Nitrogen and Biochar Addition Affected Plant Traits and Nitrous Oxide Emission From Cinnamomum camphora

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Atmospheric nitrous oxide (N₂O) increase contributes substantially to global climate change due to its large global warming potential. Soil N₂O emissions have been widely studied, but plants have so far been ignored, even though they are known as an important source of N₂O. The specific objectives of this study are to (1) reveal the effects of nitrogen and biochar addition on plant functional traits and N₂O emission of C. camphora seedlings; (2) find out the possible leaf traits affecting plant N₂O emissions. The effects of nitrogen and biochar on plant functional traits and N₂O emissions from plants using C. camphora seedlings were investigated. Plant N₂O emissions, growth, each organ biomass, each organ nutrient allocation, gas exchange parameters, and chlorophyll fluorescence parameters of C. camphora seedlings were measured. Further investigation of the relationships between plant N₂O emission and leaf traits was performed by simple linear regression analysis, principal component analysis (PCA), and structural equation model (SEM). It was found that nitrogen addition profoundly increased cumulative plant N₂O emissions (+109.25%), which contributed substantially to the atmosphere's N₂O budget in forest ecosystems. Plant N₂O emissions had a strong correlation to leaf traits (leaf TN, PN, Gn, Tr, WUEL, α, ETRmax, Ik, Fv/Fm, Y(II), and SPAD). Structural equation modelling revealed that leaf TN, leaf TP, PN, Ci, Tr, WUEL, α, ETRmax, and Ik were key traits regulating the effects of plants on N₂O emissions. These results provide a direction for understanding the mechanism of N₂O emission from plants and provide a theoretical basis for formulating corresponding emission reduction schemes.

Keywords: nitrogen addition, leaf traits, plant N₂O emissions, photosynthesis, structural equation model

INTRODUCTION

Nitrous oxide (N₂O) is a potent greenhouse gas with a sustained-flux global warming potentials (SGWPs) 270 times greater than that of carbon dioxide (CO₂) over a 100-year scale (Neubauer and Megonigal, 2015). N₂O is also the dominant ozone depleting substance (Ravishankara et al., 2009; Wu et al., 2021).
Soils are considered the major source of N$_2$O in forest ecosystems (Chapuis-Lardy et al., 2007). So far, the N$_2$O emission between forest and atmosphere is only based on the calculated N$_2$O exchange between soil and atmosphere (Butterbach-Bahl et al., 1997). However, plants have been shown to be involved in N$_2$O emissions from soil–plant systems (Chen et al., 1999; Muller, 2003).

Soil microorganisms and plants are two possible sources of N$_2$O emissions from plants. One idea is that N$_2$O emitted by plants is produced by soil microbes. Plants can transport N$_2$O produced by soil to stems and leaves and emit it into the atmosphere (Rusch and Rennenberg, 1998; Pihlatie et al., 2005; Machacova et al., 2013; Diaz-Pines et al., 2016). Another view is that plants produce and emit nitrous oxide, which produce N$_2$O during N assimilation processes (Smart and Bloom, 2001). However, the exact mechanism of N$_2$O production in plants remains unclear (Goshima et al., 1999; Lenhart et al., 2019).

Methods such as isotopic studies can provide more support for distinguishing the source of nitrous oxide. Based on stable isotope measurement research methods, studies have shown that the dual isotopocule fingerprint of N$_2$O released by plants is different from that of all known microbial or chemical processes, indicating that N$_2$O released by plants is produced in plant cells (Lenhart et al., 2019). The findings in field conditions challenge the idea that plants may transport N$_2$O produced by soil microbes. The site preference (SP) results of soil and plant N$_2$O emissions showed that plant cells N$_2$O release contributed to total N$_2$O emissions under field conditions (Timilsina et al., 2022). Therefore, attention should be paid to the plant N$_2$O emissions in forest ecosystems. Understanding the contribution of plants to total N$_2$O emissions is also crucial to accurately estimating the global N$_2$O budget and identifying possible mitigation options.

China produces about 20 million dead pigs every year, and this number is still rising every year (He et al., 2018). However, there is a lack of research concerning biochar made by dead animals. Pyrolysis of pig carcasses into biochar is an efficient and environmentally friendly option for waste disposal (Yang et al., 2005), while its effects on plant N, P content and physiological activity in leaves remain unclear (Machacova et al., 2019). As mentioned above, existing studies have greatly advanced our understanding of the correlation between N content, P content and physiological activity in leaves. However, specific studies on the relationship between plant N$_2$O emissions and these leaf traits under nitrogen and biochar addition are still lacking.

In this study, we used the closed box method and C. camphora seedlings to monitor the changes in plant N$_2$O emissions as affected by N and biochar addition. In addition, we also investigated the deep relationship between plant N$_2$O emissions and the functional traits of C. camphora seedlings. The objectives of this study were to:

1) Study nitrogen and dead pig-derived biochar effects on plant N$_2$O emissions of C. camphora; 2) explore plant traits and physiological parameters that influence plant N$_2$O emissions.

