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Optimizing the Incorporated Amount of Chinese Milk Vetch (*Astragalus sinicus* L.) to Improve Rice Productivity without Increasing CH_4 and N_2O Emissions

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Abstract: Chinese milk vetch (CMV) is a leguminous green manure that is commonly cultivated in paddy fields and can partially substitute synthetic nitrogen fertilizer. However, the impacts of incorporating CMV on CH_4 and N_2O emissions are still a subject of controversy. Therefore, we conducted a field experiment over three years to investigate emissions under different substitution ratios: urea only (CF); incorporating a traditional amount of CMV (MV); and with incorporation ratios of 1/3 (MV1/3), 2/3 (MV2/3), and 4/3 (MV4/3) of MV for partial urea substitution. Compared with CF, MV2/3, MV, and MV 4/3 resulted in increased yields. MV and MV4/3 reduced N_2O emissions but increased CH_4 emissions by 28.61% and 85.60% (2019), 32.38% and 103.19% (2020), and 28.86% and 102.98% (2021), respectively, resulting in an overall increase in total global warming potential (except for MV in 2021). MV2/3 exhibited a low greenhouse gas intensity value ranging from 0.46 to 0.47. Partial least-squares-path model results showed that CH_4 and N_2O emissions were influenced by substitution ratios, which indirectly regulated the gene abundances of *mcrA* and *nosZ*. Overall, the impact of CMV on CH_4 and N_2O emissions was determined by substitution ratios. MV2/3, which involved partial substitution of synthetic N fertilizer with 15.0 t ha^{-1} of CMV, resulted in improved rice productivity without increasing CH_4 and N_2O emissions, making it a recommended approach in the study area.

Keywords: Chinese milk vetch; methane; nitrous oxide; paddy; global warming potential; greenhouse gas intensity

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1. Introduction

Rice is a fundamental food source for over 50% of the world's population, and with a harvest area of 1.46×10^8 ha, it accounts for more than 30% of the worldwide cereal cultivation area [1]. However, paddy fields are some of the most significant sources of greenhouse gases (GHGs), especially methane (CH_4) and nitrous oxide (N_2O) emissions, accounting for 11% and 10% of anthropogenic emissions, respectively [2]. Therefore, balancing the food supply and environmental benefits of rice production is a challenging and important research topic.

To improve soil quality and increase rice productivity, the combined application of green manure and synthetic fertilizer in paddy fields is encouraged [3]. In agroecosystems, the production and consumption of CH_4 and N_2O are usually driven by microorganisms associated with the carbon (C) and nitrogen (N) cycles in farmlands, such as methanogenic archaea, methanotrophic bacteria, ammonia oxidizers, and denitrifiers [4]. The combination of organic material and synthetic fertilizer affects the above-mentioned microorganisms by changing the substrate composition, soil nutrients, pH, and redox potential, which will likely alter CH_4 and N_2O emissions from paddy fields [5]. Therefore, confirming the specific impacts of the combined application on GHGs is significant for determining the application value of green manure.

Chinese milk vetch (*Astragalus sinicus* L.; CMV) is a leguminous green manure and winter cover crop that is widely grown in paddy fields in most Asian countries and is an important N source that can partially substitute for synthetic fertilizer [3]. Because CMV plays an important role in N management and yield increases in paddy fields, many studies have recently focused on the impacts of CMV incorporation on GHG emissions from paddy fields [6,7]. However, the current conclusions are ambiguous, limiting the promotion of CMV.

Some reported results have indicated that CMV incorporation does not significantly stimulate CH_4 emissions compared to treatments without residue amendment [8–10]. CMV shows a low C/N ratio (~15) and has a much weaker impact on CH_4 emissions than green manure with a high C/N ratio, such as ryegrass (~36) and oilseed rape (~25). The cumulative emissions under CMV treatments are 57.36–64.42% and 73.11–78.86% of those under ryegrass and rape treatments, respectively [10,11]. However, some studies have suggested that CMV incorporation remarkably increases CH_4 emissions by 24.66–508.68% compared with chemical fertilizer treatment, which may be due to the rich substrates provided by CMV and suitable environmental conditions for soil methanogenic archaea [12–14].

N_2O emissions are related to nitrification–denitrification processes and are directly influenced by the inorganic N applied during agricultural cultivation [15]. Water management in paddy fields, such as flooding and drainage, will shift the balance between nitrification and denitrification and then affect N_2O emissions [16]. Soil temperature is another nonnegligible factor affecting N_2O emissions [17]. Moreover, the mineralization process of organic amendments produces N_2O [18]. CMV has typically been considered to restrict N_2O emissions by partially replacing inorganic N fertilizer and due to its relatively slow mineralization rate [7,19]. However, other studies indicate that CMV incorporation does not affect N_2O emissions compared with the application of only chemical fertilizer, but the microprocesses related to N_2O emissions after green manure incorporation are still unclear [20–22].

In addition to the type of green manure, paddy soil properties are also directly affected by the incorporation volume of green manure. The incorporation volume regulates soil pH and SOC stocks, which elicit alterations in the composition and abundance of soil microorganisms, especially the functional genera related to C and N cycling [23,24]. Thus, the inconsistent conclusions above may have been caused by the single organic-inorganic fertilizer substitution ratio utilized in most studies. The influence of different proportions of organic and inorganic fertilizers on CH_4 and N_2O emissions is meaningful for the application of CMV and needs to be further verified. The current study is expected (1) to assess the CH_4 and N_2O emissions from paddies with different incorporation amounts of CMV to substitute synthetic N fertilizer, (2) to reveal the microbial mechanisms that affect CH_4 and N_2O emissions, and (3) to explore a rational incorporated amount of CMV that is beneficial for improving rice productivity without increasing CH_4 and N_2O emissions.

2. Materials and Methods

2.1. Experimental Site

The experimental field plots were located in Yijiang town, Wuhu city, China (30°55′ N 118°29′ E), which has a subtropical monsoon climate. Monthly precipitation and daily ambient data were collected by a tintype meteorological station (Weather Hawk Station; Campbell Scientific, Logan, UT, USA) and are presented in Figure S1. The experimental soil is classified as Gleyi-Stagnic Anthrosol (CRGCST 2001). The soil background values (0–20 cm) were as follows: pH 5.91, soil organic carbon (SOC) 16.90 $g \cdot kg^{-1}$, dissolved organic carbon (DOC) 102.26 $mg \cdot kg^{-1}$, total nitrogen (TN) 1.73 $g \cdot kg^{-1}$, alkaline hydrolysis nitrogen (AN) 130.27 mg·kg^{-1}, available phosphorus (AP) 9.31 mg·kg^{-1}, and available potassium (AK) 70.38 mg kg^{-1}.

