditions with natural light, temperature, and precipitation.

In May 2023, we carried out foliar herbivory treatment at the peak stage of feeding on leaves using insect larvae of the 4th to 6th instar. We used *Dendrolimus houi Lajonquiere* to feed on *Cryptomeria fortunei*, *Dendrolimus punctatus* to feed on *Pinus massonianna*, and *Eriogyna pyretorum* to feed on *Cinnamomum camphora* and *Liquidambar formosana*, respectively, considering their different foliage-feeding predilection. Two to three larvae were placed on leaves in each seedling, and the canopy was covered with nylon mesh bags (3 mm size) to prevent larvae from escaping. The intensity of foliar herbivory was about 50% in LH treatment, considering almost 30% of leaf productivity of seedlings can be eaten by insect herbivory in the subtropical region [28], and there is increasing insect herbivory under global climate change. For LD treatments, the artificial leaf cutting was conducted

weekly to the same intensity in order to compare the effects of physical loss and chemical induction of insect foliar herbivory. After foliar herbivory for 1 month, the nylon mesh bags and larvae were removed, and all tree seedlings grew under natural field conditions, with watering and fertilization regularly carried out to avoid limiting water and nutrients.

2.3. Soil N_2O Emission Analysis

Soil N_2O emission flux was measured using a static chamber method, and gas samples were determined using a gas chromatograph (GC-2014, Shimadzu Corporation, Kyoto, Japan). The chamber base (10 cm diameter, 10 cm high PVC ring) was installed 2 weeks prior to the first gas sampling. Then, the gas samples were collected immediately after insect foliar herbivory treatment and in each month during the whole experiment period. For each gas sampling, the chamber cover was placed on the chamber base and sealed, and then 10 mL of gas was collected using a syringe at 0 min, 20 min, and 40 min. Soil N_2O emission flux was calculated using the following equation [29].

$$F = \rho \times h \times \frac{d_c}{d_t} \times \frac{273}{273 + T}$$

where F is the N_2O emission flux ($\mu g \cdot m^{-2} \cdot h^{-1}$); ρ is the density of N_2O in the standard state (1.25 $kg \cdot m^{-3}$); h is the height of the chamber (m); d_c/d_t is the change in N_2O concentration in the chamber during gas recovery; and T indicates temperature (°C) in the chamber.

2.4. Soil pH, NH_4^+-N, NO_3^--N, DON, MBN, and Enzyme Activity

In December 2023, all soils in each pot were collected. Soils were passed through 2 mm sieves and keep in 4 °C conditions for measurements of soil pH, soil reactive nitrogen indicators, and soil enzyme activity.

Soil inorganic nitrogen content was determined by the extraction method [30]. Field moisture soil (equivalent to 10 g dry soil) was weighed in a 100 mL conical flaska and extracted using 40 mL 2 mol/L KCL solution for 1 h. Soil NH_4^+–N and NO_3^-–N content in the solution was determined using a discrete auto analyzer (SmartChem 200, AMS Allianc, Rome, Italy).

Microbial biomass N was determined by the fumigation-extraction method and calculated as the difference in N content in fumigated and non-fumigated samples (E_N) using the k_{EN} coefficient (microbial biomass N = E_N:k_{EN}). The value of k_{EN} = 0.54 was used to calculate microbial biomass N [31]. The measurement of soil DON content followed the method described by Jones and Willett [32].

The activities of soil acid protease and urease enzymes were determined using reagent kits [33]. Soil pH was measured in water (water/soil ratio 1:2.5) with a pH meter (Remo, pHS-3C, Shanghai, China).

2.5. Data Analysis

Data statistical analysis was performed using IBM SPSS statistics software (SPSS 27.0, IBM). The normal distribution of the data and homogeneity of variances were tested before performing statistical analyses. The repeated measured ANOVA was used to test the effects of insect foliar herbivory treatment on soil N_2O emission flux in each tree species, respectively. The two-way ANOVA was used to test the effects of insect foliar herbivory, tree species, and their interaction on the average soil N_2O emission flux, and soil NH_4^+–N and NO_3^-–N, DON, MBN, pH, acid protease activity, urease activity, and multiple comparisons were tested using the LSD method (α = 0.05). Pearson correlation analysis was used to test the correlation between the average soil N_2O emission fluxes and soil

N indicators. Moreover, the random forest analysis was conducted using the 'rfPermute' package in R 4.2.3 software to test the relative factors affecting soil N_2O emission.