**MATERIALS AND METHODS**

**Pot Experiment Design**

This study was conducted in Jiangxi Agricultural University, Jiangxi, China (28$^\circ$46'05.0"N, 115$^\circ$50'22.9"E) from November 2017 (seeds collection) to November 2018 (seedling harvest). According to the Chinese classification system of Quaternary Red Clay, the soil in the pot experiment is classified as typical red soil. Seeds of C. camphora were planted in January 2018. After seeds germinated in early April, C. camphora seedlings were transplanted into a plastic pot filled with 2 kg soil passed through a 2 mm sieve. At the end of July 2018 (growth period for C. camphora), seedlings of the same size for experimentation were selected. The growth period of C. camphora in the experiment was consistent with that in this area. In this pot experiment, eight treatments with four replicates were carried out.

A full factorial randomized design with four N (N$_0$, 0 mg N kg$^{-1}$ dry soil; N$_1$, 100 mg N kg$^{-1}$ dry soil; N$_2$, 200 mg N kg$^{-1}$ dry soil; and N$_3$, 300 mg N kg$^{-1}$ dry soil) and two pig carcass biochar levels (BC$_0$, control; and BC$_1$, 1% pig biochar, w/w) (Chen et al., 2020) was employed. Nitrogen addition was performed by spraying the same volume of urea [CO(NH$_2$)$_2$] solution (1 g N L$^{-1}$, 2 g N L$^{-1}$ and 3 g N L$^{-1}$) and was applied twice on August 2 and September 1, 2018. The biochar was derived from pig carcasses (Huzhou Industrial and Medical Waste Treatment Center, Zhejiang, China) and was ground to
pass through a 2 mm sieve before application. Biochar was dissolved in water and applied to the soil of *C. camphora* seedlings on 2 August 2018. Both soil and biochar characteristics are shown in Supplementary Table 1. See Table 1 for a list of measured *C. camphora* plant traits with their abbreviations and units.

**Measurement of Plant N₂O Emissions**

Plant N₂O fluxes were measured by using a closed transparent chamber (diameter × height = 17 cm × 80 cm) (Figure 1). The sampling chamber was a cylinder made of PVC tube. On the top of the cylinder, there was a small hole, into which a thermometer with a rubber plug was placed. Then they inserted a rubber hose in the middle of the barrel, which was connected by a three-way valve on the outside of the hose (Figure 1). Aluminum foam was used to cover the outer surface to reduce temperature change during sampling. Before gas collection, distilled water was injected into the collar groove with a syringe for airtight sealing (Figure 1). Overall, this experiment consisted of 32 pots with four replicates and 14 times measurements of plant N₂O emissions (4N × 2 biochar × 4 replicates × 14 times). When plant N₂O emissions were measured, soil was wrapped by plastic bag (Figure 1). During the gas collection, the chamber was sealed to ensure it was airtight. A 60 ml syringe was used to collect gas samples at 0, 10, 20, and 30 min after the chambers were closed. Gas samples were immediately transferred into a 100 mL aluminum foil gas bag. Gas samples were collected between 9:00 and 11:00 (China Standard Time). The collected gas samples were immediately taken back to the laboratory for concentration determination. N₂O concentrations were determined within 24 h after sampling using a gas chromatograph (Agilent 7890B, Santa Clara, CA, United States) equipped with an electron capture detector (ECD). Because most of the plant N₂O emissions are emitted by leaves (Li and Chen, 1993), the plant N₂O emissions of *C. camphora* were calculated based on leaf area. After each gas collection, leaf area was measured by a hand-held laser blade area meter (CID, CI-203, America). The leaf area was measured 14 times. The plant N₂O emissions were shown as µg m⁻² leaves h⁻¹ (Pihlatie et al., 2005). Plant nitrous oxide fluxes (F, µg m⁻² leaves h⁻¹) were calculated by the following equation (Bowatte et al., 2014):

\[
F = P \times V \times \frac{\Delta c}{\Delta t} \times \frac{1}{RT} \times M \times \frac{1}{s} 
\]  

(1)

where \(P\) is the standard atmospheric pressure (Pa); \(V\) and \(S\) are the cylindrical chamber volume \((m^3)\) and seeding leaf area \((m^2)\); \(\Delta c/\Delta t\) means the rate of N₂O (ppb) concentration change with time based on linear regressions; \(R\) indicates the universal gas constant; \(T\) is the absolute air temperature (K) when the gas sample was aspirated.

Cumulative plant N₂O emissions \((E, \mu g m^{-2})\) were calculated by Abalos et al. (2018):

\[
E = \sum_{i=1}^{n} \frac{(F_i + F_{i+1})}{2} \times (t_{i+1} - t_i) \times 24
\]

where \(E\) is the cumulative plant N₂O emissions \((\mu g m^{-2} leaves\); \(F\) and \(i\) are the plant N₂O emission rates \((\mu g m^{-2} leaves h^{-1})\) and \(i\) gas collection, respectively; \((t_{i+1} - t_i)\) refers to the interval time of two gas collection; \(n\) is the total number of gas collection times. Fluxes of N₂O were measured 14 times from 22 July 2018 to 17 November 2018 at days 1, 11, 13, 16, 19, 35, 43, 46, 49, 57, 74, 88, 106, 119. During the experiment, *C. camphora* seedlings were regularly irrigated with equal amounts of distilled water. During the gas collection periods, *C. camphora* seedlings were irrigated 3 days in advance.

