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ORIGINAL PAPER

Cropping Systems Mediate Carbon and Nitrogen Substrate Effects on Soil Carbon Dioxide and Nitrous Oxide Emissions

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Abstract

Agricultural management practices fundamentally influence soil greenhouse gas emissions, yet the mechanisms governing these emissions across different cropping systems remain incompletely understood. This study investigated how carbon (C) substrates and nitrogen (N) additions regulate carbon dioxide (CO_2) and nitrous oxide (N_2O) emissions in soils from contrasting agricultural systems. Using a factorial design, we examined the effects of C substrates (glucose, citric acid, glutamine) and N addition (KNO_3) on greenhouse gas emissions from soils under three-year continuous wheat (*Triticum aestivum*) or lucerne (*Medicago sativa*) cultivation. Lucerne soils showed consistently higher N_2O emissions (0.42 ± 0.04 nmol m^{-2} s^{-1}) compared to wheat soils (0.35 ± 0.03 nmol m^{-2} s^{-1}). CO_2 emissions showed substrate-specific responses, with glutamine treatment yielding the highest emissions (1.35 ± 0.12 µmol m^{-2} s^{-1}), followed by citric acid (1.12 ± 0.09 µmol m^{-2} s^{-1}) and glucose (0.98 ± 0.08 µmol m^{-2} s^{-1}), all significantly exceeding the water control (0.82 ± 0.07 µmol m^{-2} s^{-1}). Structural equation modeling revealed that substrate effects were mediated through distinct pathways in each system, with iron availability and enzyme activities explaining 37% and 29% of emission variations in lucerne and wheat soils, respectively. Network analysis suggested strong correlations between N_2O emissions and soil iron fractions ($r = 0.64$–0.69) in both systems. Citric acid enhanced N_2O emissions by 31% through pH-mediated effects on denitrification, while glucose and glutamine suppressed emissions by 24% and 18%, respectively, through enhanced N immobilization. The contrasting responses between systems reflected fundamental differences in microbial resource utilization strategies, with lucerne soils showing stronger coupling between C and N cycling processes (path coefficient = 0.45). These findings suggest that greenhouse gas mitigation strategies should consider both cropping system legacy effects and substrate-specific response patterns. System-specific approaches targeting both C input quality and N availability may offer effective pathways for emission reduction in agricultural soils.

Keywords Enzymatic activities · Crop rotation · Priming effect · Organic acids

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1 Introduction

Greenhouse gas emissions, particularly carbon dioxide (CO_2) and nitrous oxide (N_2O), play a critical role in climate change and sustainable agricultural practices (Liang et al. 2023; Moinet et al. 2023). Agricultural soils, through their interactions with crop management practices, substantially influence the production and emission of these gases by altering soil organic carbon (SOC) and nitrogen (N) cycling processes (Means et al. 2022; Smith et al. 2016; Xu et al. 2023). Because SOC represents a significant global C pool and plays a key role in mediating soil fertility, understanding how different cropping systems influence SOC dynamics and their effects on greenhouse gas emissions is essential for promoting both agricultural productivity and environmental sustainability (Foley et al. 2011; Jobbágy and Jackson 2000).

Cropping systems that differ markedly in plant type, such as perennial legumes compared with annual cereals, create various soil environments, each differently impacting soil biochemical properties, microbial activities, and nutrient cycling dynamics (Means et al. 2022; Oliveira et al. 2021; Pan et al. 2025; Siddique et al. 2023; Zhang et al. 2022). For example, perennial legume systems, such as lucerne (*Medicago sativa*), can substantially modify soil organic matter inputs and microbial community structure due to high biomass production and biological N fixation (Sprunger et al. 2019; Yuan et al. 2016). In contrast, annual cereals such as winter wheat (*Triticum aestivum*) are often managed with inorganic fertilizers, resulting in distinct soil N dynamics and microbial responses (Huang et al. 2024; Raiesi 2012). Despite the known general effects of crop types on soil properties and microbial communities, explicit mechanisms by which different cropping systems regulate the response of soil greenhouse gas emissions to exogenous C and N substrates remain unclear.

The addition of exogenous C substrates can substantially influence microbial activity and alter CO_2 and N_2O emissions by providing readily available energy and nutrients. These substrates differ significantly in their chemical complexity, nutrient content, and bioavailability, factors critical in determining their impact on microbial metabolism and gas emissions (Blagodatskaya et al. 2011; Daly et al. 2024; Gunina et al. 2014). For instance, glucose, a simple carbohydrate, is rapidly utilized by microbes, potentially promoting microbial respiration and affecting greenhouse gas emissions differently from more complex organic acids or amino acids (Abinandan et al. 2025; Daly et al. 2024; Gunina et al. 2014). Additionally, N availability, either from exogenous N inputs or substrate-induced changes in microbial N dynamics, strongly influences microbial community structure and enzymatic processes governing nitrification and denitrification, key pathways responsible for N_2O production (Baggs 2011; Cai et al. 2010; Chen et al. 2024; Morley et al. 2014). While previous studies have assessed how individual substrates influence greenhouse gas emissions, comprehensive understanding of interactive effects between cropping system legacies and multiple substrate types remains limited. Furthermore, the mechanistic pathways through which soil iron fractions and enzymatic activities mediate substrate-induced emission responses remain poorly characterized.

Therefore, the primary aim of our study was to evaluate how different cropping systems (continuous lucerne and continuous wheat) influence soil CO_2 and N_2O emissions following the addition of different C substrates (glucose, citric acid, and glutamine) and N inputs, with particular focus on identifying the key soil physicochemical properties and enzymatic activities driving differential emission responses. We hypothesized that: (1) soils from lucerne systems would exhibit greater responses in CO_2 and N_2O emissions to exogenous substrates compared to wheat systems due to differences in microbial activity associated with higher background N availability and organic matter quality; and (2) additions of exogenous C substrates and N would increase soil CO_2 and N_2O emissions compared to water-only controls, with the magnitude and direction of responses varying by substrate type through their differential effects on microbial metabolism and soil biogeochemistry.

2 Materials and Methods

2.1 Field Site Characterization and Experimental Design

Soil samples were collected from a long-term experimental field station located at the National Field Scientific Observation and Research Station of Grassland Agro-Ecosystems in Gansu Qingyang, China (35°39'N, 107°51'E; elevation 1298 m). The region experiences a continental monsoon climate with a mean annual precipitation of 609 mm and a mean annual temperature of 9.9 °C (Deng et al. 2021). The soil is classified as a Heilu soil (FAO classification system), characterized by a silty loam texture (Deng et al. 2021).

The experimental plots for soil sampling comprised two cropping systems: three years continuous winter wheat (WWW) and three years continuous lucerne (LLL), conducted from 2018 to 2021. The plots, each 20 m^2, were arranged in a randomized complete block design with four replicates for each cropping systems. Management practices differed between the two cropping systems, where wheat received more frequent fertilizer applications, while both systems relied on rainfed conditions. Field management

histories differed between systems, wheat system received split applications of N fertilizer (urea, 46% N, total 150 kg N ha^{-1} yr^{-1}) at seeding and jointing stages, while the lucerne system relied on biological N fixation with periodic biomass removal through 3 cuts annually (Chu et al. 2023). Detailed information on harvests for grain and forage, along with other management practices, can be found in our previous study (Chu et al. 2023).

Soil sampling occurred in the fall of 2021 following wheat harvest and the final lucerne cut of the season. Surface residues were carefully removed, and soil samples were collected from the 0–15 cm depth using a stainless-steel auger. For each plot, samples were collected from at least four randomly selected locations and combined to form a composite sample. A portion of the fresh soil was immediately used for inorganic N extraction and determination. The remaining soil was air-dried and sieved to <2 mm for basic soil property analyses, including SOC, total N, total and available phosphorus, and soil pH (Table 1).