3. Results

3.1. Soil N_2O Emission Flux

Soil N_2O emission flux showed similar dynamics among foliar herbivory treatments in four tree species, with gradually declining N_2O emission over time and even N_2O uptake occurring during November and December (Figure 2). Soil N_2O fluxes differed significantly among foliar herbivory treatments in both *Cinnamomum camphora* and *Pinus massonianna* (Figure 2a,c). Moreover, the averaged N_2O fluxes were significantly affected by foliar herbivory treatment, tree species, and their interaction (Figure 3). The means of soil N_2O fluxes in LH and LD treatment were 218% and 213% higher than CK treatment in *Cinnamomum camphora*, respectively. The means of soil N_2O emission fluxes decreased significantly in the order of LH > LD > CK in *Pinus massonianna* (Figure 3), but not for *Liquidambar formosana* or *Cryptomeria fortunei*. In addition, the averaged soil N_2O fluxes over foliar herbivory treatments were significantly higher in both *Cinnamomum camphora* and *Liquidambar formosana* compared to both *Pinus massonianna* and *Cryptomeria fortunei* (Figure 3).

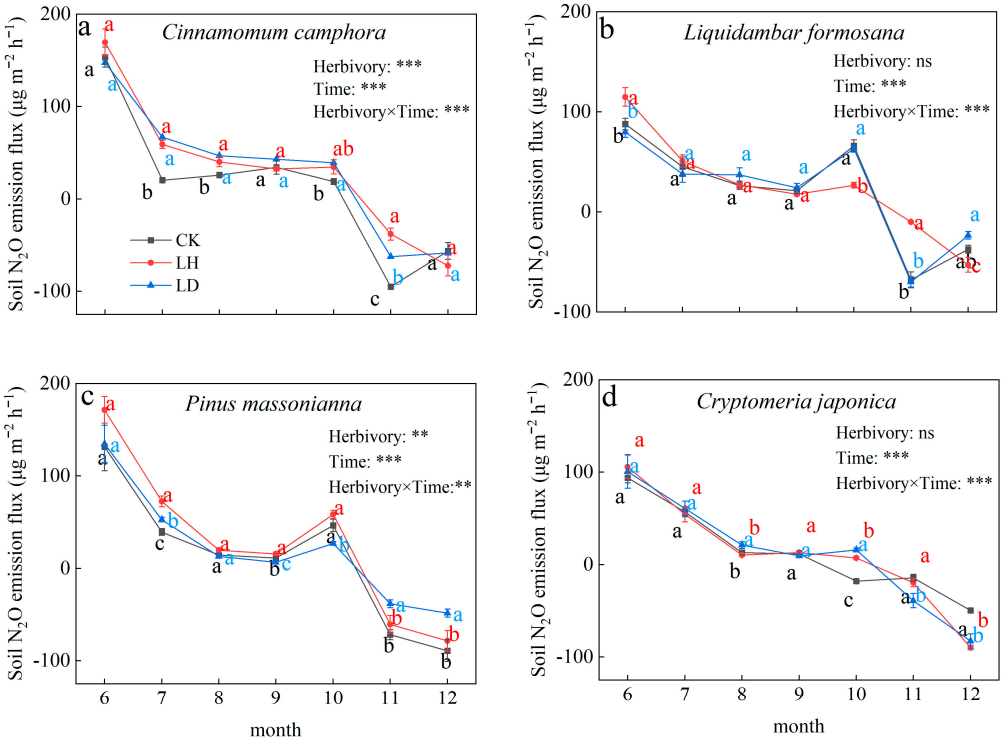

Figure 2. Dynamics of soil N_2O emission flux among insect foliar treatments in *Cinnamomum camphora* (**a**), *Liquidambar formosana* (**b**), *Pinus massonianna* (**c**), and *Cryptomeria japonica* (**d**). CK, control treatment; LH, leaf herbivory; LD, artificial defoliation. Different lowercase letters indicate significant differences between insect foliar herbivory treatments at the level of $p < 0.05$. ns, no significant difference; ** and *** represent significant differences at the levels of $p < 0.01$ and $p < 0.001$, respectively.