**Measurement of Leaf Gas Exchange and Chlorophyll Fluorescence Parameters**

Before harvesting, the gas exchange parameters, chlorophyll fluorescence parameters, and relative chlorophyll content of seedlings were measured. In this pot experiment, 32 pots were carried out for measurement. The net photosynthetic rate \(P_n\) (µmol CO₂ m⁻² s⁻¹), stomatal conductance \(G_s\) (mol H₂O m⁻² s⁻¹), intercellular CO₂ concentration \(C_i\) (µmol CO₂ mol⁻¹) and transpiration rate \(Tr\) (mmol H₂O m⁻² s⁻¹) of the fourth fully expanded leaf were measured by a LI-6400XT photosynthesis system (LI-COR, Lincoln, NE, United States) between 8:30 and 11:30 a.m. on a sunny day. For each *C. camphora* seedling, three fully expanded leaves were selected for the photosynthetic measurement. The parameters were set as follows: the measured light was 1,000 µmol m⁻² s⁻¹, the CO₂ concentration in the leaf chamber was 400 µmol mol⁻¹, the temperature in the leaf chamber was 25°C, and the air flow rate was 500 ml min⁻¹ (Gong et al., 2022). Leaf instantaneous water use efficiency WUE₂ (µmol CO₂ mmol H₂O⁻¹) was calculated as follows: WUEL = \(P_n/Tr\) (Fang et al., 2018).

For each *C. camphora* seedling, three representative leaves were selected for the chlorophyll fluorescence measurement. Chlorophyll fluorescence parameters of representative leaves were measured by a Pulse-Amplitude-Modulation (PAM) fluorometer (PAM 2500, Walz GmbH, Nuremberg, Germany). The minimum \((F_o)\) and maximum \((F_m)\) fluorescence were recorded after sufficient dark adaptation (at least 30 min) of the sample with a dark adaptation clip. The leaves were given saturated pulsed light \((2,000 \mu mol m^{-2} s^{-1})\) for 3 s and actinic light \((300 \mu mol m^{-2} s^{-1})\) for 10 min. The chlorophyll fluorescence peak was recorded as \(F_{m'}\), and before the saturation pulse was turned off, the recorded fluorescence value was \(F\). The maximum quantum yield of photosystem II \(F_{v'/f_{m'}} = (F_{m'} - F_0)/F_{m'}\). The effective quantum yield of photosystem II, \(Y(II)=\Delta F/F_{m'} = (F_{m'}-F)/F_{m'}\) (Kitajima and Butler, 1975; Genty et al., 1989). At night, after the samples had passed sufficient dark adaptation, the actinic light with light intensities of 2, 6, 31, 101, 141, 271, 474, 785, 1,160, and 1,663 µmol m⁻² s⁻¹ was turned on in turn. The irradiation time of actinic light for each intensity was 10 s. Using the Pam Win-3 software, the fast light response curve was fitted with the formula (Eilers and Peeters, 1988). \(\alpha\), initial slope of fast light curve \((electrons photons^{-1}); ETR_{max}\), potential maximum relative electron transfer rate \((\mu mol m^{-2} s^{-1}); I_k\), half full
TABLE 1 | List of Cinnamomum camphora plant traits measured, their abbreviations and units.

| Abbreviation | Plant trait                   | Unit     |
|--------------|-------------------------------|----------|
| PH           | Plant height                  | cm       |
| GD           | Ground diameter               | mm       |
| LN           | Leaf number                   |          |
| LM           | Leaf mass                     | g        |
| SM           | Stem mass                     | g        |
| RM           | Root mass                     | g        |
| TM           | Total mass                    | g        |
| R:S          | Root shoot ratio              |          |
| Leaf TN      | Leaf total nitrogen content   | g kg⁻¹   |
| Leaf TP      | Leaf total phosphorus content | g kg⁻¹   |
| Leaf TK      | Leaf total potassium content  | g kg⁻¹   |
| Stem TN      | Stem total nitrogen content   | g kg⁻¹   |
| Stem TP      | Stem total phosphorus content | g kg⁻¹   |
| Stem TK      | Stem total potassium content  | g kg⁻¹   |
| Root TN      | Root total nitrogen content   | g kg⁻¹   |
| Root TP      | Root total phosphorus content | g kg⁻¹   |
| Root TK      | Root total potassium content  | g kg⁻¹   |
| $P_n$        | Net photosynthetic rate       | μmol CO₂ m⁻² s⁻¹ |
| $G_s$        | Stomatal conductance          | mol H₂O m⁻² s⁻¹ |
| $C_i$        | Intercellular CO₂ concentration |       |
| $Tr$         | Transpiration rate            | μmol H₂O m⁻² s⁻¹ |
| WUE_L        | Leaf instantaneous water use efficiency |       |
| $\alpha$     | The initial slope of the fast light response curve | Electrons photons⁻¹ |
| $ETR_{max}$  | Potential maximum relative electron transfer rate | μmol m⁻² s⁻¹ |
| $I_h$        | Half full and light intensity | μmol m⁻² s⁻¹ |
| $F_v/F_m$    | Maximum quantum yield of photosystem II |       |
| Y(II)        | Effective quantum yield of photosystem II |       |
| SPAD         | Relative chlorophyll content |          |

and light intensity (μmol m⁻² s⁻¹) (Ralph and Gademann, 2005). The SPAD-502 Plus (Konicaminolta, Japan) was used to determine the relative chlorophyll content (SPAD). For each C. camphora seedling, four fully expanded leaves were selected for measurement.