2.2 Experimental Design and Treatments

A factorial incubation experiment used soils from the two cropping systems treatments (WWW and LLL). The design matrix included four replicates and investigated the effects of two N addition levels (deionized water as control and 120 μg N as KNO_3 g^{-1} dry soil), three C substrates types (glucose, citric acid, and glutamine, each contributing 80 μg C g^{-1} dry soil; this standardized approach enabled direct comparison of substrate effects while acknowledging that differential priming responses may occur between systems with varying baseline SOC concentrations), and added deionized water that served as a control for both the C and N treatments and their impacts on soil CO_2 and N_2O emissions. We selected glucose, citric acid, and glutamine as exogenous C substrates. Glucose is a readily available energy source for microbial metabolism commonly found in root exudates (Keiluweit et al. 2015). Citric acid, an organic acid, represents more complex C compounds involved in soil chelation and mineral dissolution. And is also a commonly released C from roots (Gunina et al. 2014; Morley et al. 2014). Glutamine, an amino acid, is chosen to simulate the N-containing organic compounds commonly found in soil systems and contributes both C and N to the soil system (Gunina et al. 2014). These substrates collectively, represent a range of complexities and functionalities, offering a comprehensive insight into how different C substrates can influence soil CO_2 and N_2O emissions.

For the incubation, approximately 120 g of soil (oven-dry weight equivalent) from each cropping system was packed into Mason jars (5.6 cm diameter × 10 cm height) at a bulk density of 1.1 g cm^{-3}. The jars were fitted with a rubber septum for headspace gas sampling and pre-incubated at 25 °C for 6 days to stabilize the soil microbial community. Soil moisture was adjusted to approximately 75% of water-filled pore space by daily addition of deionized water following gas measurements. The target water-filled pore space was selected to enable both nitrification and denitrification processes while favoring denitrification (Li et al. 2021). The 14-day incubation duration was chosen based on previous studies showing this timeframe captures the primary response period of soil microbial communities to substrate additions (Gunina et al. 2014; Keiluweit et al. 2015; Li et al. 2021; Liang et al. 2023). These studies demonstrated that the most significant changes in microbial activity and greenhouse gas emissions typically occur within the first 7–14 days following substrate addition.

For substrate addition, following the pre-incubation period, 1 mL N solution was injected once on the first day of the experiment at five different points within each soil core using a precision microliter syringe equipped with a 51 mm needle. Subsequently, 1 mL C substrates was similarly injected into the same five locations daily throughout the 14-day experimental period. Injection points were carefully mapped to ensure that N and C substrates were consistently applied to the same locations on consecutive days. Although this approach does not achieve a perfectly homogeneous distribution, it effectively simulates field conditions, where substrate inputs commonly produce localized concentration gradients in soil. The experiment spanned 14 days, with gas emissions measured on days 1, 2, 3, 4, 5, 6, 7, 8, 9, 11, 13 and 14, and destructive soil sampling conducted on days 3, 8, and 14 for further soil analyses. In summary, the experimental design encompassed 192 units, calculated as four replicates × two cropping systems × (three C substrates + water control) × two N levels × three destructive samplings.

Table 1 Soil properties in the three-year continuous winter wheat (WWW) and Lucerne (LLL) treatments

Crop	pH	SOC (g kg^{-1})	NH_4^+-*N* (mg kg^{-1})	NO_3^--*N* (mg kg^{-1})	TN (g kg^{-1})	AP (mg kg^{-1})	TP (g kg^{-1})
WWW	8.04 ± 0.02^a	9.21 ± 0.40^a	1.78 ± 0.08^b	15.25 ± 0.41^b	0.71 ± 0.06^b	26.51 ± 0.85^b	0.36 ± 0.02^b
LLL	7.92 ± 0.04^a	9.54 ± 0.16^a	3.32 ± 0.22^a	22.43 ± 1.23^a	0.80 ± 0.04^a	40.07 ± 3.01^a	0.50 ± 0.03^a

Note: Data are mean ± standard deviation, $n = 4$. Mean values for each treatment were compared using ANOVA, with letters indicating homogeneous groups obtained from post-hoc analysis. Significance levels are given for differences between cropping systems ($p < 0.05$) and means denoted by different letters. SOC, soil organic carbon (g kg^{-1}); NH_4^+-N, ammonium nitrogen (mg kg^{-1}); NO_3^--N, nitrate nitrogen (mg kg^{-1}); TN, total soil nitrogen (g·kg^{-1}); AP, available phosphorus (mg kg^{-1}); TP, total phosphorus (g kg^{-1})

2.3 Measurements of Soil CO_2 and N_2O Emissions

Measurements of soil CO_2 and N_2O emissions from the jars were carried out using a combination of methods. For soil N_2O emissions, a tunable diode laser absorption spectrometer (Li-Cor 7820 N_2O/H_2O, Nebraska, USA) was connected to the sampling ports to monitor N_2O concentrations (ppb) in the chamber headspace, while a Li-Cor 850 (LI-Cor, Nebraska, USA) was used to measure soil CO_2 emissions. To validate flux measurements, we conducted system tests using known gas standards before experimental measurements and verified linear increases in headspace concentrations during the measurement period. Greenhouse gas emissions were calculated from the linear increase in headspace concentration over 30 to180 seconds after sealing the jars, regressions with $R^2 > 0.90$ for concentration increase over time were included in the analysis.

2.4 Soil Analyses

On days 3, 8 and 14 following N addition, a set of replicate soils ($n = 4$ per treatment) were sampled destructively for subsequent soil analyses. The soil from each jar was thoroughly mixed, and subsamples were taken for various measurements.

The gravimetric soil moisture content was determined by oven-drying subsamples at 105 °C for 8 h. Soil pH and oxidation-reduction potential were measured using a smart portable oxidation-reduction potential meter (LD-QX6530, Leyende, China).

SOC was quantified using the potassium dichromate-sulfuric acid oxidation method, with external heating applied to facilitate the reaction. Dissolved organic C (DOC) was extracted from soils with 0.5 M K_2SO_4 and quantified using an ultraviolet spectrophotometer (Deflandre and Gagné 2001). Permanganate oxidizable C was measured following Blair et al. (1995). Following established convention, this fraction is hereafter referred to as easily oxidizable C (EOC), though it is acknowledged that this operationally defined pool (also termed POXC) may include lignin-derived compounds in addition to labile C fractions (Margenot et al. 2024).

Soil total N concentration was determined using the Kjeldahl method on a Kjeltec 8400 analyzer (Foss, Denmark). Inorganic N, specifically nitrate (NO_3^--N) and ammonium (NH_4^+-N), was extracted from fresh soils with 2 M KCl (5 g soil in 25 mL extractant).

Available phosphorus was extracted with 0.5 M $NaHCO_3$ and measured via segmented flow analysis using a SMARCHEM 450 auto-analyst (AMS, Italy). Extractable Fe(II) and Fe(III) were quantified colorimetrically after extraction with 0.5 M HCl. Iron-bound organic carbon (OC) was determined by dithionite-citrate-bicarbonate extraction, followed by OC analysis of the extracts.

Free iron oxides (Fe_d), crystalline iron oxides (Fe_p), and poorly crystalline iron oxides ((Fe_o) were determined by extraction with NH_2OH ·HCl/acetic acid, sodium dithionite-citrate-bicarbonate, and ammonium oxalate/oxalic acid, respectively (McKeague and Day 1966). Iron concentrations in the extracts were measured by atomic absorption spectrometry using the M6AA system (Massachusetts, USA).

Soil microbial biomass C was determined by chloroform fumigation extraction (CFE), according to the method described by Voroney et al. (2007). Hydrogen peroxidase (CAT) and polyphenol oxidase (PPO) activities were measured using a Solabio kit, following the manufacturer's instructions (DeForest 2009; Yang et al. 2022).

β-Glucosidase and N-acetyl-*β*-glucosaminidase activities were assayed using 4-methylumbelliferyl (4-MUB)-linked substrates. For these assays, soil samples were incubated with the substrates, and the release of 4-methylumbelliferone was detected fluorometrically (Chen et al. 2017; DeForest 2009).

2.5 Statistical Analysis

The effects of cropping systems, C substrate addition, N addition, and their interactions on measured variables (e.g., SOC, DOC, TN, NO_3^--N, NH_4^+-N, CAT) and soil daily or cumulative CO_2 and N_2O emissions were analyzed using three-way ANOVA. Prior to ANOVA, normality and homogeneity of variance were examined. The significance ($P < 0.05$) main effects or interactions were found, led to Tukey's HSD post-hoc test for multiple comparisons of means.