2.2. Experimental Description

A field experiment was conducted from 2019 to 2021. CMV was planted in the fallow season (winter) and then incorporated into the soil before transplanting in the following season. There were five treatments with three replications in this experiment: urea only (CF); incorporating a traditional amount of CMV (sowing seeds with 30 kg ha^{-1} and incorporating CMV with 22.5 t ha^{-1} fresh weight) to partially substitute for urea (MV); and incorporating 1/3 (MV1/3), 2/3 (MV2/3), and 4/3 (MV4/3) of MV to partially substitute for urea (Table 1). CF is a common management approach in paddy fields, and MV has been repopularized in recent years. The other treatments are newly developed methods that need to be further explored. Before incorporation, CMV samples were collected to test the actual fresh weight in each plot. When the fresh weight in a plot was deficient, CMV was supplemented from other fields. When it was superfluous, some CMV was removed. The urea application amounts were designed based on the N inputs of CMV incorporation to ensure that the total N input was equal among treatments. Substitution ratios of synthetic N fertilizer with CMV were represented by the ratios of N from CMV to N from urea. The dosages of P_2O_5 (80 kg ha^{-1}) and K_2O (120 kg ha^{-1}) remained the same for all treatments. All inorganic fertilizers were applied as basic fertilizer before transplanting. Fifteen experimental plots (10 m × 4 m) were established using a randomized complete block design separated by a 35 cm high ridge with an impermeable membrane. The CMV and mono-rice varieties were "Yijiang zi" and "Y Liangyou 957", respectively.

Table 1. Incorporation amounts of Chinese milk vetch and N inputs under different treatments.

Treatment	CMV		Urea			Total N (kg ha^{-1})	MV:CF (%)
	Application Rate (t ha^{-1})	N Input (kg ha^{-1})	Application Rate (kg ha^{-1})	Substituted by CMV (kg ha^{-1})	N Input (kg ha^{-1})		
CF	0.0	0.0	480.0	0.0	220.8	220.8	0.0
MV25	7.5	17.7	441.4	38.6	203.1	220.8	8.7
MV50	15.0	35.5	402.9	77.1	185.3	220.8	19.1
MV75	22.5	53.2	364.3	115.7	167.6	220.8	31.7
MV100	30.0	70.9	325.8	154.2	149.9	220.8	47.3

CMV denotes Chinese milk vetch. MV:CF denotes the ratio of N from Chinese milk vetch to N from urea.

2.3. Gas Sampling and Analysis

CH_4 and N_2O emissions were measured using static chamber-gas chromatography [11]. Gases were sampled once every seven days during the rice season from 2019 to 2021. The sampling frequency was appropriately increased during the peak emission period. The gas emission rates were calculated from four continuous sample concentrations collected every 10 min. The gas fluxes were calculated using Equation (1) [10].

$$F = \rho \times h \times \frac{\Delta C}{\Delta T} \times \frac{273}{273 + T} \tag{1}$$

where ρ is the density of gas under a standard state (kg m^{-3}), h is the height of the chamber (m), $\frac{\Delta C}{\Delta T}$ is the change rate of gas concentration per unit time in the chamber (mg m^{-3} h^{-1}), and T is the temperature in the chamber (°C).

The cumulative emissions were calculated using Equation (2) [10].

$$E = \sum_{i=1}^{n} \frac{F_i + F_{i+1}}{2} \times 24 \times D_i \tag{2}$$

where F_i and F_{i+1} represent two consecutive days of gas fluxes (g m^{-2} h^{-1}) and D_i stands for two consecutive sampling intervals (days).

The global warming potential (*GWP*) and greenhouse gas intensity (*GHGI*) were calculated according to Equations (3) and (4), respectively [7].

$$GWP = 29.8 \times CH_4 + 273 \times N_2O \tag{3}$$

$$GHGI = {}^{GWP}/_{Yield} \tag{4}$$

2.4. Soil Sampling and Analysis

At the rice tillering stage in 2021, soils (0–20 cm) were collected from each plot using a five-point sampling method. After removing the roots and impurities, the soils were thoroughly mixed to form a composite sample. The soil sample was then divided into two portions, one for chemical analysis and the other for microbiological analysis.

Soil pH was detected by a pH meter (water:soil: 1:2.5) (DZS-708, Leici, China). SOC, DOC, and TN were measured as described by Lin, et al. [25] using an elemental analyzer (Elementar Vario MAX; Elementar Scientific Instruments, Hanau, Germany). AN, AP, and AK were determined as described by Cai, et al. [26].

The total soil genomic DNA was extracted from 0.5 g of fresh soil using the Fast DNA spin method with the Soil Toolkit (MP Biomedical, LLC, OH, USA). The expression of functional genes was analyzed by polymerase chain reaction (q-PCR). The PCR primers of *mcrA*, *pmoA*, AOA-*amoA*, AOB-*amoA*, *nirS*, *nirK*, and *nosZ* were MLf/MLr, A189F/mb661R, nirS-cd3aF/nirS-R3cd, nirK-F1aCu/nirK-R3Cu, and nosZ-2F/nosZ-2R, respectively. Detailed information can be found in Li, et al. [27] and Wang, et al. [28].

2.5. Rice Yield Determination

At the mature stage every year, rice plants were harvested in each plot. The grains were separated from straw by a plot thresher. After sun drying, rice grains were weighed to determine the yield in each plot.

2.6. Statistical Analysis

SPSS version 20.0 (SPSS INC, Armonk, New York, NY, USA) was used for statistical analysis. Mean values for each variable under different treatments were compared by one-way ANOVA, followed by an LSD test ($p < 0.05$). Pearson's correlations were analyzed to assess the relationships between the abundances of functional genes and soil nutrients. Partial least-squares-path modeling (PLS-PM) is a useful statistical method for exploring the cause-effect relationships among observed and latent variables [29]. Estimates of the path coefficients and the coefficients of determination (R^2) in the current path model were validated using SmartPLS 4 (SmartPLS GmbH, Oststeinbek, Schleswig-Holstein, Germany).

3. Results

3.1. Rice Yields and Soil Properties

Compared with the CF treatment, the MV2/3, MV, and MV4/3 treatments significantly increased rice yields during the experimental period (2019–2021) (Figure 1). The differences between the MV2/3 and MV treatments were not statistically significant and the two treatments increased yields by 38.29% and 40.92% (2019), 28.37% and 25.13% (2020), and 26.82% and 28.76% (2021), respectively, compared to the yield in the CF treatment. There were slight decreases in yields under the MV4/3 treatment compared with those under the MV treatment in 2019 and 2020. The results indicated that partial substitution of synthetic N fertilizer with a certain amount of CMV could increase the rice yield. Decreasing the traditionally applied amount of CMV (MV) to two thirds (MV2/3) did not affect its yield-increasing effect.