Measurement of Seedling Biomass and Nutrient
At the end of the study, whole plant seedlings were harvested in November 2018. In this pot experiment, 32 C. camphora seedlings were harvested. All seedlings were gently processed by removing soil. Then seedling height, ground diameter, leaf number, and leaf area were measured and recorded. The leaves, stems, and roots of seedlings were cleaned with pure water and dried to a constant weight, which was weighed separately. The aboveground mass (leaf mass+stem mass), underground mass (root mass), total mass (underground mass+aboveground mass) and root-shoot ratio (R: S = underground mass: aboveground mass) could be obtained by calculation (Zhang et al., 2013; Deng et al., 2019). After the above data were recorded, the roots, stems and leaves of seedlings were crushed to pass through 0.149 mm sieve for determination of N, P, and K. The total phosphorus content was determined by molybdenum blue colorimetry, the total nitrogen content was determined by indophenol blue colorimetry after removing part of the digestion liquid and adjusting to neutral pH, and the total potassium in the digestion liquid was determined by flame photometry (Chen et al., 2022).

Statistical Analyses
Analysis of variance (ANOVA) was used to examine the dependence of plant N₂O emission rate and leaf area as affected by N and biochar and their interactions as fixed effects and sampling time as random effects (Xu et al., 2020). Two-way ANOVA was used to examine the dependence of cumulative plant N₂O emissions and plant trait parameters on N, biochar, and their interactions. Tukey post-hoc tests were used to examine differences among means with significant results. Pearson correlation analysis to explore the linear relationship between leaf traits and plant N₂O emissions (Chen et al., 2015). Origin 2021 was used to conduct principal component analysis (PCA) to analyze leaf traits and determine the main functional traits of N₂O emission in plants (Li et al., 2022). AMOS 26.0 (IBM Corp, Armonk, NY, United States) was employed to perform structural equation modelling (SEM) to detect the influences among the
variables. The model was constructed using our hypotheses about plant functional traits affecting plant N\textsubscript{2}O emissions. The quality of the SEM model was assessed by using the chi-square goodness-of-fit statistic $\chi^2/df$, Akaike's information criterion (AIC), the Bayesian information criteria (BIC), the root mean square error of approximation value (RMSEA), the comparative fit index (CFI), and the standardized root mean square residual (SRMR) (Grace, 2006). We used JMP 9.0 (Cary, NC, United States) for data analysis.

RESULTS

Plant Traits as Effected by N and Biochar Addition

Plant height, ground diameter, and leaf number differed significantly among different levels of N and between two different levels of biochar addition ($P < 0.01$; Table 2). The same was true for leaf mass, stem mass, root mass, and total mass. These differences could be ascribed to the absorption and utilization of different nutrients by C. camphora. Plant height, ground diameter, leaf number, and each organ mass showed an increasing trend with the addition of N supplemental level regardless of adding biochar or not. However, root shoot ratio had no significant difference among different levels of N and between two different levels of biochar addition (Supplementary Figure 1).

Leaf TN, stem TN, and root TN differed significantly between levels of N addition ($P < 0.0001$; Table 3). Leaf, stem and root TN content of all treatments ranged from 11.73 g kg\textsuperscript{−1} to 17.70 g kg\textsuperscript{−1}, 7.84 g kg\textsuperscript{−1} to 14.84 g kg\textsuperscript{−1}, and 9.76 g kg\textsuperscript{−1} to 14.85 g kg\textsuperscript{−1} (Supplementary Figure 2). Leaf TP, TK, stem TP, TK, root TP and TK differed significantly between levels of biochar addition ($P < 0.01$; Table 3). Compared with control, biochar increased TP content in leaves and roots (+20.63 and +14.62%), respectively ($P < 0.05$; Supplementary Figure 2). Biochar increased TK content in leaves, stems, and roots (+19.78, 32.44, 33.00%), respectively ($P < 0.05$; Supplementary Figure 2).

Gas exchange parameters and chlorophyll fluorescence parameters had significant responses to nitrogen addition. At the same time, biochar affects most physiological indicators ($P < 0.01$; Table 4). The mean values of $P_n$, $G$, $Tr$, and $WUE_L$ became larger as N addition increased (Supplementary Figure 3). Nitrogen and biochar had significant interaction on $P_n$ and $Tr$ ($P < 0.001$; Table 4 and Supplementary Figure 3). For all nitrogen and biochar levels, the maximum mean values of $P_n$ and $Tr$ were 7.82 \(\mu\text{mol CO}_2 \text{m}^{-2} \text{s}^{-1}\) and 1.99 mmol H\textsubscript{2}O m\textsuperscript{−2} s\textsuperscript{−1} as compared to control (Supplementary Figure 3). $\alpha$, $ETR_{\text{max}}$, $I_k$, $Fv/Fm$, $Y(II)$, and SPAD differed significantly among different levels in N and between two different levels in biochar addition ($P < 0.05$; Table 4 and Supplementary Figure 4). The maximum mean values of $\alpha$, $ETR_{\text{max}}$, $I_k$, $Fv/Fm$, $Y(II)$, and SPAD are 0.18 electrons photons\textsuperscript{−1}, 35.08 \(\mu\text{mol m}^{-2} \text{s}^{-1}\), 199.90 \(\mu\text{mol m}^{-2} \text{s}^{-1}\), 0.84, 0.36, and 42.73 (Supplementary Figure 4).