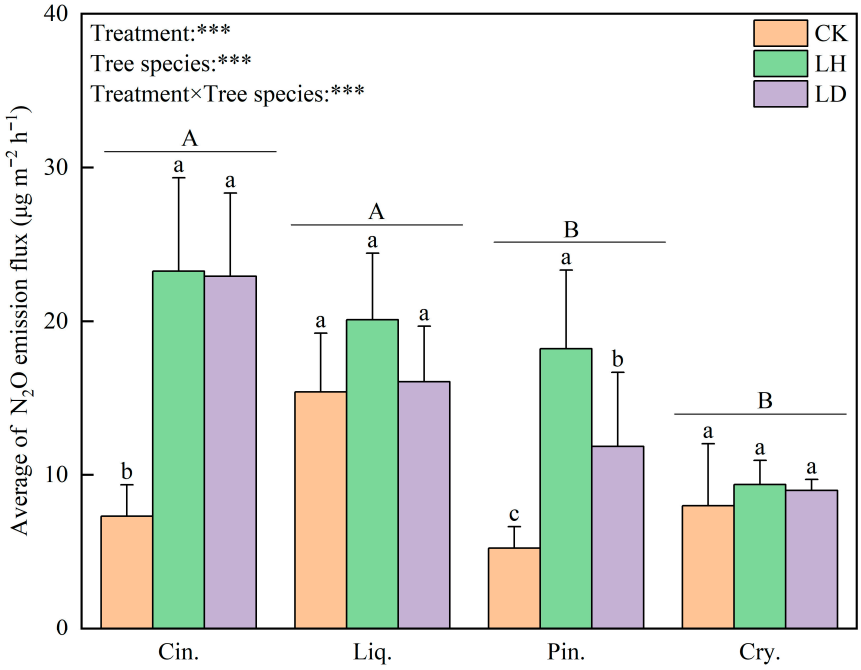

Figure 3. Effects of insect foliar herbivory and tree species on the mean values of soil N_2O emission flux. CK, control treatment; LH, leaf herbivory; LD, artificial defoliation. Cin.: *Cinnamomum camphora*, Liq.: *Liquidambar formosana*, Pin.: *Pinus massonianna*, Cry.: *Cryptomeria japonica*. Different lowercase letters indicate significant differences between insect foliar herbivory treatments in each tree species at the level of $p < 0.05$. Different uppercase letters indicate significant differences between tree species at the level of $p < 0.05$. *** represents a significant difference at the level of $p < 0.001$.

3.2. Soil NH_4^+-N, NO_3^--N, DON Contents and pH

Soil inorganic N (i.e., NH_4^+–N and NO_3^-–N) and DON contents were significantly affected by insect foliar herbivory treatment and tree species, but there were no interactive effects of treatment and tree species (Table 1; Figure 4a–c). The averaged soil NH_4^+–N, NO_3^-–N, and DON contents over tree species were significantly higher in LH than CK treatment, but no significant differences were found between CK and LD treatments (Figure 4a–c). Furthermore, soil NO_3^-–N and DON contents were generally higher in broad-leaved trees than in conifer trees, and soil NH_4^+–N content in *Cryptomeria japonica* was the lowest than in the other three species. Soil pH was not altered by insect foliar herbivory, whereas soil pH differed significantly among tree species, with lower soil pH in both broad-leaved trees compared to conifer trees (Table 1; Figure 4d).

Table 1. Two-way ANOVA of the impacts of foliar herbivory and tree species on soil NH_4^+-N, NO_3^--N, DON, pH, MBN, and enzyme activities.