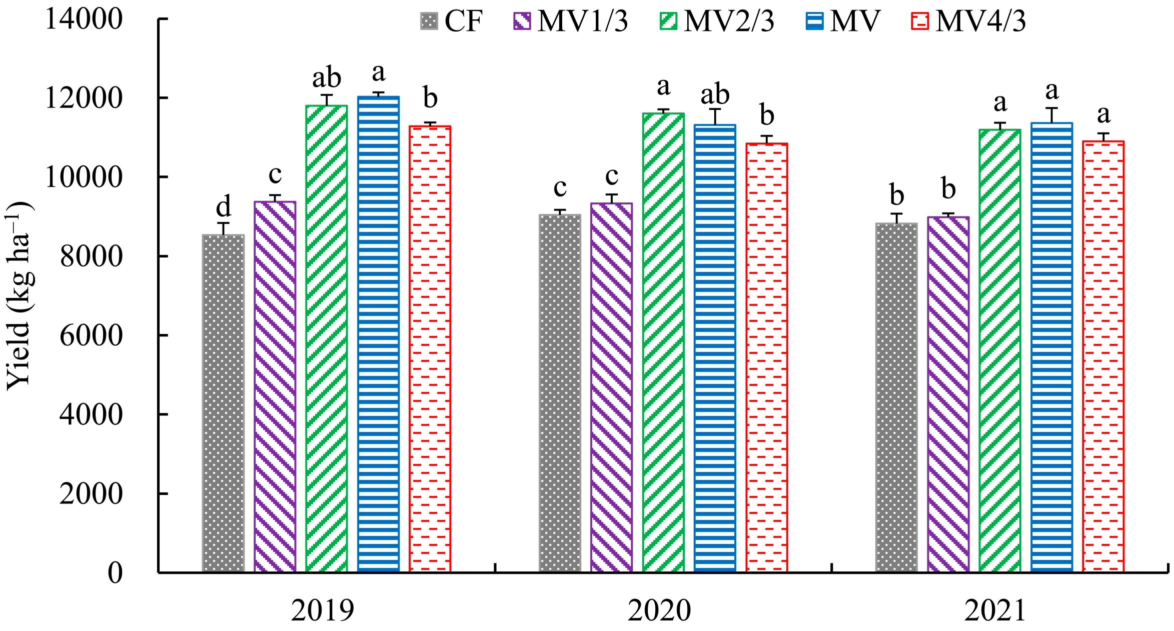

Figure 1. Rice yields affected by the substitution of synthetic N fertilizer with different amounts of Chinese milk vetch. The vertical bars denote the standard errors of the means (n = 3). The different lowercase letters indicate significant differences among the means under different treatments in the same year ($p < 0.05$). CF, MV1/3, MV2/3, MV, and MV4/3 indicate incorporating 0, 7.5, 15.0, 22.5, and 30.0 t ha^{-1} of Chinese milk vetch, respectively, to partially substitute for urea.

After the different fertilization treatments, the soil pH, SOC, DOC, AP, and AK concentrations showed an upward trend with increasing CMV incorporation (Table 2). Different fertilization management practices did not significantly affect the soil TN and AN concentrations. The results indicated that incorporating a certain amount of CMV could ameliorate soil acidification and benefit soil nutrients.

Table 2. Soil properties affected by the substitution of synthetic N fertilizer with different amounts of Chinese milk vetch.

Treatment	**pH**	**SOC [1] (g kg^{-1})**	**DOC (mg kg^{-1})**	**TN (g kg^{-1})**	**AN (mg kg^{-1})**	**AP (mg kg^{-1})**	**AK (mg kg^{-1})**
CF	6.03 ± 0.07 ^{c}	16.11 ± 0.78 ^{c}	98.29 ± 13.13 ^{c}	1.88 ± 0.12 ^{a}	159.31 ± 4.23 ^{a}	11.28 ± 0.66 ^{b}	74.2 ± 2.05 ^{b}
MV1/3	6.14 ± 0.06 ^{bc}	18.17 ± 0.53 ^{bc}	104.19 ± 6.32 ^{c}	1.86 ± 0.09 ^{a}	159.38 ± 10.46 ^{a}	14.21 ± 0.53 ^{b}	80.42 ± 4.26 ^{ab}
MV2/3	6.17 ± 0.09 ^{bc}	20.77 ± 1.34 ^{ab}	142.10 ± 24.01 ^{bc}	1.95 ± 0.1 ^{a}	155.7 ± 5.13 ^{a}	15.63 ± 1.8 ^{ab}	88.16 ± 3.85 ^{a}
MV	6.31 ± 0.02 ^{ab}	20.83 ± 1.08 ^{ab}	173.55 ± 8.15 ^{ab}	2.08 ± 0.03 ^{a}	157.61 ± 14.77 ^{a}	18.64 ± 2.16 ^{a}	89.78 ± 5.45 ^{a}
MV4/3	6.44 ± 0.07 ^{a}	21.53 ± 1.25 ^{a}	218.92 ± 30.41 ^{a}	2.00 ± 0.25 ^{a}	164.44 ± 13.19 ^{a}	19.16 ± 1.00 ^{a}	91.46 ± 2.99 ^{a}

Samples were collected in 2021. Values are reported as means ± SEs. Different lowercase letters indicate significant differences ($p < 0.05$) in the variable means among treatments in the same year. [1] SOC: soil organic carbon, DOC: dissolved organic carbon, TN: total nitrogen, AN: available nitrogen, AP: available phosphorus, AK: available potassium. CF, MV1/3, MV2/3, MV, and MV4/3 indicate incorporating 0, 7.5, 15.0, 22.5, and 30.0 t ha^{-1} of Chinese milk vetch, respectively, to partially substitute for urea.

3.2. CH_4 and N_2O Emissions

The dynamics of CH_4 emission fluxes were similar among the different treatments in different years (Figure 2). The CH_4 fluxes exhibited obvious seasonal variations, reaching a peak at the tillering stage. The peaks under the MV and MV4/3 treatments were observed to be higher than those under the other treatments. Seasonal patterns of N_2O emissions were consistent across all treatments during the rice growing season (Figure 3). N_2O fluxes peaked at the ineffective tillering stage; however, the peak value varied. The N_2O fluxes showed a small peak 90 days after transplanting in 2021 under the CF, MV1/3, and MV2/3 treatments (Figure 3c).