Relationships of Plant N\textsubscript{2}O Emissions and Leaf Traits

Plant N\textsubscript{2}O emissions were significantly affected by N addition and increased with N addition levels (Table 4 and Figure 2). No significant differences in cumulative plant N\textsubscript{2}O emissions were recorded between biochar and control conditions (Figure 2). In addition, the leaf area of C. camphora seedlings was also increased by N and biochar during the study based on the dynamics (Supplementary Figure 5). Plant N\textsubscript{2}O emission rates were significantly affected by N addition and increased with N addition levels (Supplementary Figure 6). However, the plant N\textsubscript{2}O emission rates had no significant difference between the two different levels of biochar addition (Supplementary Figure 6).
**TABLE 2** | Dependence of plant growth and biomass on N (0, 100, 200, and 300 mg N kg$^{-1}$ dry soil) and biochar addition (control and biochar addition) and their interactions in two-way ANOVAs.

| Variables         | Nitrogen | Biochar | Nitrogen × Biochar |
|-------------------|----------|---------|--------------------|
|                   | DF | F   | P     | DF | F   | P     | DF | F   | P     |
| Plant height      | 3 | 41.5 | <0.0001 | 1 | 25.6 | <0.0001 | 3 | 1.6 | 0.229 |
| Ground diameter   | 3 | 93.8 | <0.0001 | 1 | 55.1 | <0.0001 | 3 | 0.8 | 0.494 |
| Leaf number       | 3 | 50.0 | <0.0001 | 1 | 18.4 | 0.0003 | 3 | 1.6 | 0.231 |
| Leaf mass         | 3 | 57.0 | <0.0001 | 1 | 7.9  | 0.011  | 3 | 0.61| 0.617 |
| Stem mass         | 3 | 55.5 | <0.0001 | 1 | 17.3 | 0.0004 | 3 | 1.0 | 0.401 |
| Root mass         | 3 | 100.2| <0.0001 | 1 | 15.2 | 0.001  | 3 | 0.2 | 0.897 |
| Total mass        | 3 | 136.2| <0.0001 | 1 | 26.0 | <0.0001 | 3 | 0.9 | 0.463 |
| Root shoot ratio  | 3 | 0.8  | 0.52   | 1 | 0.03 | 0.867  | 3 | 0.4 | 0.761 |

Significant results are shown in bold.

**TABLE 3** | Dependence of TN, TP, and TK in leaf, stem and root on N (0,100,200 and 300 mg N kg$^{-1}$ dry soil) and biochar addition (control and biochar addition) and their interactions in two-way ANOVAs.

| Variables         | Nitrogen | Biochar | Nitrogen × Biochar |
|-------------------|----------|---------|--------------------|
|                   | DF | F   | P     | DF | F   | P     | DF | F   | P     |
| Leaf TN           | 3 | 29.1 | <0.0001 | 1 | 0.03 | 0.854  | 3 | 1.3 | 0.287 |
| Leaf TP           | 3 | 0.01 | 0.998 | 1 | 26.2 | <0.0001 | 3 | 0.4 | 0.742 |
| Leaf TK           | 3 | 0.5  | 0.686 | 1 | 14.1 | 0.002  | 3 | 1.7 | 0.226 |
| Stem TN           | 3 | 85.8 | <0.0001 | 1 | 0.2  | 0.686  | 3 | 1.1 | 0.375 |
| Stem TP           | 3 | 0.1  | 0.976 | 1 | 5.3  | 0.03   | 3 | 0.3 | 0.793 |
| Stem TK           | 3 | 0.1  | 0.964 | 1 | 37.0 | <0.0001 | 3 | 1.9 | 0.162 |
| Root TN           | 3 | 17.6 | <0.0001 | 1 | 0.5  | 0.508  | 3 | 0.3 | 0.811 |
| Root TP           | 3 | 1.4  | 0.271 | 1 | 21.6 | 0.0001 | 3 | 1.7 | 0.187 |
| Root TK           | 3 | 1.8  | 0.181 | 1 | 353.0| 0.0001 | 3 | 2.9 | 0.056 |

Significant results are shown in bold. TN, total nitrogen; TP, total phosphorus; TK, total potassium.