Pearson's correlation analysis was used to evaluate relationships between soil CO_2 emissions, N_2O emissions, and soil properties. Stepwise multiple linear regression analysis was performed to identify factors controlling soil CO_2 and N_2O emissions. Variation partitioning analysis (VPA) was conducted to quantify the relative importance of soil properties in affecting CO_2 and N_2O emissions. These analyses were undertaken in the 'Agricola' package (De Mendiburu 2014) or the 'vegan' package, and plotted using the 'ggplot2' package (Wickham 2016) in R (v4.1.3).

Additional multivariate analyses were conducted to assess complex relationships between soil properties and crop systems, and greenhouse gas emissions. Random forest analysis was performed using the 'randomForest' package (Liaw and Wiener 2002) to identify the relative importance of soil properties and crop systems in predicting CO_2 and N_2O emissions. Variable importance was calculated as the percent increase in mean squared error (%IncMSE) when

each predictor was randomly permuted. Redundancy analysis (RDA) was conducted using the 'vegan' package to visualize relationships between soil properties and CO_2 and N_2O emissions. RDA was performed separately for each cropping system to identify system-specific patterns. Data were standardized prior to analysis, and results were visualized using the first two RDA axes which captured most of the explained variance.

Piecewise structural equation modeling (SEM) using the 'lavaan' package (Adane 2011) was used to determine how soil properties C substrates, and N addition under different cropping systems influenced soil CO_2 and N_2O emissions. Prior to SEM construction, the principal component analysis was used to reduce the number of variables and enhance interpretability. Model performance was evaluated using the Akaike information criterion (AIC) and Fisher's C statistic, selecting the optimal model based on the lowest AIC. Variables in the VPA or SEM were categorized based on their hypothesized or established roles in affecting CO_2 and N_2O emissions in agricultural soils. Specifically: C substrate addition (e.g., soil pH, ORP, EOC, DOC, AP): directly influenced by or influencing C substrate on the availability and dynamics in soil; N addition (e.g., NO_3^--N, NH_4^+-N): different forms of N introduced to the soil, critical for microbial nitrification and denitrification processes, influencing soil N_2O emissions; Cropping systems (e.g., SOC, TN, TP): encapsulate the impact of cropping systems on SOC, TN, and TP concentrations; Soil iron availability and enzyme activity (e.g., Fe_o, Fe_p, Fe_d, Fe (III), Fe (II), CAT, PPO): various forms of iron and their availability in the soil interact with redox conditions, influencing microbial and enzymatic activities related to C and N cycling. CAT and PPO activities reflect microbial functional diversity and metabolic capacity, crucial for understanding soil biochemical responses and their impact on greenhouse gas emissions. The statistical analyses were performed in R (v4.1.3).

3 Results

3.1 Soil CO_2 and N_2O Emissions

During the 14-day incubation period, soil CO_2 and N_2O emissions showed distinct temporal patterns (Fig. 1A, B). CO_2 emissions in soils of lucerne system were initially high and then generally declined (Fig. 1A). These emissions were profoundly influenced by the C substrate type (Table 2; Fig. 1C). Specifically, the control treatment (water) yielded consistently lower CO_2 emissions than any C substrate. For instance, on the first day of incubation, soils from the lucerne system treated with glutamine recorded an average CO_2 emission of 1.35 µmol m^{-2} s^{-1}, exceeding the 0.82 µmol m^{-2} s^{-1} observed in the WWW system with control treatment ($P<0.05$, Fig. 1A). Conversely, on day 8, WWW soils with N addition had CO_2 emissions of approximately 0.48 µmol m^{-2} s^{-1}, surpassing the 0.43 µmol m^{-2} s^{-1} recorded for LLL soils without N addition ($P<0.05$, Fig. 1A). Notably, N addition significantly reduced daily soil CO_2 emissions compared to control (Fig. 1E), and cropping system type (LLL or WWW) did not significantly influence daily and cumulative CO_2 emissions (Table 2; Fig. 1G).

Soil N_2O emissions fluctuated during the incubation period (Fig. 1B). The type of C substrate played a critical role in shaping these emissions, with the citric acid treatment and control indicating the highest N_2O emissions (Fig. 1D). On day 1, soil N_2O emissions ranged from 0.16 nmol m^{-2} s^{-1} to 0.43nmol m^{-2} s^{-1} in LLL soils treated with citric acid (Fig. 1B). Similar to its effect on CO_2 emissions, N addition did not significantly increase N_2O emissions (Fig. 1F); however, N addition significantly affected the cumulative N_2O emissions (Table 2). Cropping systems significantly impacted daily and cumulative N_2O emissions, with LLL soils consistently recording higher N_2O emissions than the WWW system (Table 2; Fig. 1H).

3.2 Key Soil Properties Affecting Soil CO_2 and N_2O Emissions

Network analysis revealed complex relationships between soil properties and greenhouse gas emissions in both cropping systems (Fig. 2). In WWW soils, CO_2 emissions significantly associated with soil EOC, SOC, Fe_o, Fe_p, Fe_d, Fe (II), inorganic N (NO_3^--N and NH_4^+-N), AP, and CAT and PPO activities (Fig. 2A). Soil N_2O emissions in these soils correlated with soil ORP, EOC, DOC, Fe_o, Fe_p, Fe_d, and CAT and PPO activities ($P<0.05$, Fig. 2A). The RDA suggested that these soil properties collectively explained 60% of the observed variance in CO_2 and N_2O emissions in WWW soils (Fig. 3B).

In LLL soils, CO_2 emissions correlated with various soil properties, including ORP, Ph, EOC, SOC, Fe_o, Fe_p, Fe_d, dithionite extracted Fe (Fe (III) and Fe (II)), and inorganic N (NO_3^--N and NH_4^+-N), TN, AP, TP, and the CAT and PPO activities ($P<0.05$, Fig. 2B). Similarly, N_2O emissions in LLL soils correlated with soil OPR, EOC, Fe_o, Fe_p, Fe_d, and CAT and PPO activities (Fig. 2B). The RDA suggested that these soil properties collectively accounted for 61% of the observed variability in CO_2 and N_2O emissions in LLL soils (Fig. 3C).

Moreover, the random forest analysis highlighted the pivotal role of specific soil properties in modulating soil greenhouse gas emissions across both cropping systems (Fig. 3A). For CO_2 emissions, soil NO_3^--N, pH, AP, PPO activity, and C substrates type emerged as primary regulators (Fig. 3A).