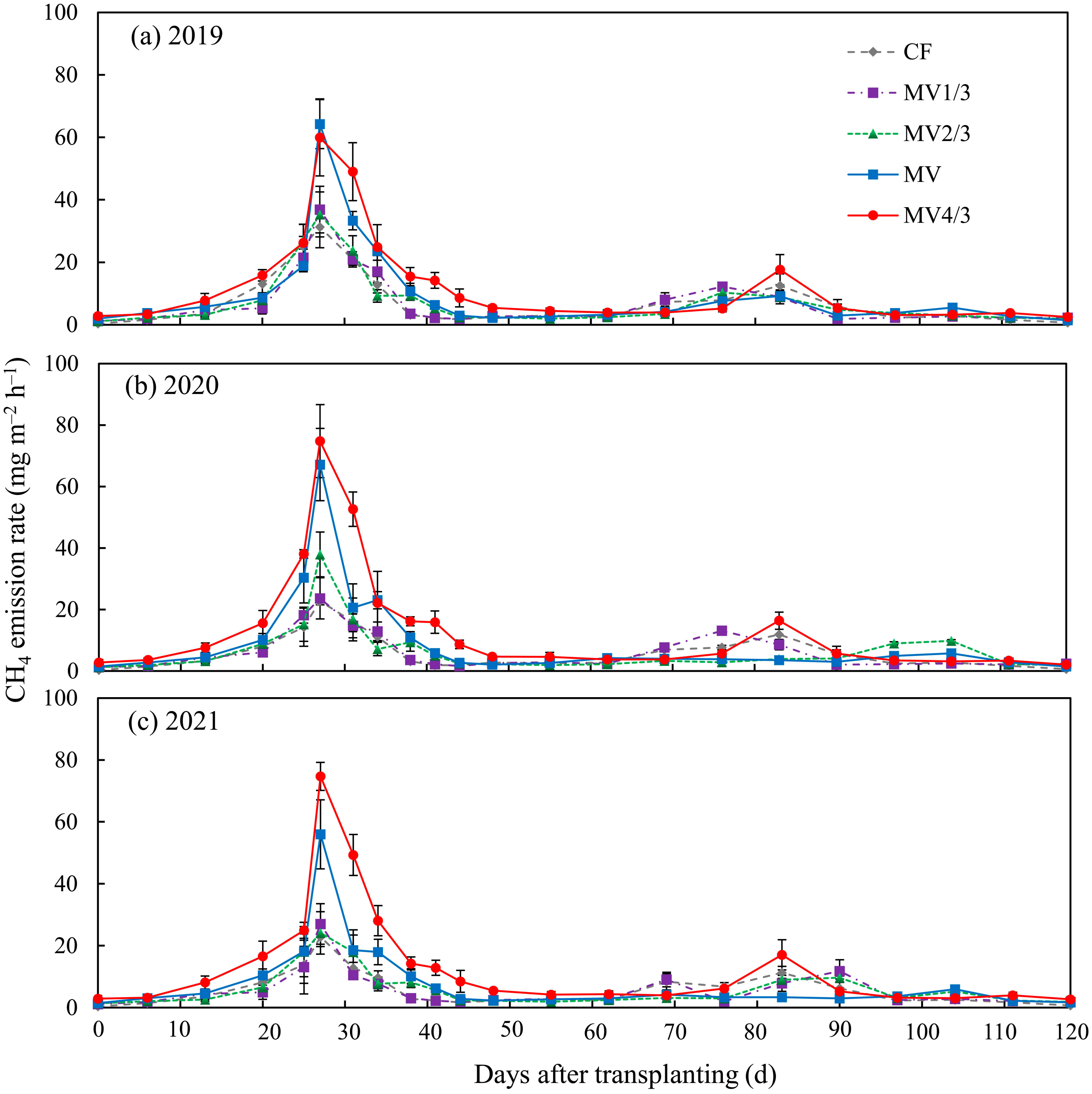

Figure 2. CH$_4$ emission rate affected by the substitution of synthetic N fertilizer with different amounts of Chinese milk vetch. The vertical bars denote the standard errors of the means (*n* = 3). CF, MV1/3, MV2/3, MV, and MV4/3 indicate incorporating 0, 7.5, 15.0, 22.5, and 30.0 t ha^{-1} of Chinese milk vetch, respectively, to partially substitute for urea.

Compared to the cumulative emissions of CH$_4$ under the CF treatments, those under the MV1/3 and MV2/3 treatments did not show a significant change, but those under the MV and MV4/3 treatments increased by 28.61% and 85.60% (2019), 32.38% and 103.19% (2020), and 28.86% and 102.98% (2021), respectively (Figure 4). The results suggested that when the CMV incorporation amounts were equal to or higher than the traditional incorporation levels (MV), the paddy soils promoted CH$_4$ emissions. However, the increased emissions were mitigated when the incorporated amounts decreased to one third (MV1/3) or two thirds (MV2/3). The cumulative emissions of N$_2$O ranked in the following order: CF ≈ MV1/3 > MV2/3 > MV ≈ MV4/3 (2019–2020) and CF ≈ MV1/3 > MV2/3 ≈ MV > MV4/3 (2021) (Figure 5). N_2O emissions tended to decrease with increasing CMV incorporation.