Factor		NO_3^-–N	NH_4^+–N	DON	pH	MBN	Urease Activity	Acid Protease Activity
Herbivory	F	8.193	5.691	4.637	2.181	6.217	19.147	36.977
	p	0.001	0.005	0.013	0.122	0.004	<0.001	<0.001
Species	F	7.508	8.095	9.569	29.865	5.119	1.705	2.565
	p	<0.001	<0.001	<0.001	<0.001	0.003	0.175	0.063
Herbivory × Species	F	0.807	1.314	0.357	0.174	0.787	1.527	2.814
	p	0.569	0.265	0.903	0.983	0.583	0.185	0.018

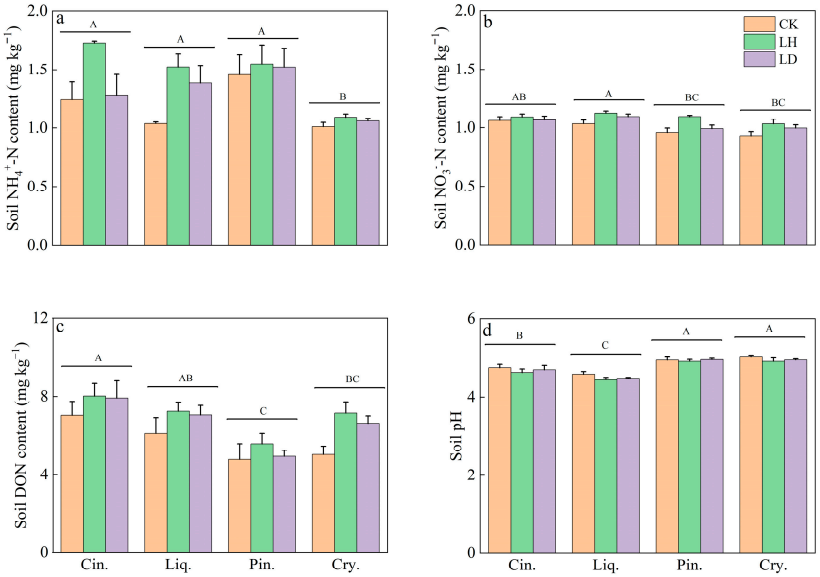

Figure 4. Effects of insect herbivory and tree species on soil NH_4^+- N content (**a**), NO_3^-- N content (**b**), soil DON content (**c**), and soil pH (**d**).

3.3. Soil Microbial Biomass Nitrogen, Acid Protease, and Urease Activities

Soil MBN content was significantly affected by insect foliar herbivory and tree species, but there were no interactions between insect foliar herbivory and tree species (Table 1; Figure 5a). The average MBN content was significantly higher in LH than in both CK and LD treatments, but there was no difference in MBN between CK and LD treatments. In addition, the soil MBN content in *Liquidambar formosana* was higher than in both *Pinus massonianna* and *Cryptomeria japonica* (Figure 5a). Soil acid protease and urease activities were significantly affected by leaf insect herbivory but not based on tree species (Table 1; Figure 5b). Soil urease activity in LH treatment was higher than that in both CK and LD treatments (Figure 5b). There was an interactive effect of insect foliar herbivory and tree species on soil acid protease activity (Table 1), with the higher acid protease activities in both LH and LD treatments compared to CK treatment in *Cinnamomum camphora*, *Liquidambar formosana*, and *Pinus massonianna*, but not for *Cryptomeria japonica* (Figure 5c).

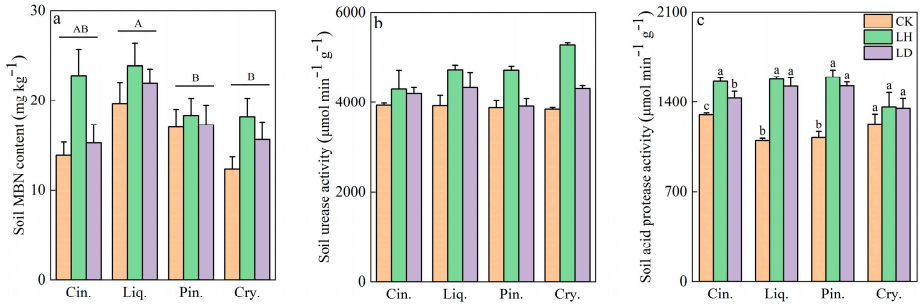

Figure 5. Effects of insect foliar herbivory on soil MBN content (**a**), soil acid protease activity (**b**), and soil urease activity (**c**) in four tree species. CK, control treatment; LH, leaf herbivory; LD, artificial defoliation. Cin.: *Cinnamomum camphora*, Liq.: *Liquidambar formosana*, Pin.: *Pinus massonianna*, Cry.: *Cryptomeria japonica*. Different uppercase letters indicate significant differences between tree species at the level of $p < 0.05$. Different lowercase letters indicate significant differences between insect foliar herbivory treatments in each tree species at the level of $p < 0.05$.

