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ORIGINAL PAPER

Procyanidin inhibited N_2O emissions from paddy soils by affecting nitrate reductase activity and *nirS*- and *nirK*-denitrifier populations

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Abstract

A 28-day microcosm experiment was conducted using three paddy soils (an alluvial paddy soil, a loess-formed paddy soil, and a yellow clayey paddy soil) to investigate the impact of procyanidin on N_2O emissions and associated microbial mechanisms. The efficacy of procyanidin on N_2O emissions varied among the paddy soils tested, with an average inhibition rate ranging from 2.7% in the alluvial paddy soil to 57.1% in the loess-formed paddy soil. Furthermore, suppression of N_2O emissions by procyanidin occurred alongside fluctuations in nitrate reductase activity and *nirS*- and *nirK*-type denitrifiers abundance. The correlation analysis indicates that nitrate reductase, clade I *nirS*-denitrifiers, clade I or II, and clade III *nirK*-denitrifiers were closely linked to N_2O emissions. These findings provide evidence that procyanidin is capable of limiting N_2O emissions in paddy soils by inhibiting nitrate reductase and different clades of *nirS*-/*nirK*-denitrifiers.

Keywords Nitrous oxide · Procyanidin · Nitrate reductase activity · Denitrifiers

Introduction

Nitrous oxide (N_2O) as a potent greenhouse gas that greatly contributes to global warming and stratospheric ozone depletion (Montzka et al. 2011; Ravishankara et al. 2009). Agricultural soils are a predominant source of anthropogenic N_2O, which accounts for about 60% of the global emissions (Bhatia et al. 2010). Production of N_2O in soils is principally associated with microbially-mediated nitrification and denitrification processes, occurring under aerobic and anaerobic conditions, respectively (Volpi et al. 2017; Zhang et al. 2015b). Paddy soils are submerged during most of the growing season; the submergence induces an anaerobic environment for microorganisms, resulting in denitrification as the dominant N_2O-producing process (Mathieu et al. 2006). The denitrification-derived N_2O emissions across paddy soils have caused massive losses of agricultural N (Lan et al. 2015; Wang et al. 2017) and exacerbated global warming. Therefore, developing optimal management strategies to curb N_2O emissions from paddy soils has been extensively researched (Linquist et al. 2015; Shaukat et al. 2019; Song et al. 2017; Xia et al. 2020).

Application of inhibitors, including both synthetic and biological origin, has been recommended as a potential option to mitigate agricultural N_2O emissions (IPCC 2014) and has been widely investigated across diverse agroecosystems (Akiyama et al. 2010; Qiao et al. 2015). The most regularly used and best understood are synthetic nitrification inhibitors to mitigate nitrification-induced N_2O emissions (Li et al. 2021a; Rodrigues et al. 2018). In contrast, very few inhibitors have been proven to be effective in regulating denitrification-derived N_2O emissions. Recently, a novel biological denitrification inhibitor (BDI) has been discovered from the roots of *Fallopia* spp. (Bardon et al. 2014). Bardon et al. (2016a) demonstrated that BDI from root extracts, a B-type procyanidin, can suppress soil microbial denitrification, by inhibiting denitrifiers (Galland et al. 2019). The addition of procyanidin to soil decreased N_2O emissions from denitrification (Bardon et al. 2017), but its efficacy

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depended on soil properties (Bardon et al. 2016a). However, the inhibitory efficacy of procyanidin on N_2O emissions has only been tested in a riparian soil (Bardon et al. 2017), but not in diverse agricultural soils, especially in paddy soils.

Denitrification is the fundamental biogeochemical pathway through which N_2O is produced (Tao et al. 2018), and it involves the transformations of NO_3^- and NO_2^- into gaseous forms of N, such as NO, N_2O, and N_2 (Zumft 1997). A key step in the process is the reduction of NO_3^- to NO_2^-, which is catalyzed by the nitrate reductase enzyme (NaR) (Enwall et al. 2005), and it is inhibited by procyanidin, likely by perturbing membrane stability (Bardon et al. 2016b). Among the other denitrification reactions, the reduction of NO_2^- to gaseous forms is catalyzed by the nitrite reductase enzyme (NiR). Functional genes encoding NiR (i.e., *nirS* and *nirK*) are commonly used as biomarkers in the analysis of the denitrifier population (Kuypers et al. 2018). Denitrifying organisms harboring the *nirS* or *nirK* gene are closely connected to N_2O emissions in soils (Cui et al. 2016; Fan et al. 2019; Qiu et al. 2018; Yin et al. 2017). Procyanidin inhibiting denitrification also affects the abundance of *nirS*- and *nirK*-containing denitrifiers (Galland et al. 2019). Therefore, procyanidin can regulate N_2O emissions during the denitrification process, both at the enzyme and soil population levels. However, the traditional primer pairs targeting *nirS* and *nirK* poorly cover some potentially abundant denitrifiers such as Burkholderiales and Rhizobiales bacteria in rice paddies (Yoshida et al. 2009), but not all functional clades involved in N_2O production (Jones et al. 2008; Saito et al. 2008; Wei et al. 2015).