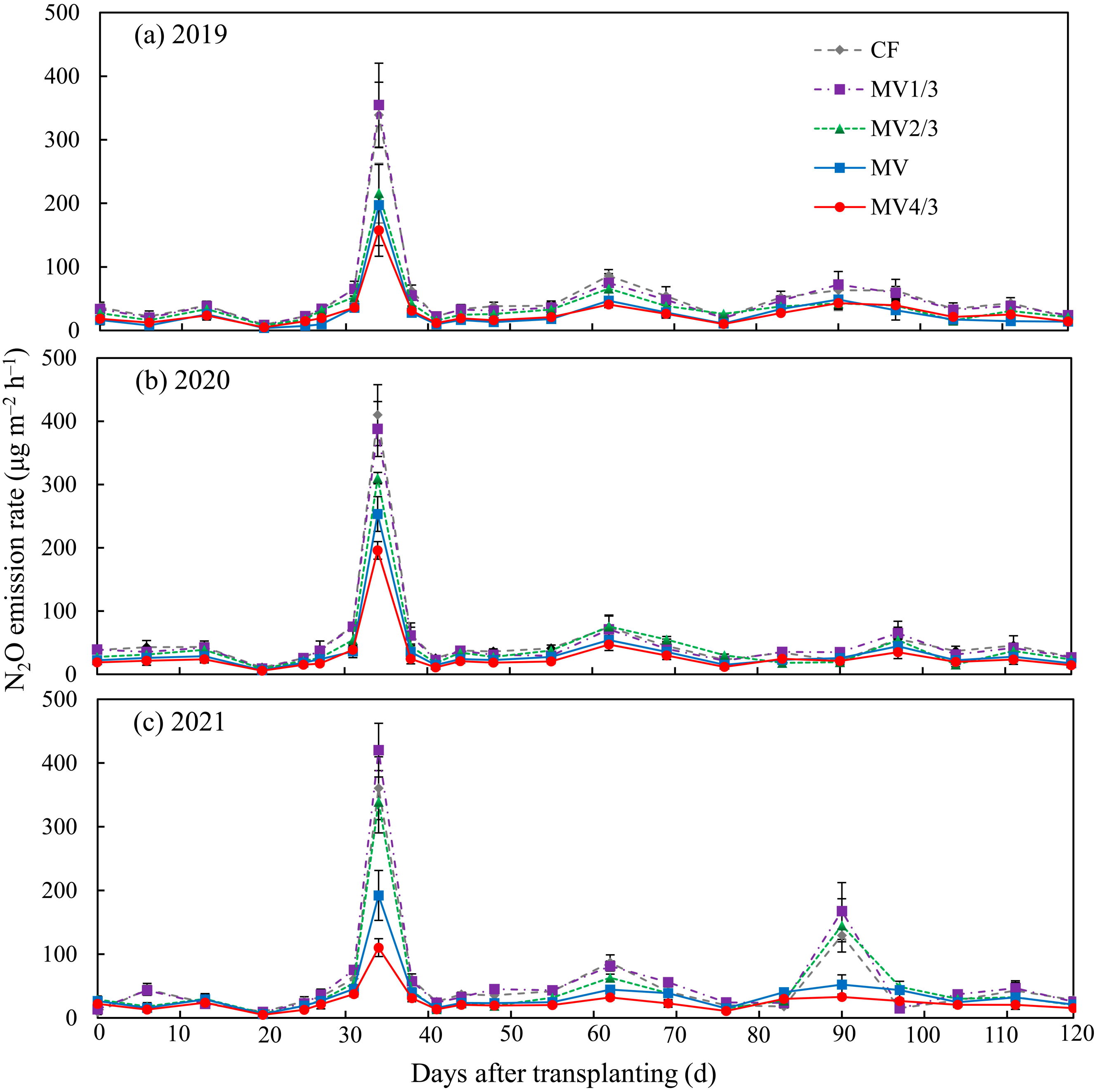

Figure 3. N_2O emission rate affected by the substitution of synthetic N fertilizer with different amounts of Chinese milk vetch. The vertical bars denote the standard errors of the means (n = 3). CF, MV1/3, MV2/3, MV, and MV4/3 indicate incorporating 0, 7.5, 15.0, 22.5, and 30.0 t ha^{-1} of Chinese milk vetch, respectively, to partially substitute for urea.

3.3. Global Warming Potential and Greenhouse Gas Intensity

The contribution of CH_4 emissions to the total GWP (92.38–98.23%) was much higher than that of N_2O emissions (1.77–7.62%) (Table 3). Although the MV and MV4/3 treatments significantly decreased the N_2O-induced GWP compared to the CF treatment, the former two treatments significantly increased the CH_4-induced GWP, leading to a higher total

GWP (except for the MV treatment in 2021). There was no significant difference in the total GWP among the CF, MV1/3, and MV2/3 treatments. However, due to the higher grain yield, the GHGI under the MV2/3 treatment showed a low value, ranging from 0.46 to 0.47 (Figure 1, Table 3).

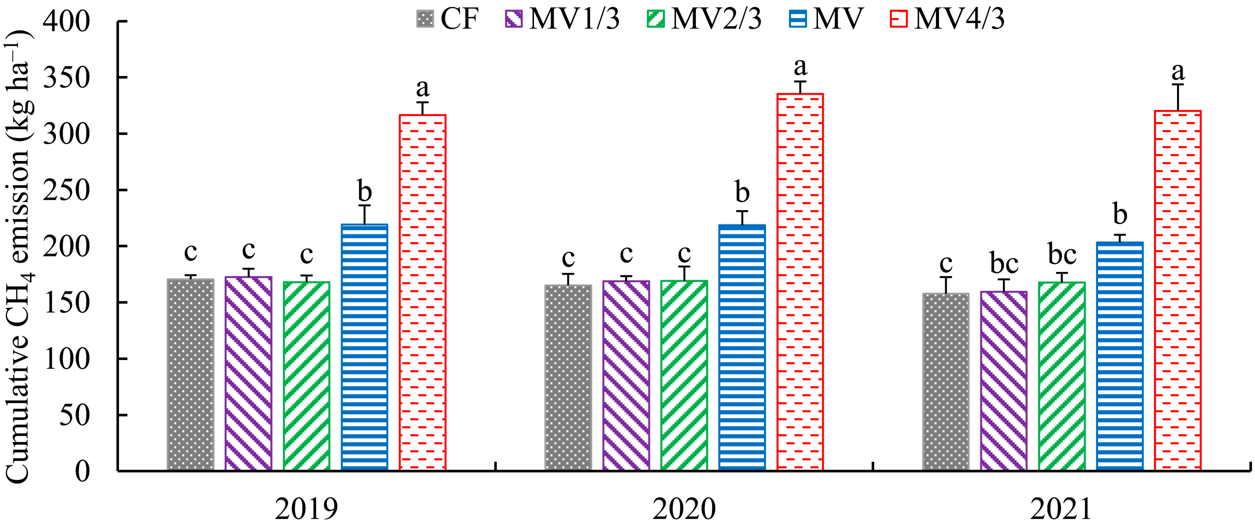

Figure 4. Cumulative emissions of CH_4 affected by the substitution of synthetic N fertilizer with different amounts of Chinese milk vetch. The vertical bars denote the standard errors of the means ($n = 3$). Different lowercase letters indicate significant differences among the means under different treatments in the same year ($p < 0.05$). CF, MV1/3, MV2/3, MV, and MV4/3 indicate incorporating 0, 7.5, 15.0, 22.5, and 30.0 t ha^{-1} of Chinese milk vetch, respectively, to partially substitute for urea.

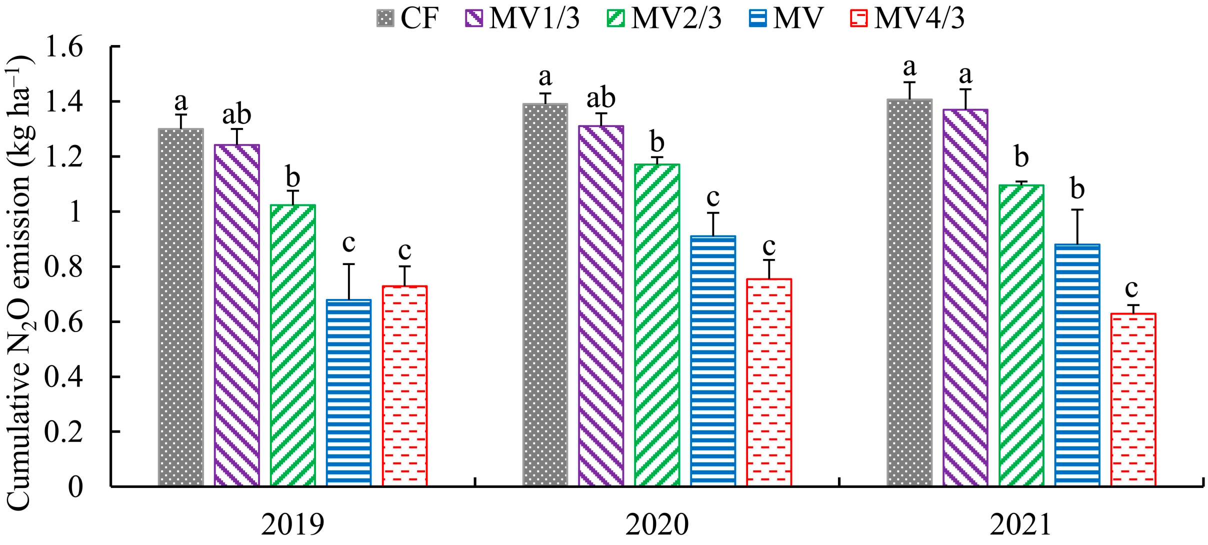

Figure 5. Cumulative emissions of N_2O affected by the substitution of synthetic N fertilizer with different amounts of Chinese milk vetch. Vertical bars denote the standard error of the mean ($n = 3$). Different lowercase letters indicate significant differences among the means under different treatments in the same year ($p < 0.05$). CF, MV1/3, MV2/3, MV, and MV4/3 indicate incorporating 0, 7.5, 15.0, 22.5, and 30.0 t ha^{-1} of Chinese milk vetch, respectively, to partially substitute for urea.