3.4. Relationships Between Soil N_2O Emission and Soil Available N

Soil N_2O emission flux showed significantly positive correlations with soil NO_3^-–N and DON and MBN contents and soil acid protease activity but was negatively correlated

with soil pH (Figure 6a). In addition, soil acid protease activity was positively correlated with soil DON, NO_3^-–N, and MBN contents, and soil urease activity had a positive correlation with soil NH_4^+-N content. Furthermore, a random forest analysis showed that the change in soil N_2O emission flux was mainly explained by soil NO_3^-–N content and followed by soil NH_4^+–N, pH, and MBN content (Figure 6b).

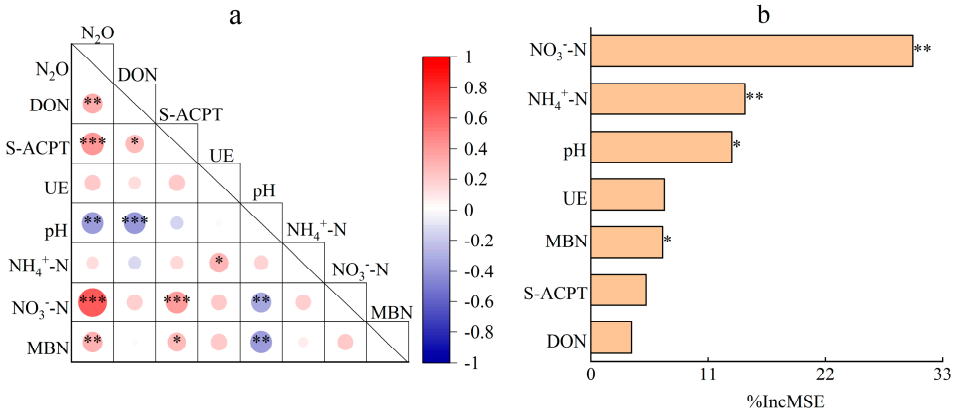

Figure 6. Correlations of soil N_2O emission flux and soil available N indicators (**a**) and relative factor importance (**b**). S-ACPT: acid protease activity, UE: urease activity. *, **, and *** represent significance levels of $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

4. Discussion

4.1. Effect of Insect Foliar Herbivory on Soil Available N Contents and Soil Enzyme Activities

Our results showed that insect foliar herbivory increased soil MBN content and enzymatic activities, which is consistent with several previous studies [34]. This result supported our first hypothesis. Insect foliar herbivory can induce the compensatory response of photosynthesis rate and aboveground growth, synthesizing more carbon substances that are transported to belowground roots [35]. One of the previous studies reported that insect foliar herbivory shortened the lifespan of fine roots and accelerated root turnover [36], increasing nutrient supplies to soil microorganisms [37]. In addition, several previous studies suggested that insect foliar herbivory stimulates plants to allocate more photosynthesized carbon to root exudates, serving as energy sources for microorganisms and thereby enhancing extracellular enzyme activities [38]. Consequently, we observed that leaf insect herbivory significantly increased soil inorganic and organic N (i.e., NH_4^+–N, NO_3^-–N, and DON) contents as compared to the control treatment. Moreover, plant leaf biomass and canopy transpiration were reduced by insect foliar herbivory [18], resulting in increases in soil temperature and moisture that improve soil N mineralization. In addition, leaf insect herbivory increased the number of soil nematodes that hindered root growth and reduced plant N uptake [39]. However, some studies found that insect foliar herbivory increased mycorrhizal infestation of plant roots [40] and plant N-transport protein genes [41], leading to increases in plant N uptake and decline in soil mineral N contents. In this present study, we observed the lack of interaction between leaf insect herbivory and tree species in affecting soil available N content, implying the consistent declined effects of insect foliar herbivory on soil available N in subtropical forests. We only conducted insect foliar herbivory in four tree seedlings, and the small-scale nature of the pot experiment might limit the complex responses of adult trees to insect foliar herbivory in natural forests.