**TABLE 4** | Dependence of plant physiological parameters and plant N$_2$O emissions on N (0, 100, 200, and 300 mg N kg$^{-1}$ dry soil) and biochar addition (control and biochar addition) and their interactions in two-way ANOVAs.

| Variables         | Nitrogen | Biochar | Nitrogen × Biochar |
|-------------------|----------|---------|--------------------|
|                   | DF | F   | P     | DF | F   | P     | DF | F   | P     |
| $P_n$             | 3 | 238.2| <0.0001 | 1 | 59.0 | <0.0001 | 3 | 9.2 | 0.0003 |
| $G_s$             | 3 | 31.3 | <0.0001 | 1 | 6.4  | 0.018  | 3 | 1.0 | 0.4    |
| $C_i$             | 3 | 5.3  | 0.006  | 1 | 0.4  | 0.528  | 3 | 2.5 | 0.087  |
| $Tr$              | 3 | 86.2 | <0.0001 | 1 | 60.2 | <0.0001 | 3 | 8.5 | 0.0005 |
| WUE$_c$           | 3 | 35.9 | <0.0001 | 1 | 0.3  | 0.615  | 3 | 2.4 | 0.09   |
| $\alpha$          | 3 | 8.5  | 0.0005 | 1 | 6.4  | 0.018  | 3 | 0.6 | 0.626  |
| ETR$_{max}$       | 3 | 15.7 | <0.0001 | 1 | 6.5  | 0.017  | 3 | 0.05| 0.984  |
| $I_s$             | 3 | 10.2 | 0.0002 | 1 | 2.7  | 0.116  | 3 | 0.2 | 0.903  |
| Fv/Fm             | 3 | 35.9 | <0.0001 | 1 | 14.1 | 0.001  | 3 | 2.9 | 0.055  |
| Y(II)             | 3 | 25.5 | <0.0001 | 1 | 4.1  | 0.054  | 3 | 0.5 | 0.656  |
| SPAD              | 3 | 48.0 | <0.0001 | 1 | 8.8  | 0.007  | 3 | 1.0 | 0.397  |
| C-plant N$_2$O    | 3 | 255.2| <0.0001 | 1 | 0.9  | 0.353  | 3 | 1.9 | 0.151  |

Significant results are shown in bold. $P_n$, net photosynthetic rate; $G_s$, stomatal conductance; $C_i$, intercellular CO$_2$ concentration; $Tr$, transpiration rate; WUE$_c$, leaf instantaneous water use efficiency; $\alpha$, the initial slope of the fast light response curve; ETR$_{max}$, potential maximum relative electron transfer rate; $I_s$, half sat light intensity; Fv/Fm, maximum quantum yield of photosystem II; Y(II), effective quantum yield of photosystem II; C-plant N$_2$O, cumulative plant N$_2$O emissions.
Plant N₂O emissions were positively correlated with leaf mass (Figure 3, $R^2 = 0.74, P < 0.0001$), leaf TN ($R^2 = 0.69, P < 0.0001$), total mass ($R^2 = 0.80, P < 0.0001$), leaf area ($R^2 = 0.76, P < 0.0001$), $P_n$ ($R^2 = 0.75, P < 0.0001$), $G_s$ ($R^2 = 0.63, P < 0.0001$), $Tr$ ($R^2 = 0.61, P < 0.0001$), WUE$_l$ ($R^2 = 0.67, P < 0.0001$), $\alpha$ ($R^2 = 0.34, P = 0.0005$), $ETR_{max}$ ($R^2 = 0.49, P < 0.0001$), $I_k$ ($R^2 = 0.44, P < 0.0001$), $Fv/Fm$ ($R^2 = 0.59, P < 0.0001$), $Y(II)$ ($R^2 = 0.61, P < 0.0001$), and SPAD (Figure 3, $R^2 = 0.74, P < 0.0001$). However, it was negatively correlated with $C_i$ (Figure 3, $R^2 = 0.28, P = 0.0018$).

To further clarify the relationship between leaf traits and plant N₂O emissions, a principal component analysis (PCA) was performed using leaf area, leaf biomass, leaf TN, leaf TP, and physiological indicators (Figure 4). PC1 and PC2 accounted for 68.10 and 9.30% of the investigated variation, respectively. Leaf area, LM, leaf TN, $P_n$, $G_s$, $Tr$, WUE$_l$, $\alpha$, $ETR_{max}$, $I_k$, $Fv/Fm$, $Y(II)$, and SPAD were more influenced by PC1, while leaf TP and $C_i$ were more influenced by PC2. At the same time, each treatment has a good degree of differentiation.

Relationships Among Functional Traits and Mechanisms Linking Leaf Traits and Plant N₂O Emissions

The results are exhibited in Figure 5. As for the model fit indices, which are shown in the lower right, all indices indicate that our hypothesized model was acceptable [$X^2/df = 7.586 (P > 0.001)$, AIC = 636.604; BIC = 708.425; CFI = 0.461; RMSEA = 0.461; SRMR = 0.244]. Additionally, the modification indices were low, indicating that our model could not be further improved by adding omitted relationships.