Table 3. Global warming potential and greenhouse gas intensity affected by the substitution of synthetic N fertilizer with different amounts of Chinese milk vetch.

Treatment	CH_4-Induced GWP		N_2O-Induced GWP		Total GWP (T CO_2 eq ha^{-1})	GHGI (kg CO_2 eq kg^{-1} Yield)
	GWP (T CO_2 eq ha^{-1})	Account for Total GWP (%)	GWP (T CO_2 eq ha^{-1})	Account for Total GWP (%)		
2019						
CF	5.08 ^{c}	93.47	0.36 ^{a}	6.53	5.44 ^{c}	0.64 ^{b}
MV1/3	5.14 ^{c}	93.81	0.34 ^{ab}	6.19	5.48 ^{c}	0.58 ^{b}
MV2/3	5.01 ^{c}	94.72	0.28 ^{b}	5.28	5.29 ^{c}	0.47 ^{c}
MV	6.54 ^{b}	97.27	0.19 ^{c}	2.73	6.72 ^{b}	0.56 ^{bc}
MV4/3	9.43 ^{a}	97.94	0.20 ^{c}	2.06	9.63 ^{a}	0.82 ^{a}
2020						
CF	4.92 ^{c}	92.77	0.38 ^{a}	7.23	5.3 ^{c}	0.59 ^{b}
MV1/3	5.15 ^{c}	93.35	0.36 ^{ab}	6.65	5.39 ^{c}	0.58 ^{b}
MV2/3	5.04 ^{c}	93.98	0.32 ^{b}	6.02	5.36 ^{c}	0.46 ^{c}
MV	6.35 ^{b}	96.34	0.25 ^{c}	3.66	6.76 ^{b}	0.60 ^{b}
MV4/3	10 ^{a}	97.99	0.21 ^{c}	2.01	10.2 ^{a}	0.94 ^{a}
2021						
CF	4.70 ^{c}	92.38	0.38 ^{a}	7.62	5.08 ^{b}	0.58 ^{b}
MV1/3	4.75 ^{bc}	92.69	0.37 ^{a}	7.31	5.13 ^{b}	0.57 ^{b}
MV2/3	4.99 ^{bc}	94.32	0.30 ^{b}	5.68	5.29 ^{b}	0.47 ^{b}
MV	6.06 ^{b}	96.21	0.24 ^{b}	3.79	6.3 ^{b}	0.56 ^{b}
MV4/3	9.54 ^{a}	98.23	0.17 ^{c}	1.77	9.71 ^{a}	0.89 ^{a}

Different lowercase letters indicate significant differences in the variable means among treatments in the same year at $p < 0.05$. CF, MV1/3, MV2/3, MV, and MV4/3 indicate incorporating 0, 7.5, 15.0, 22.5, and 30.0 t ha^{-1} of Chinese milk vetch, respectively, to partially substitute for urea.

3.4. Abundances of Functional Genes

Compared with the CF treatment, the MV and MV4/3 treatments significantly increased the *mcrA* gene abundances by 143.84% and 174.04%, respectively (Figure 6a). Moreover, the two treatments significantly increased the *pmoA* gene abundances by 61.01% and 57.80%, respectively (Figure 6b). The copy numbers of *mcrA* were more than ten times higher than those of *pmoA* in all the treatment groups, indicating that methanogens were dominant in paddy soils. The MV and MV4/3 treatments significantly decreased the copy numbers of AOA by 63.03% and 62.43%, respectively, compared to those in the CF treatment (Figure 6c). Compared with the CF treatment, CMV incorporation treatments did not significantly affect the copy numbers of AOB (Figure 6d), *nirK* (Figure 6e), and *nirS* (Figure 6f), whereas the MV2/3, MV, and MV4/3 treatments increased the *nosZ* gene abundances by 159.42%, 250.50%, and 338.26%, respectively (Figure 6e).

3.5. Partial Least-Squares-Path Model and Correlation Analysis

To explore the cause-effect relationships among the substitution ratios of urea with CMV, soil properties, abundances of functional genes, and GHG emissions, a partial least-squares-path model (PLS-PM) was constructed (Figure 7). The model was assessed using a goodness-of-fit (GoF) statistic, and the GoF value was 0.721, indicating that the model fit well. The results showed that *mcrA* gene abundances rather than the *pomA* gene had a strong and positive effect (0.930) on CH_4 emissions. The *nosZ* gene abundances negatively regulated N_2O emissions (−0.878). The *mcrA*, *pmoA*, and *nosZ* gene abundances were positively affected by soil nutrients with path coefficients of 0.592, 0.437, and 0.640, respectively. Moreover, *mcrA* and *nirS* gene abundances were positively affected by the soil pH, with path coefficients of 0.365 and 0.437, respectively. CMV-urea ratios positively regulated the pH (0.827) and soil nutrients (0.889). Overall, by directly affecting the soil pH and nutrients, the partial substitution of urea with CMV indirectly regulated the *mcrA* and *nosZ* gene abundances and thus affected CH_4 and N_2O emissions.

A Pearson correlation was performed to reveal the relationships between the abundances of functional genes and soil nutrients (Figure S2). The *mcrA* gene abundances were positively associated with DOC, TN, AP, and AK. The *pmoA* gene abundances were positively correlated with DOC, TN, and AP. The *nosZ* gene abundances were positively correlated with SOC, DOC, AP, and AK.