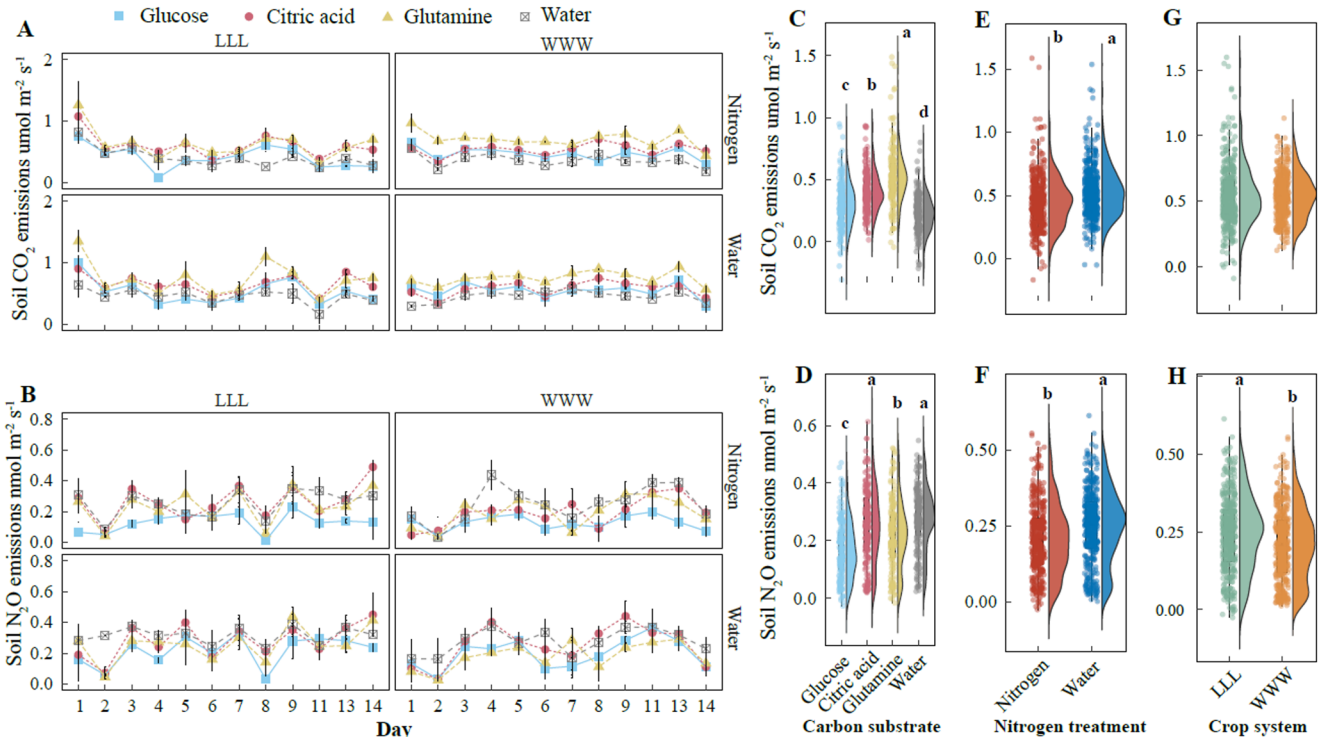

Fig. 1 Soil carbon dioxide (CO_2) and nitrous oxide (N_2O) emissions influenced by carbon substrate, nitrogen, and cropping systems over a 14-day incubation. Temporal variations in (**A**) daily soil CO_2 emissions and (**B**) daily soil N_2O emissions; effects of carbon substrate on (**C**) daily soil CO_2 emissions and (**D**) daily soil N_2O emissions; effects of nitrogen addition on (**E**) daily soil CO_2 emissions and (**F**) daily soil N_2O emissions; effects of cropping systems on (**G**) daily soil CO_2 emissions and (**H**) daily soil N_2O emissions. Dots represent means ($n=4$) and error bars indicate standard errors. Raincloud plots show the distribution of data for each treatment group. Plots depict raw data (dots), box plots display the extrema (whisker tails), interquartile range (box boundaries), and median (horizontal line); violin plots show the probability density of the data. Carbon substrates include glucose, citric acid, glutamine, and water control; nitrogen treatments include nitrogen addition versus no nitrogen; cropping systems include three continuous years of lucerne (LLL) and or winter wheat (WWW). Statistical comparisons between treatments are based on cumulative emissions (Table 2) rather than daily values to ensure appropriate handling of temporal non-independence. Raincloud plots show the distribution patterns of daily measurements for descriptive purposes only

Table 2 Cumulative soil CO_2 and N_2O emissions under different carbon substrate, nitrogen, and cropping systems over the 14 incubation days. All values shown are mean ± standard deviation, $n=4$. Significance levels: *** $P<0.001$

Crop	Carbon	Cumulative CO_2-C emissions (g C m^{-2})		Cumulative N_2O-N emissions (g N m^{-2})	
		Nitrogen	Water	Nitrogen	Water
WWW	Glucose	12.33 ± 6.63	14.18 ± 8.00	0.17 ± 0.10	0.24 ± 0.16
	Citric acid	13.19 ± 7.89	14.08 ± 8.69	0.22 ± 0.16	0.31 ± 0.22
	Glutamine	18.52 ± 10.02	18.65 ± 11.18	0.23 ± 0.16	0.21 ± 0.15
	Water	9.73 ± 5.24	11.12 ± 6.97	0.30 ± 0.21	0.35 ± 0.23
LLL	Glucose	11.45 ± 5.80	14.38 ± 7.18	0.16 ± 0.11	0.27 ± 0.17
	Citric acid	16.14 ± 8.15	16.84 ± 9.02	0.32 ± 0.20	0.34 ± 0.22
	Glutamine	16.92 ± 8.28	19.42 ± 10.04	0.30 ± 0.18	0.31 ± 0.20
	Water	11.77 ± 5.41	12.41 ± 6.48	0.32 ± 0.19	0.42 ± 0.24
Carbon		**		***	
Nitrogen		**		***	
Crop		-		***	

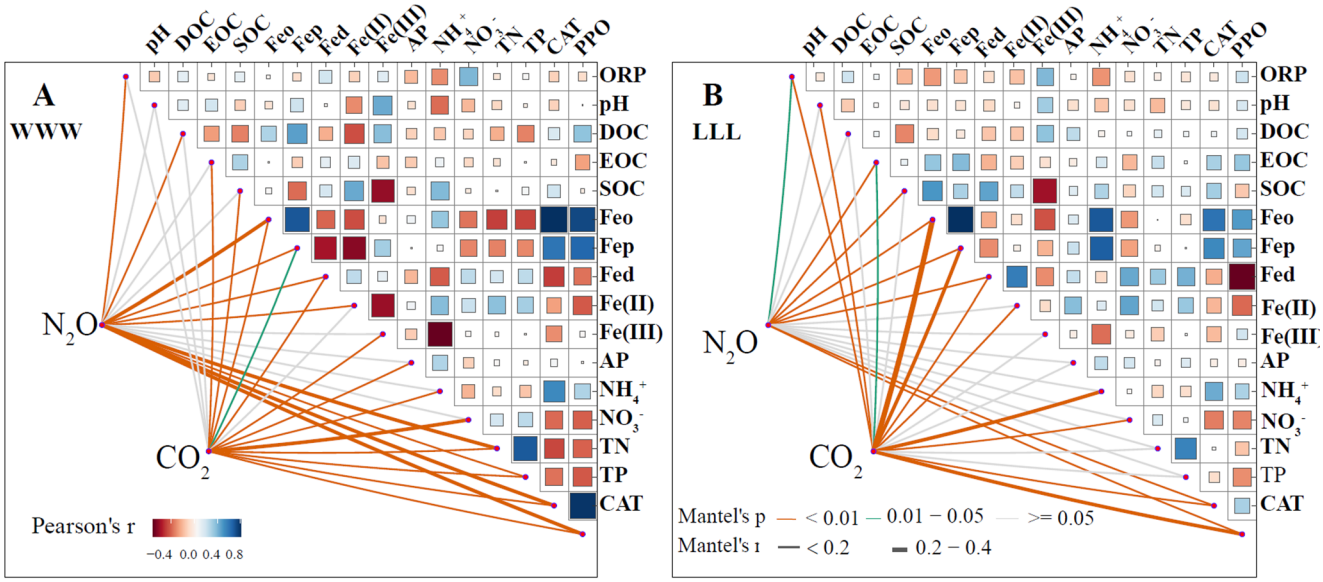

Fig. 2 Effects of soil properties on daily soil carbon dioxide (CO_2) and nitrous oxide (N_2O) emissions three years of (**A**) continuous winter wheat (WWW) and (**B**) continuous lucerne (LLL). Edge width indicates Mantel's r value, and edge color denotes statistical significance. Pairwise correlations are color-coded with Pearson's r values. Soil properties included oxidation-reduction potential (ORP), pH, dissolved organic carbon (DOC), extractable organic carbon (EOC), soil organic carbon (SOC), polyphenol oxidase (Fe_o), oxalate-extracted (Fe_p), pyrophosphate-extracted Fe (Fe_d), dithionite extracted Fe (Fe (III) and Fe (II)), available phosphorus (AP), ammonium nitrogen (NH_4^+-N), nitrate nitrogen (NO_3^--N), total nitrogen (TN), total phosphorus (TP), hydrogen peroxidase (CAT) and polyphenol oxidase (PPO)

Conversely, Fe_d, Fe_o, NO_3^--N, EOC, and CAT activity were the key determinants of soil N_2O emissions (Fig. 3A).