It is interesting that we observed higher values of soil NO_3^--N and MBN content and urease activity in LH than in LD treatment. Moreover, soil inorganic N (i.e., NH_4^+–N, NO_3^-–N), DON, and MBN contents did not differ between CK and LD treatments, except

for a higher acid protease activity in LD than in CK treatment. Our results indicated that insect herbivory had greater impacts on soil available N contents in subtropical forests, compared to artificial defoliation. Insects feeding on leaves not only caused the physical damage of plant leaves, but insect oral secretions can also induce the chemical defense of plants [42]. Moreover, one previous study suggested that the effects of artificial defoliation were generally short term, whereas herbivore damage could induce a long-term and delayed effect [43]. Though the artificial defoliation method has been widely used to simulate insect foliar herbivory in several previous studies [5,40,43], our results indicated that the impacts of insect foliar herbivory on the soil nitrogen cycle might be underestimated when leaves are only artificially cut off.

4.2. Effect of Insect Herbivory on Soil N_2O Emissions

Soil nitrous oxide, as the product of multiple nitrogen cycling processes, was influenced by many environmental factors, such as soil available nitrogen and soil pH [44]. Our study found that soil N_2O emission flux was significantly increased by insect foliar herbivory in *Cinnamomum camphora* and *Pinus massonianna*. There were several mechanisms that might explain the increase in soil N_2O emission. Firstly, insect foliar herbivory increased soil NH_4^+–N and NO_3^-–N contents (Figure 4a,b), directly improving soil nitrification and denitrification that release N_2O gas [45]. Secondly, insect foliar herbivory increased MBN and N-converting enzyme (i.e., acid protease and urease) activities (Figure 5b,c), which increased the functional gene abundances of soil nitrification and denitrification [46]. In this present study, our results suggest that the enhanced soil N_2O emission flux is related to the increasing soil N availability induced by insect foliar herbivory. In addition, soil acidification can inhibit the conversion of N_2O to N_2 [47]. In this present study, soil pH was a key explaining factor and negatively correlated with soil N_2O emission flux, implying that the increase in soil N_2O emission in insect foliar herbivory treatment may be caused by the slight soil acidification. In contrast, soil N_2O emission flux was not altered by foliar herbivory in either *Cryptomeria fortunei* or *Liquidambar formosana*. Our results indicate that the impacts of insect foliar herbivory on soil N_2O emission vary among tree species, which supported our second hypothesis. The effect of foliar herbivory on soil acid protease activity differed among tree species, which might account for the inconsistent response of soil N_2O emission among tree species [48,49].

In addition, our study found that tree species had significant influences on soil available N contents and N_2O emission. Soil N_2O emission were generally higher in broad-leaved trees (i.e., *Cinnamomum camphora* and *Liquidambar formosan*), compared to both *Pinus massonianna* and *Cryptomeria japonica*. Though soil urease and acid protease activities were not significantly different among four tree species, the higher soil N_2O emission in broad-leaved trees might be attributed to the higher DON and MBN. Generally, the lifespan of broad-leaved tree fine roots was shorter than for conifer trees [50]. Therefore, the greater input of dead root residues and faster decomposition can provide more sources for microorganisms, resulting in the increases in soil available N and N_2O emissions. On the other hand, we found that soil pH was significantly lower in broad-leaved trees than in conifer trees, accounting for soil N_2O emission. Considering the warming potential effect of N_2O gas, the management of forest pest control is necessary to sequester global warming. In this study, we only compared the responses of four tree species to insect foliar herbivory, but we need to pay more attention to reach a conclusion about the varied responses among subtropical tree species.

5. Conclusions

Our study clearly demonstrated that insect foliar herbivory increased soil available nitrogen and thereby stimulated soil N_2O emission. Moreover, our results indicated that the chemically induced defense by insect foliar herbivory had more serious impacts on soil nitrogen cycles compared to leaf physical damage. In addition, our results suggested that the impacts of insect foliar herbivory on soil N_2O emission differed among tree species. In this study, the short-term responses of tree seedlings to insect foliar herbivory were conducted in a pot experiment, and field-based studies are needed to compare the impacts of the long-term foliar herbivory on soil N_2O emission and the potential changes in soil microbial communities in forest ecosystems.

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Conflicts of Interest: The authors declare that there are no competing interests.

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