The results revealed that leaf traits do affect plant N₂O emissions in a direct or indirect way. As a multi-staged path model, we could read from the graph that leaf TN significantly affected $P_n$, $G_s$, $C_i$, $Tr$, WUE$_l$, $\alpha$, $ETR_{max}$, $I_k$, $Fv/Fm$, $Y(II)$. Among them, Leaf TN would significantly increase the $P_n$, $G_s$, $Tr$, WUE$_l$, $\alpha$, $ETR_{max}$, $I_k$, $Fv/Fm$, $Y(II)$, while significantly decreasing the $C_i$. For Leaf TP, it has a significant influence on $P_n$, $Tr$, $\alpha$, $ETR_{max}$, and $Fv/Fm$. Leaf TP would significantly increase the above indicators. $P_n$, $C_i$, $Tr$, WUE$_l$, $\alpha$, $ETR_{max}$, and $I_k$ all had a significant impact on leaf area. $P_n$ and $ETR_{max}$ would significantly decrease the leaf area, while the rest would significantly increase the leaf area. Leaf area influences leaf mass and cumulative plant N₂O emissions significantly and positively, while leaf mass doesn’t have a significant influence on cumulative plant N₂O emissions. The relationship between the remaining variables was not significant, but improved the model fitting.

DISCUSSION

Plant Traits and N₂O Emissions as Affected by N and Biochar Addition

Nitrogen is an essential macronutrient and plays an important role in plant growth and development (Galloway et al., 2002). Biochar has positive effects on plant growth (Dong et al., 2015; Purakayastha et al., 2019). Plant biomass was increased by animal carcass-derived biochar addition (Chen et al., 2020). These results support that N and biochar addition significantly increased the growth indexes (plant height, ground diameter, leaf number, biomass, etc.) of C. camphora seedlings (Supplementary Figure 1). In this study, both nitrogen and biochar increased leaf mass, stem mass, and root mass (Supplementary Figure 1), indicating that nitrogen and biochar’s effects on root-shoot ratio might have been offset by their effects on aboveground biomass and underground biomass. Recent studies have shown that net photosynthesis rate ($P_n$) and transpiration rate ($T_r$) increased by 19 and 40% in the biochar treatments, respectively, compared to control (Zulfiqar et al., 2021). Biochar significantly increased net photosynthetic rate, transpiration rate, stomatal conductance, and water use efficiency during the plant growth period, relative to control (Wang et al., 2021). Chlorophyll fluorescence parameters can effectively reflect the absorption, utilization, and transformation of light energy. $Fv/Fm$ stands for the maximum quantum yield of PSIII, which can reflect the potential maximum light energy conversion efficiency of plants. $Y(II)$ represents the actual photosynthetic quantum yield of PSII, which can reflect the current actual light energy conversion efficiency of photosynthetic organs (Baker, 2008). Research has shown that biochar has great potential in improving chlorophyll fluorescence (Wang et al., 2021). That’s probably because biochar has the effect of increasing the chlorophyll content of leaves (Feng et al., 2021), which can ensure the synthesis of various enzymes and electron transporters in the process of carbon assimilation in photosynthesis, thereby improving the function of leaf photosynthesis (Hou et al., 2021).

Plant N₂O emissions were affected by N but not by biochar addition (Figure 2). With the increase of N addition levels, plant N₂O emissions increased consistently (Figure 2). Since leaves have been proved to be the main place emitting N₂O, factors influencing leaf area might also impact plant N₂O emissions. In this study, both biochar and N increased leaf area (Supplementary Figure 5). Biochar had no effect on plant N₂O
emissions (Table 4, Figure 2, and Supplementary Figure 6), indicating biochar's effects on plant N₂O emissions might have been offset by its effects on N₂O production and leaf area. Indeed, plant N₂O emissions may be a phenomenon emitting N₂O that was produced by plants or emitting soil originated N₂O to atmosphere (Chang et al., 1998). Even though it was not studied here, plant production of N₂O by C. camphora seedlings could be possible since a ¹⁵N isotopic labelling study

FIGURE 3 | Scatterplot of the relationship of plant N₂O emissions versus leaf mass, leaf TN, total mass, leaf area, Pₙ, Gₛ, Cₛ, Tr, WUEₛ, α, ETRₘₐₓ, Iₛ, Fv/Fm, Y(II), and SPAD. Lines indicate significant relations.
revealed that N$_2$O released from wheat leaves originated from NO$_3$$^-$ assimilation by wheat plants instead of microorganisms in the rhizosphere (Smart and Bloom, 2001). Plant-mediated N$_2$O emissions have been reported in agricultural, wetland, and forest plants (Pihlatie et al., 2005). N$_2$O produced in soil could also be transported to atmosphere by transpiration streams of plants (Pihlatie et al., 2005). In our study, biochar mitigated soil N$_2$O emissions while increasing leaf area (unpublished...
Therefore, N₂O produced in the soil and emitted by plants could not be the important source of N₂O emissions by C. camphora seedlings. However, N could increase plant N₂O emissions by providing more N substrate readily available for plant, increasing NO₃⁻ and NO₂⁻ assimilation by plant roots and leaves, respectively, which will directly enhance plant N₂O emissions (Smart and Bloom, 2001; Pihlatie et al., 2005). The cumulative N₂O emissions of plants increase with the increase of nitrogen levels (Figure 2). Leaf nitrogen content is directly proportional to the N₂O emission of plants (Figure 3). The rate of N₂O release by plants is related to the utilization of nitrogen by plants, which may be due to the release of nitrogen absorbed by plants, especially NO₃⁻. After being reduced to NO₂⁻ by nitrate reductase (NR), part of it is further reduced to N₂O and released. NR is a substrate inducible enzyme. When nitrogen is added to the soil, plants grow well, have high NR activity, and produce more N₂O. With the increase in nitrogen levels, plants grow fast and produce a large amount of N₂O (Li and Chen, 1993). Soybean and maize seedlings can release N₂O by themselves, and the N₂O release is related to the amount of nitrogen and phosphorus application. The direct emission of N₂O by plants may be a physiological defence of plants to avoid excessive accumulation of NO₃⁻ under the restriction of other growth factors, which will lead to a decline in the nitrogen use efficiency of plants (Chen et al., 1995).