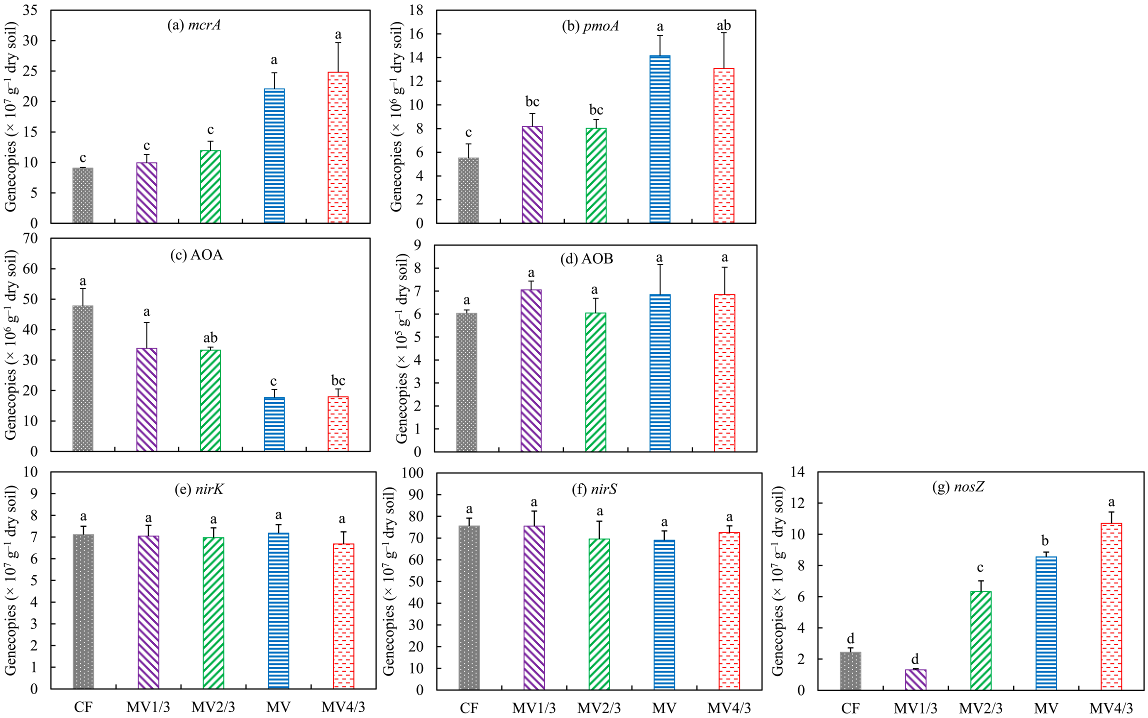

Figure 6. Abundances of the functional genes affected by the substitution of synthetic N fertilizer with different amounts of Chinese milk vetch. Samples were collected in 2021. The vertical bars denote the standard errors of the means (n = 3). Different lowercase letters indicate significant differences among the means under different treatments ($p < 0.05$). CF, MV1/3, MV2/3, MV, and MV4/3 indicate incorporating 0, 7.5, 15.0, 22.5, and 30.0 t ha^{-1} of Chinese milk vetch, respectively, to partially substitute for urea.

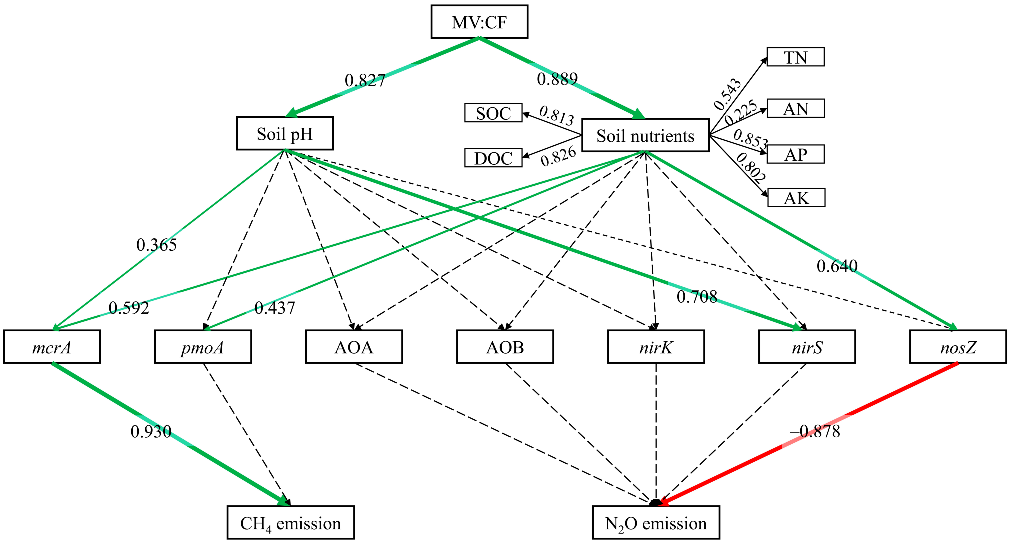

Figure 7. Directed graph of the partial least-squares-path model. MV:CF denotes the ratio of N from Chinese milk vetch to N from urea. SOC: soil organic carbon, DOC: dissolved organic carbon, TN: total nitrogen, AN: available nitrogen, AP: available phosphorus, AK: available potassium. Each box denotes an observed or latent variable. SOC, DOC, TN, AN, AP, and AK were loaded for the latent variable of soil nutrients. The numbers on the arrows and the arrow widths represent the path coefficients. The green and red arrows reflect positive and negative effects, respectively ($p < 0.05$). The dashed arrows indicate that the coefficients do not differ significantly from 0 ($p > 0.05$).

4. Discussion

4.1. Optimizing the Substitution Ratios of Urea with CMV to Improve Rice Productivity without Increasing GHG Emissions

The incorporation of green manure improves rice productivity and soil nutrients [30], which the current study confirmed (Figure 1, Table 2). Possible reasons for the enhancement of rice yield are as follows: (1) CMV increases soil nutrient availability and rice nutrient uptake [31]. (2) Although CMV did not affect the AN concentration in the soil in the present study (Table 2), CMV incorporation can prolong N availability and synchronize N release with rice demands [32]. (3) Substituting chemical fertilizer with green manure enhances various physiological indicators of the rice leaves (such as chlorophyll contents, leaf area indexes, and net photosynthetic rates) and promotes the accumulation of photosynthetic products and their transportation to grains [30].

The CH_4 and N_2O emissions in all treatments peaked at the rice tillering stage (Figures 2 and 3), which was consistent with the results of other paddy field experiments [21,33,34]. The emission characteristics suggested that GHG mitigation strategies in paddy fields should be focused on this stage. The results from the current study indicated that a relatively high CMV-urea substitution ratio promoted CH_4 emissions and mitigated N_2O emissions, but a low ratio did not affect emissions (Figures 4 and 5). This may be the reason why the previous research results were inconsistent. The results also suggested that it was feasible to improve rice productivity without increasing GHG emissions by substituting synthetic N fertilizer with a suitable amount of green manure. Moreover, previous studies observed a trade-off correlation between CH_4 and N_2O in paddies [35,36], which was confirmed by our results (Figure S3).

The GWP is considered an important indicator of the relative importance of CH_4 and N_2O. The CH_4-induced GWP accounted for more than 90% of the total GWP (Table 3), confirming that CH_4 is the dominant GHG emitted from paddies [37]. To further mitigate GHG emissions, future research should focus on reducing CH_4 emissions. Substitution of synthetic N fertilizer with a great amount of CMV increased the total GWP because the deleterious effects of increased CH_4 emissions exceeded the beneficial effects of decreased N_2O emissions (Table 3). Although MV and MV4/3 significantly increased rice yields compared with CF, the two treatments significantly increased CH_4 emissions and the total GWP (except for MV in 2021) (Figures 1 and 4, Table 3). MV1/3 did not significantly increase the total GWP, but its effect on rice yield was unsatisfactory compared with other CMV incorporation treatments. MV2/3 presented an excellent yield-increasing effect and did not increase GHG emissions, which consequently resulted in a low GHGI value, ranging from 0.46 to 0.47. Therefore, incorporating CMV at 15.0 t ha^{-1} as a substitute for 77.1 kg ha^{-1} of urea (MV2/3) seems to be the optimal nutrient management strategy in the study area.