3.3 Contributions of Independent Variables To Soil CO_2 and N_2O Emissions

An analysis of the mean effects of different variables was conducted to ascertain their relative contributions to soil CO_2 and N_2O emissions (Fig. 4). In WWW soils, the addition of various C substrates, manifesting changes in soil pH, ORP, EOC, DOC, AP, exerted a notable impact. These factors collectively accounted for 7% of the total variance in soil CO_2 emissions (Fig. 4A). Conversely, N additions (as indicated by changes in NH_4^+-N, NO_3^--N) play a more substantial role in influencing CO_2 emissions in LLL soils, explaining 9% of the observed variance (Fig. 4C).

Additionally, soil iron availability and enzymatic activity emerged as key factors in both cropping systems. Specifically, various forms of Fe (e.g., Fe_o, Fe_p, Fe_d, Fe (III), Fe (II)) and CAT and PPO activities were crucial determinants of soil CO_2 and N_2O emissions, which explained 13 ~ 37% of the total variance in soil CO_2 or N_2O emissions (Fig. 4). Moreover, the interactions among variables of N addition, iron availability, and enzyme activity significantly influenced soil greenhouse gas emissions; For instance, in WWW soils, the interactive effects explained as much as 27% of the variance in CO_2 emissions (Fig. 4A), underscoring the complexity of factors driving soil CO_2 and N_2O emissions.

3.4 Divergent Mechanisms Governing Soil CO_2 and N_2O Emissions in Different Cropping Systems

The SEM models offered insights into the interactions among distinct soil biochemical and physical properties, and their cumulative effects on daily soil CO_2 and N_2O emissions within LLL and WWW systems (Fig. 5). In WWW soils, increased EOC and DOC due to C substrate addition, suppressed CO_2 emissions (Fig. 5A), while favorably impacting N_2O emissions in LLL soils (Fig. 5B). Moreover, C substrate addition triggered a positive influence of soil iron availability on soil C availability such as EOC and DOC in the LLL system. However, the soil iron availability affected CO_2 emissions differently in the LLL and WWW systems.

The SEMs suggested contrasting responses to N addition across the two systems. In WWW, N addition negatively correlated with CO_2 and N_2O emissions (Fig. 5A), whereas, a positive correlation occurred in LLL, highlighting the clear interplay of nutrient cycling and greenhouse gas emissions across different cropping systems (Fig. 5B).

Furthermore, the SEMs highlighted the distinct impact of cropping systems on soil properties influenced by C substrate addition, alongside soil iron availability and enzymatic activities (Fig. 5). Specifically, CAT activity negatively correlated with N_2O emissions in both systems, only positively correlating with CO_2 emissions in LLL soils. In contrast, PPO activity positively correlated with CO_2 and

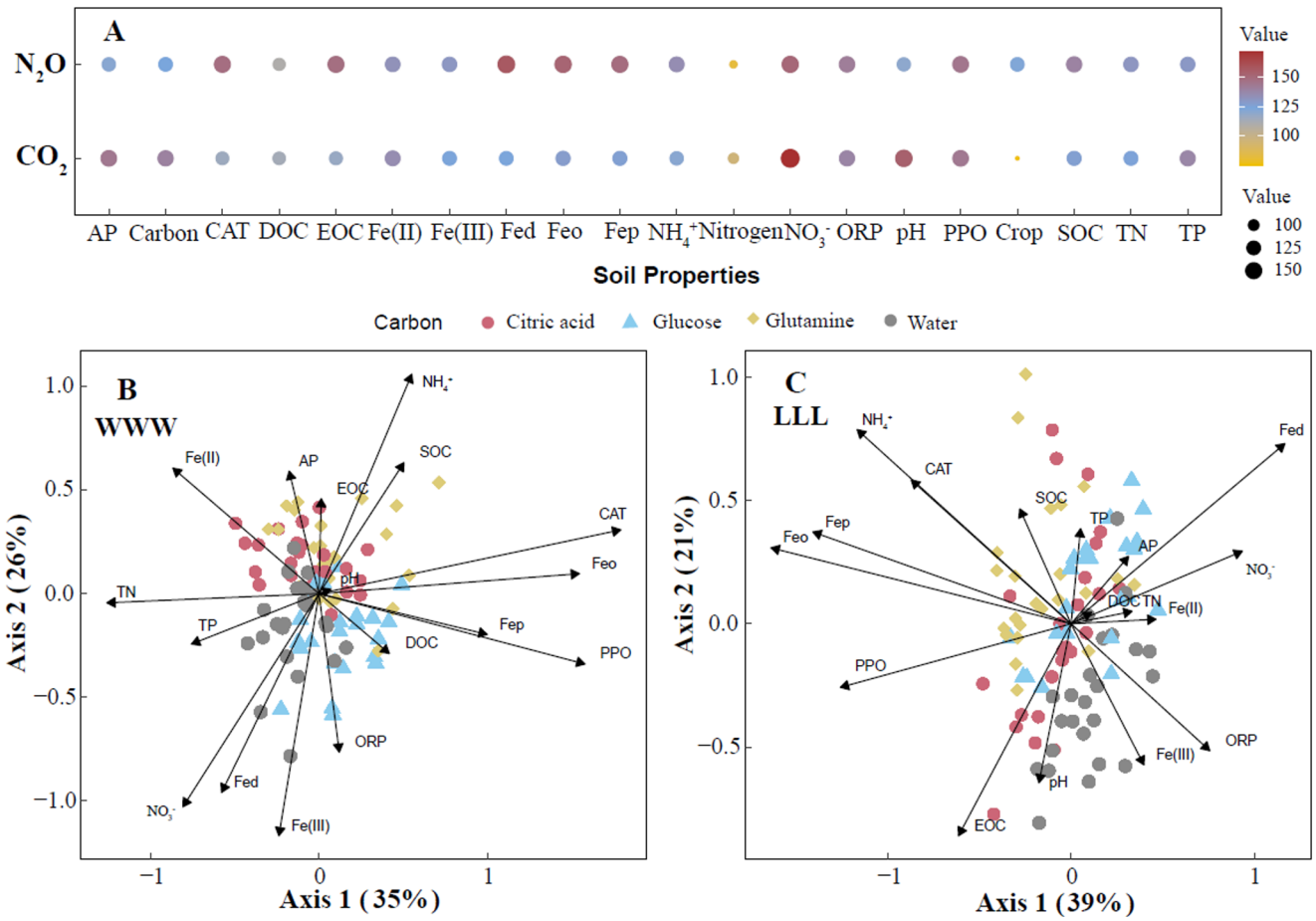

Fig. 3 Impact of soil properties and crop rotation on daily soil carbon dioxide (CO_2) and nitrous oxide (N_2O) emissions: (**A**) Variable importance from random forest analysis showing the relative contribution of measured soil properties attributed to variability in daily soil CO_2 and N_2O emissions (higher IncMSE% indicates means greater importance). Redundancy analysis (RDA) ordination plots showing the relationships between soil properties and daily soil CO_2 and N_2O emissions under three years of (**B**) continuous winter wheat (WWW) and (**C**) continuous lucerne (LLL). Symbols denote individual soil samples; vectors indicate the strength and direction of correlation between soil properties and gas emissions. Soil properties as described in Fig. 2

N_2O emissions in WWW soils and negatively correlated with N_2O emissions in LLL soils.

In line with these SEM outcomes (Fig. 5), a significant positive linear relationship was observed between soil N_2O and CO_2 emissions in WWW soils ($R^2=0.06$, $P<0.01$, Fig. 6A). However, no significant relationship between the two gases occurred in LLL soils ($P=0.056$, Fig. 6B), highlighting crop-specific intricacies in soil greenhouse gas dynamics.