**Relationships Between Plant N₂O Emissions and Various Leaf Traits**

Our results provide powerful data for understanding the N₂O emission patterns of seedlings during their growing period (Supplementary Figure 6). Importantly, we found a close relationship between leaf traits and plant N₂O emissions (Figures 3–5). Our continuous emission measurement method is an effective improvement over the traditional indoor study because it overcomes the influence of the device during seedling growth and better reflects the temporal variation of N₂O emission flux (Supplementary Figure 6). Therefore, our method is suitable for measuring plant N₂O emissions during seedling growth and can be used to explore deeper association analysis.

Leaf photosynthetic capacity is related to leaf N concentration (Figures 4, 5). N-rich compounds [ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco)] play an important role in the biochemical fixation of carbon dioxide (Field and Mooney, 1986). The formation of N₂O in plants is a complex physiological process, including photosynthesis, nitrogen assimilation, and transpiration (Hu et al., 2021). Leaf TN, Tr, α, ETRmax, I k, Fv/Fm, Y (II), and SPAD were all positively correlated with plant N₂O emissions (Figure 3). The relationship between stomatal conductance, leaf nitrogen content, and nitrogen oxide emission rate can explain the variation of plant capacity to release atmospheric nitrogen oxides (Teklemariam and Sparks, 2006). A correlation between transpiration rates and nitrogen emissions was found in nitrogen compounds released by plants (Stutte et al., 1979). Studies have shown that transpiration rate and other physiological processes affect the transport and emission of N₂O in plants (Chang et al., 1998; Pihlatie et al., 2005; Borah and Baruah, 2016). The above research results are similar to our results. Leaf nitrogen content, stomatal conductance, and transpiration rate are significantly positively correlated with plant N₂O emission (Figure 3). Nitrite assimilation in chloroplasts can produce intermediates that react to produce N₂O. At the same time, there is a negative correlation between N₂O emission and NO₃⁻ assimilation (Dean and Harper, 1986; Hakata et al., 2003). Many scholars have discussed the possible sites, mechanisms, and enzymes involved in N₂O production in plant cells. The mitochondria of plants have a protective mechanism to increase NO scavenging. NADH might act as an electron donor to reduce cytochrome c oxidase (CcO), leading to the conversion of NO to N₂O (de Oliveira et al., 2008; Gupta et al., 2016; Timilsina et al., 2020). The phenomenon that photosynthesis is closely related to plant N₂O emission does not only appear in forest ecosystems. In aquatic ecosystems, algae contribute significantly to N₂O emissions. The green microalga Chlamydomonas reinhardtii reduces NO into N₂O using photosynthetic electron transport and is catalyzed by flavodiiron proteins. The above research provides a new mechanistic understanding of N₂O production by eukaryotic phototrophs (Burlacot et al., 2020).

Structural equation modelling revealed the process of leaf traits affecting plant N₂O emissions (Figure 5). Nitrogen and phosphorus in leaves are essential nutrients for carbon assimilation in photosynthesis, which can affect the plant’s photosynthesis (Mo et al., 2019). Photosynthesis is closely related to chlorophyll fluorescence (Mareckova et al., 2019). Leaf area was affected by photosynthesis, and plant N₂O emission was closely related to leaf area (Figure 5). The formation of plant N₂O emission is a complex physiological process closely linked with various steps, and the enzymes and pathways involved need to be further studied.

**CONCLUSION**

In conclusion, our study indicates that nitrogen and animal carcass-derived biochar addition affect the functional traits of C. camphora seedlings. Nitrogen addition substantially increases plant N₂O emissions. All seedling biomass was consistently increased by biochar addition, indicating pig carcass biochar will potentially benefit the leaf-harvesting C. camphora industry. However, while seedling leaf area was increased by biochar, plant N₂O emissions were not influenced by biochar. As an important source of atmospheric N₂O, plant N₂O emissions deserve more attention. Plant N₂O emission may be closely related to leaf TN, leaf TP, Pn, Ci, Tr, WUEi, α, ETRmax, and I k. Future studies on the mechanisms underlining N and biochar’s effects on plant N₂O emissions should be conducted, especially in plantations with intensive N fertilization practices.

**DATA AVAILABILITY STATEMENT**

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.
AUTHOR CONTRIBUTIONS
CZ, HL, and LZ conceived and designed the study. LL, KW, YL, SZ, and SH collected the samples and performed the physiological measurements. XG commented on the manuscript. CZ and HL wrote the manuscript. LZ supervised the whole work. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL
The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.905537/full#supplementary-material

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