Incorporation of CMV with 22.5 t ha^{-1} in the MV treatment is also utilized in many other regions in China. A similar incorporation level (25.8 t ha^{-1}) has been reported in South Korea [8,31]. Therefore, the present results may provide a reference for these regions. However, GHG emissions from paddies are affected by many factors, such as climate, soil properties, rice variety, fertilizer type, and water management [38,39]. Therefore, the optimal incorporation amount of CMV (15.0 t ha^{-1}) should be validated in these regions. Considering that the decomposition rate and products of other cover crops differ from those of CMV, further experiments are needed to confirm whether the substitution ratio affects GHG emissions.

4.2. Effects of Substituting Urea with CMV on Soil Properties and Functional Gene Abundances

Urea application can acidify paddy soils due to the processes of nitrification and nitrate leaching [40,41]. This study confirmed that CMV incorporation can ameliorate acidification (Table 2, Figure 7) because (1) urea application was reduced and (2) the organic residue possessed a high pH buffering capacity [42,43]. The return of green manure has long been advocated and practiced to enhance soil fertility and increase soil C. External application of CMV can directly contribute to soil C input, increasing SOC and DOC content [44]. Moreover, the application of organic residues benefits rice growth and causes rice root exudates enriched with DOC to be secreted into the soil [14]. CMV roots secrete organic acids to activate soil-insoluble *p*, and the abundant root surfaces have a great attraction for K ions, converting unavailable K into available K [31], which explains the increased AP and AK values under the CMV treatments in this study (Table 2).

Soil acidification can inhibit soil microbial communities [45]. The PLS-PM results showed that a relatively high pH resulted in an increased gene abundance of *mcrA* and *nirS* (Figure 7). Methanogenic archaea are sensitive to increases in the pH of acidic paddy soil, and even a small increase from 6.3 to 6.6 will enhance the abundance of methanogens and stimulate CH_4 production [46,47], which was consistent with our results (Table 2, Figure 6a). Another study also reported that the increasing pH in soil under organic amendment positively regulates the gene abundance of *mcrA* [48]. The *nirS*-type denitrifiers were dominant and were sensitive to soil pH (Figures 6f and 7), and a significantly positive direct effect of pH on the *nirS* gene was also observed in a structural equation model study [49].

In the current study, soil nutrients had positive effects on the gene abundances of *mcrA*, *pmoA*, and *nosZ* (Figure 7). The abundance of methanogens was associated with DOC (Figure S2), indicating that this abundance was regulated by substrate availability. DOC contributes abundant C sources for methanogenic growth, and the decomposition of exogenous organic matter under flooding conditions accelerates the decrease in redox potential in paddy fields, which provides suitable environmental conditions for the rapid reproduction of methanogenic archaea [50]. As an important external source of DOC in paddies, organic residues positively regulate the dynamics of methanogens [51]. Other studies have similarly observed a significant relationship between DOC and methanotrophs [52,53], possibly because DOC promotes the proliferation of methanogens, increasing CH_4 production, and CH_4 is the sole C and energy source for methanotrophs [14,54]. Plant residue incorporation increases the assembly of nitrous oxide reductase (expression of the *nosZ* gene) by supplying labile carbon, an essential energy source for denitrifying bacteria [55]. Moreover, AP was another factor driving the increased gene abundances of *mcrA*, *pmoA*, and *nosZ* (Figure S2). As an indispensable element for nucleotides and adenosine triphosphate, the soil AP status affects microorganism proliferation [45,47,56]. We found that AK increased *mcrA* and *nosZ* gene abundances, probably because K plays a limiting factor in microorganism growth in terms of enzyme activity and cell osmotic pressure maintenance [45].

4.3. Effects of Functional Gene Abundances on GHG Emissions

CH_4 is the final product of methanogens. The *mcrA gene* encodes the alpha subunit of the enzyme methyl coenzyme M reductase, which catalyzes the terminal step in biogenic methane production. Therefore, gene copies of *mcrA* are widely used to reflect methanogen abundances and methanogenesis potentials [57]. Conversely, CH_4 can be utilized or oxidized by methanotrophs to produce organic tissue or CO_2. The *pmoA* gene encodes the subunit of the particulate methane monooxygenase that is found in all methanotrophs. Therefore, gene copies of *pmoA* are widely used to reflect the methanotroph abundances and CH_4 oxidation potentials [58]. The significant increases in both *mcrA* and *pmoA* gene abundances with higher CMV inputs implied enhanced methanogenesis and methane oxidation potentials in the paddy soil (Figure 6a,b). However, CH_4 emissions depend on the balance between methanogenesis and methane oxidation [54]. The PLS-PM results revealed that the gene abundance of *mcrA* rather than that of *pmoA* had important direct effects on CH_4 emissions (Figure 7), indicating that CH_4 production that masked CH_4 consumption was directly responsible for CH_4 emissions. Furthermore, the direct effects reported above were also observed in other studies [14,23], suggesting that the gene abundance of *mcrA* could be used as a predictor for CH_4 emissions in paddies with CMV incorporation. Moreover, in addition to the functional gene abundances, the community composition of methanogens and methanotrophs may affect CH_4 emissions, which needs to be further explored [14].

Nitrite reduction is the critical and rate-limiting step in the denitrification process. Nitrite reductase is divided into soluble copper-containing enzymes and cytochrome enzymes, encoded by *nirK* and *nirS* genes, respectively. Nitrous oxide reductase converts the greenhouse gas N_2O to harmless N_2 and determines the end product of denitrification, encoded by *nosZ* genes [59]. The *nosZ*/(*nirK* + *nirS*) ratio indicates the proportion of N_2O that is reduced to N_2 [60]. Under CMV incorporation, *nirK* and *nirS* gene abundances were not affected, while *nosZ* gene abundances increased (Figure 6e–g), suggesting fewer N_2O emissions. The PLS-PM results confirmed that *nosZ* gene abundances negatively regulated N_2O emissions (Figure 7). This finding indicated that the substitution of synthetic N fertilizer with CMV enhanced the capability of denitrifying bacteria to transform the greenhouse gas N_2O into N_2.

5. Conclusions

The impacts of CMV on rice productivity and GHG emissions were associated with substitution ratios. In conclusion, the partial substitution of synthetic N fertilizer with a large amount of CMV improved grain yield, ameliorated soil acidification, and benefited soil nutrients. However, relatively high substitution ratios indirectly increased the gene abundances of *mcrA* and *nosZ*, which stimulated CH_4 emissions and mitigated N_2O emissions. The increase in total GWP was mainly due to stimulated CH_4 emissions. Therefore, to improve rice productivity while mitigating GHG emissions in the study area, incorporating CMV at 15.0 t ha^{-1} to substitute for 77.1 kg ha^{-1} of urea is a more desirable practice than the common approach of using only synthetic fertilizer or the traditional CMV application rate (22.5 t ha^{-1}).

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13061636/s1, Figure S1: Monthly precipitation and daily ambient temperature during the experimental period. Figure S2: Pearson's correlations between the abundances of functional genes and soil nutrients.

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