4 Discussion

The contrasting greenhouse gas emission patterns observed between perennial legume and annual cereal systems reflect complex interactions among plant-specific traits, soil microbial communities, and biogeochemical processes that develop under long-term cultivation (Means et al. 2022; Oliveira et al. 2021; Siddique et al. 2023). The initially increased CO_2 emissions from lucerne soils align with enhanced microbial substrate utilization efficiency characteristic of legume-based systems (Means et al. 2022; Siddique et al. 2023). This response pattern likely reflects the development of specialized decomposer communities adapted to legume-derived organic inputs through the home-field advantage effect, whereby microbial communities become functionally optimized for processing substrates similar to those routinely encountered in their native environment (Fox et al. 2020; Jílková et al. 2023). The higher baseline soil organic carbon in lucerne systems (9.54±0.16 g kg^{-1} vs. 9.21±0.40 g kg^{-1} in wheat) provided greater substrate availability for heterotrophic respiration, contributing to the observed emission differences between cropping systems (Chen et al. 2017; Daly et al. 2024; Gunina et al. 2014).

The significantly higher N_2O emissions observed in lucerne soils (0.42±0.04 nmol m^{-2} s^{-1} vs. 0.35±0.03 nmol m^{-2} s^{-1} in wheat) can be attributed to several interacting mechanisms operating within these systems. First, the

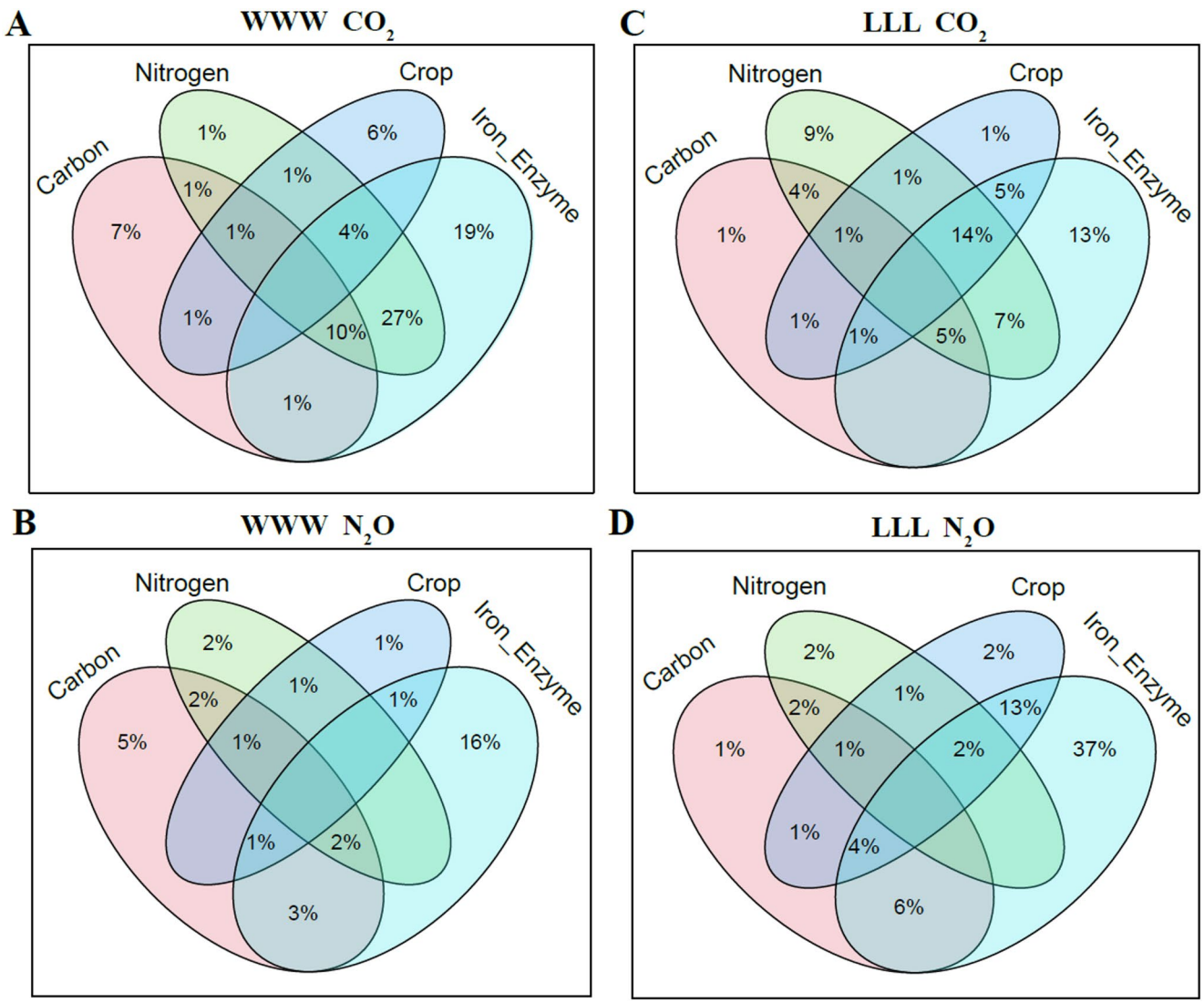

Fig. 4 Variation partitioning analysis (VPA) to analyze the effects of carbon substrate addition (e.g., soil pH, ORP, EOC, DOC, AP), nitrogen addition (e.g. NH_4^+-N, NO_3^--N), cropping systems (e.g. SOC, TN, TP), soil iron availability and enzyme activity (e.g. Fe_o, Fe_p, Fe_d, Fe (III), Fe (II), CAT, PPO), and their interactions on (**A**, **C**) mean daily soil carbon dioxide (CO_2) and (**B**, **D**) nitrous oxide (N_2O) emissions under three years of continuous winter wheat (WWW) and continuous lucerne (LLL), respectively. Each variable group's pure effects and their interactions are shown as percentages of the total variance explained. Soil properties as described in Fig. 2

cessation of active plant N uptake following lucerne termination, combined with continued mineralization of N-rich residues, creates conditions favorable for N_2O production through both nitrification and denitrification pathways (Bouwman et al. 2002; Morley et al. 2014). Second, the high water-filled pore space maintained during incubation (75%) particularly favored denitrification processes in lucerne soils, where elevated organic matter content likely created more anaerobic microsites compared to wheat soils (Jiang et al. 2025; Li et al. 2021; van Kessel et al. 2013). Third, the contrasting NO_3^- concentrations between cropping systems (lucerne: 22.43 ± 1.23 mg kg^{-1} vs. wheat: 15.25 ± 0.41 mg kg^{-1}) fundamentally influenced N_2O emission patterns through their effects on denitrification substrate availability. Under the experimental conditions, elevated NO_3^- concentrations in lucerne soils provided abundant electron acceptors for denitrifying microorganisms, particularly when coupled with readily available C substrates (Baggs 2011). These findings indicate that cropping system legacy effects fundamentally shape the biogeochemical context within which substrate additions influence greenhouse gas production.

The differential responses to C and N substrate additions show distinct regulatory mechanisms governing greenhouse gas emissions in agricultural soils. The observed increase in CO_2 emissions following C substrate addition aligns with current understanding of priming effects, where fresh organic inputs stimulate microbial activity and native soil organic C decomposition (Huo et al. 2017). However, the

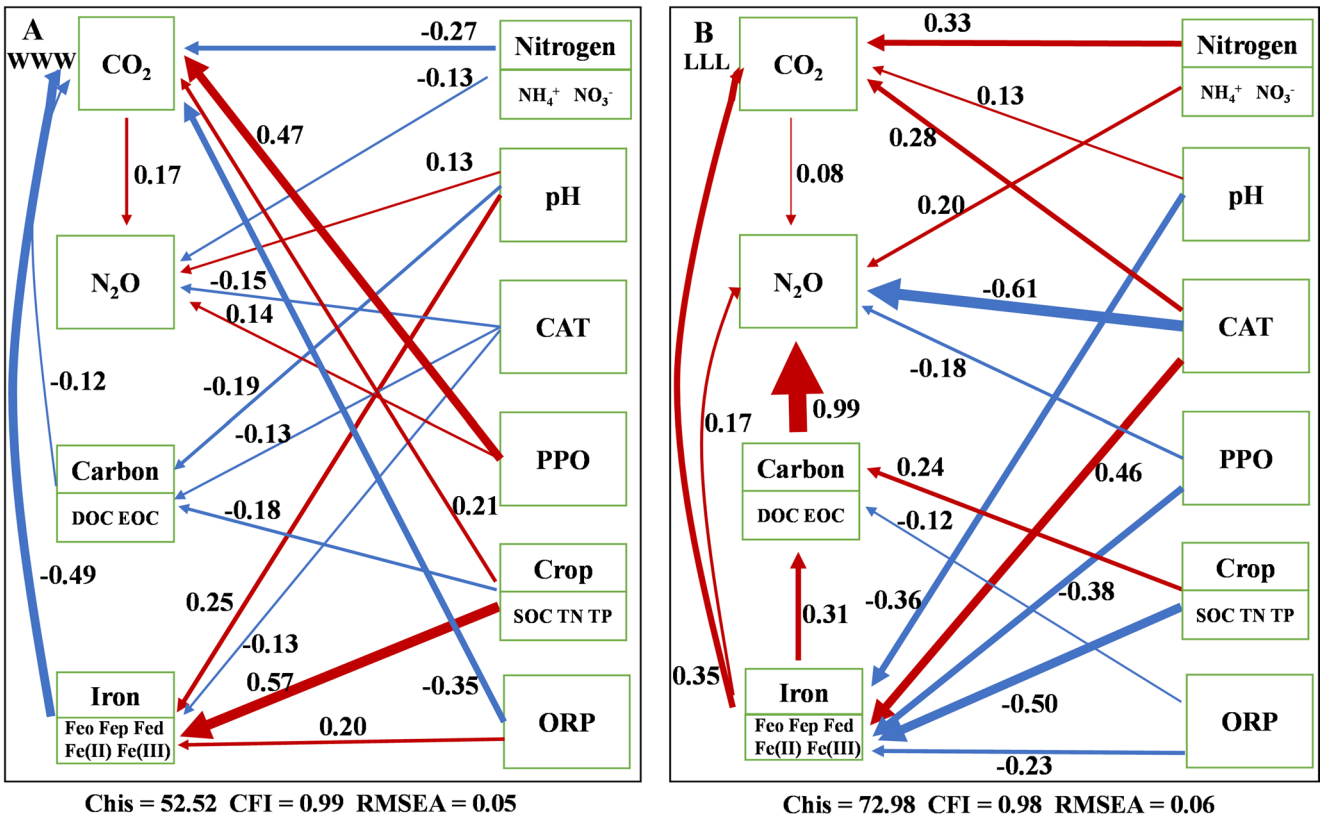

Fig. 5 Outcomes of the piecewise structural equation model analysis evaluating the effects of carbon substrate addition (e.g., soil pH, ORP, EOC, DOC, AP), nitrogen addition (e.g. NH_4^+-N, NO_3^--N), cropping systems (e.g. SOC, TN, TP), soil iron availability and enzyme activity (e.g. Fe_o, Fe_p, Fe_d, Fe (III), Fe (II), CAT, PPO), and their interactions on daily soil carbon dioxide (CO_2) and nitrous oxide (N_2O) emissions under three years of (**A**) continuous winter wheat (WWW) and (**B**) continuous lucerne (LLL), respectively. Red and blue arrows indicate significant positive and negative relationships ($P<0.05$), respectively. Standardized path coefficients (values next to arrows) varied in width and significance (*$P<0.05$; ** $P<0.01$; *** $P<0.001$). Soil properties as described in Fig. 2

substrate-specific responses, particularly the contrasting effects of citric acid versus glucose and glutamine on N_2O emissions, indicate distinct metabolic pathways and microbial community responses to different C sources (Keiluweit et al. 2015; Schmidt et al. 2011). Glutamine treatment yielded the highest CO_2 emissions (1.35±0.12 μmol m^{-2} s^{-1}), followed by citric acid (1.12±0.09 μmol m^{-2} s^{-1}) and glucose (0.98±0.08 μmol m^{-2} s^{-1}), all significantly exceeding the water control (0.82±0.07 μmol m^{-2} s^{-1}). These patterns suggest the differential bioavailability and metabolic fates of each substrate class within soil microbial communities (Chen et al. 2022; Means et al. 2022; Sprunger et al. 2019).

The reduction in N_2O emissions observed with glucose (24% reduction) and glutamine (18% reduction) additions, but not with citric acid (31% enhancement), indicates substrate-specific effects on denitrification processes operating through distinct mechanisms. For glucose and glutamine, two complementary pathways likely contributed to emission reduction: (1) the rapid assimilation of these readily metabolizable substrates promoted microbial N immobilization, effectively reducing N substrate availability for nitrification and denitrification (Baggs 2011; Curtright and Tiemann 2023; Morley et al. 2014); and (2) enhanced complete denitrification to N_2 rather than N_2O, particularly under the high WFPS conditions (75%) maintained during incubation (Fromm et al. 2021). In contrast, citric acid enhanced N_2O emissions through pH-mediated effects on denitrification; by lowering soil pH and potentially mobilizing iron through chelation, citric acid created conditions more favorable for N_2O production while simultaneously inhibiting N_2O reductase activity (Wang and Kuzyakov 2024). The absence of a significant N_2O emission increase following KNO_3 addition indicates that denitrification potential was likely limited by C availability rather than NO_3^- concentration under these experimental conditions, particularly in the wheat soils with lower organic matter content. These substrate-specific mechanisms highlight the importance of considering C input quality, not merely quantity, when developing greenhouse gas mitigation strategies for agricultural systems. These substrate effects were modulated by baseline NO_3^- availability differences between systems (as described above). The absence of significant N_2O emission increases following KNO_3 addition suggests that

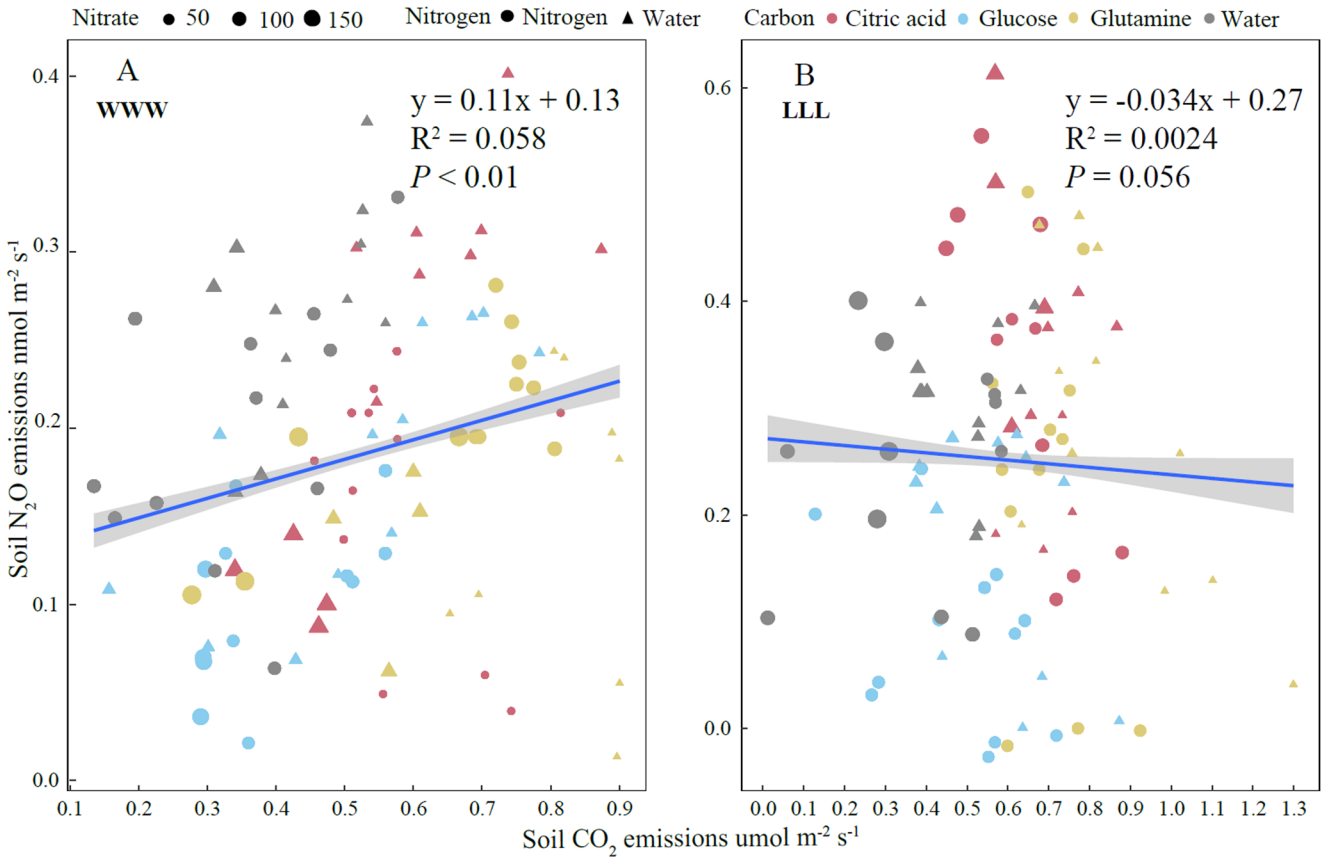

Fig. 6 Relationship between daily soil CO_2 and N_2O emissions. Values are daily means of four replicates (n=4). Soils are from the three years of (**A**) continuous winter wheat (WWW) and (**B**) continuous lucerne (LLL), respectively. Color indicates four carbon substrates: glucose, citric acid, glutamine, and water control; nitrogen treatments include nitrogen addition versus no nitrogen, represented by symbol shape; and shape size indicates soil nitrate concentrations

denitrification was primarily C-limited rather than N-limited in these systems, consistent with previous observations that C availability often constrains denitrification rates in agricultural soils (Baggs 2011; Morley et al. 2014).

The identification of distinct controlling factors for CO_2 and N_2O emissions highlights the complex biogeochemical regulation of greenhouse gas production in agricultural soils, mediated through both direct and indirect pathways (Oliveira et al. 2021; Yang et al. 2022). Random forest analysis identified soil NO_3^--N, pH, available P, and polyphenol oxidase activity as primary regulators of CO_2 emissions, indicating that microbial C metabolism is constrained by both nutrient availability and environmental conditions (Davidson and Janssens 2006). The strong influence of pH likely reflects its fundamental role in controlling microbial community composition and enzyme activities, particularly in soils where pH affects both organic matter solubility and mineral surface chemistry (Gan et al. 2023). In contrast, iron fractions (Feo, Fed) and EOC emerged as dominant regulators of N_2O emissions, with strong correlations suggesting that iron serves both as an electron acceptor supporting organic matter oxidation and as a catalyst for N_2O production through chemodenitrification (Hall and Silver 2013; Yao et al. 2023). This mechanism appears particularly relevant in the lucerne system, where higher organic matter content likely promoted the formation of Fe-organic matter complexes that can influence both C availability and electron transfer processes.

The contrasting relationships between enzyme activities and greenhouse gas emissions in the two cropping systems indicate system-specific biological regulation mechanisms shaped by long-term management practices. The negative correlation between catalase activity and N_2O emissions in lucerne soils, coupled with positive associations with CO_2 emissions, suggests that enhanced oxidative metabolism may suppress denitrification while promoting aerobic C mineralization, possibly by maintaining higher redox potentials in microsites (Hall and Silver 2013; Yao et al. 2023). Conversely, the positive correlation between polyphenol oxidase activity and both gases in wheat soils indicates that phenol oxidation may be coupled to both C mineralization and N transformation processes under these conditions. These enzymatic patterns align with recent theoretical frameworks proposing that soil enzyme activities reflect both resource availability and microbial community adaptation to long-term management

practices (Curtright and Tiemann 2023). The variation partitioning analysis quantified these relationships, revealing that iron availability and enzyme activities explained 37% and 29% of emission variations in lucerne and wheat soils, respectively, underscoring their critical regulatory roles.

Structural equation modeling indicated divergent mechanistic pathways regulating greenhouse gas emissions in the two cropping systems, providing quantitative insights into the direct and indirect effects of substrate additions. In lucerne soils, the positive relationship between C substrate addition and N_2O emissions suggests that enhanced C availability stimulates denitrification activity, particularly under the experimental conditions (75% WFPS) that favor anaerobic microsites. This mechanism is further supported by the significant interaction between soil iron availability and C substrate effects, indicating that iron-mediated electron transfer processes may facilitate both C oxidation and N_2O production (Zhuang et al. 2024). The contrasting response patterns between systems to N addition highlight fundamental differences in N cycling dynamics: the positive association of N addition with both CO_2 and N_2O emissions in lucerne soils suggests that the legume-cultivated soil environment maintains active coupling between C and N cycling, likely reflecting microbial communities adapted to higher N availability and more diverse C substrates under long-term legume cultivation (Liu et al. 2024). Conversely, the negative associations observed in wheat soils indicate potential substrate limitations or microbial community constraints on N utilization efficiency. The significant positive linear relationship between soil N_2O and CO_2 emissions in wheat soils, but not in lucerne soils, further suggests these crop-specific intricacies in soil greenhouse gas dynamics.

The controlled laboratory conditions of this study, while essential for isolating specific mechanistic relationships, necessarily differ from field environments where plant-soil-microbe interactions, seasonal temperature fluctuations, and precipitation patterns create dynamic conditions affecting greenhouse gas emissions (Smith et al. 2016). The 14-day incubation period captures the primary response phase of soil microbial communities to substrate additions (Gunina et al. 2014; Keiluweit et al. 2015; Liang et al. 2023), though longer-term studies would be valuable for assessing sustained responses and potential shifts in microbial community composition. The temporal emission patterns observed, characterized by an initial peak followed by fluctuations around a relatively stable baseline, reflect complex biogeochemical dynamics including rapid microbial utilization of readily metabolizable substrates during days 1–2, periodic responses to moisture adjustments necessary for maintaining 75% water-filled pore space, and microbial community adaptation to sustained substrate inputs through shifts in metabolic strategies and C allocation patterns. The C substrate concentration (80 μg C g^{-1} dry soil) was selected based on established methodological precedents representing typical root exudate concentrations (Gunina et al. 2014; Keiluweit et al. 2015), though differential priming effects between systems with varying baseline SOC concentrations may have contributed to the observed emission differences.

Field validation of these laboratory-derived mechanisms should account for rhizosphere effects, root exudate dynamics, and crop phenological stages that influence substrate input patterns under actual agricultural conditions. Future research would benefit from extended experimental durations to assess long-term shifts in microbial community composition and function under continuous substrate additions, manipulation of substrate addition frequencies and concentrations to elucidate dose-response relationships and threshold effects relevant to field conditions, and integration of molecular techniques to track microbial functional gene expression throughout incubation periods. Such methodological refinements would contribute to a more comprehensive understanding of how substrate-microbe interactions regulate greenhouse gas emissions in agricultural soils under different management practices. Despite these limitations, the mechanistic insights obtained provide a foundation for developing system-specific greenhouse gas mitigation strategies that account for cropping system legacy effects and substrate quality considerations.

5 Conclusions

This study indicates different mechanistic pathways regulating greenhouse gas emissions in contrasting agricultural systems. Three key findings: (1) cropping system legacy effects fundamentally affected substrate utilization patterns, with lucerne systems showing enhanced coupling between C and N cycling processes compared to wheat systems; (2) substrate-specific effects on emissions operate through distinct mechanisms, citric acid induced N_2O emissions through pH-mediated effects, while glucose and glutamine suppress emissions through enhanced N immobilization; (3) iron availability and enzyme activities serve as critical regulators of emission patterns, explaining 29–37% of observed variations across systems. These mechanistic insights suggest that effective greenhouse gas mitigation strategies should consider system-specific microbial adaptation patterns when planning C and N management, and account for substrate quality effects on emission pathways. Future research should examine these mechanisms under field conditions, particularly focusing on temporal dynamics and plant-microbe interactions.

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Declarations

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