This study dealt with the effect of procyanidin on N_2O emissions and associated microbial mechanisms in paddy soils. The main objectives of this study were to (i) assess the effect of procyanidin on N_2O emissions in different paddy soils; (ii) evaluate the inhibition of denitrifying enzymes (NaR and NiR) by procyanidin; and (iii) understand the relationship between N_2O emissions and the *nirS*- and *nirK*-type denitrifiers in the paddy soils amended with procyanidin.

Materials and methods

Site description and sample collection

The soil samples used for the experiment were collected from three geographically different sites. The three soils studied include an alluvial paddy soil, a loess-formed paddy soil, and a yellow clayey paddy soil; those soils were developed in contrasting environments and have different properties. The yellow clayey paddy soil was collected from Jinhua (JH, 29° 01′ N, 119° 28′ E), Zhejiang province; the loess-formed paddy soil was sampled from Jintan (JT, 31° 39′ N, 119° 28 ′E), Jiangsu province; and the alluvial paddy soil was collected from Qinhuangdao (QHD, 40° 7′ 34″ N, 119° 11′ 27″ E) in Hebei province. The three sites are located in typical rice-growing areas in China, with average annual precipitations of 1424 mm (JH), 1063 mm (JT), and 551 mm (QHD), and mean annual temperatures of 17.5 °C (JH), 15.3 °C (JT), and 11.2 °C (QHD). The yellow clayey paddy soil at JH was derived from Quaternary red earth, the loess-formed paddy soil at JT from Xiashu loess earth, and the alluvial paddy soil at QHD from an alluvial deposit. All three paddy soils were slightly acidic to neutral. At each sampling site, twelve cores (top 0–20 cm) were collected, mixed into a composite sample, and immediately transported to the laboratory. The soils were sieved (< 2 mm) to remove visible plant debris and coarse fragments, homogenized, then air-dried, and stored at room temperature before the construction of microcosms.

Determination of soil properties

Soil pH was determined using a glass electrode in 1:2.5 soil:water solution (w:v). Soil moisture content was measured gravimetrically after drying at 105 °C for 24 h. Soil organic matter (SOM) content was determined using the $K_2Cr_2O_7$ wet oxidation method (Kalembasa and Jenkinson 1973). Total N (TN) of soil was measured by Kjeldahl method using a Lachat flow-injection autoanalyzer (Lachat Instruments, WI, USA) (Bremner and Mulvaney 1982). Soil exchangeable NH_4^+-N and NO_3^--N were extracted by 0.01 M $CaCl_2$ and analyzed colorimetrically using an ultraviolet spectrophotometer (ThermoFisher Scientific, Massachusetts, USA) (Denmead et al. 1976; Norman et al. 1985). Total P (TP) was determined by molybdenum antimony blue colorimetry after digestion with $HClO_4$-H_2SO_4 (Thomas et al. 1967). Soil available P (Olsen P) was extracted with 0.5 M $NaHCO_3$ (Olsen et al. 1954) and measured colorimetrically (Murphy and Riley 1962). Available K (AK) was extracted by 1 M CH_3COONH_4 and quantified with a flame photometer (Inesa Instrument, Shanghai, China) (Standford and English 1949). Details of the soil physicochemical properties prior to the incubation experiment are presented in Table 1.

Soil microcosm experiment

A microcosm experiment, comprising the control (Control) and procyanidin application (BDI) treatments, was established to investigate the effect of procyanidin on N_2O emissions in paddy soils. All treatments received ammonium sulfate ($(NH_4)_2SO_4$) and potassium nitrate (KNO_3) as N substrate and were replicated three times for each sampling point (see below). Soil microcosms consisted of 125-mL glass bottles, each containing 20 g of soil (oven-dried weight basis), were pre-incubated around 30% water-filled pore space (WFPS) for 7 days at 25 °C. We used a commercial

Table 1 Physicochemical properties of three paddy soils used in this study

Site	pH (H_2O)	SOM (g kg^{-1})	TN (g kg^{-1})	Exchangeable NH_4^+-N (mg kg^{-1})	NO_3^--N (mg kg^{-1})	TP (g kg^{-1})	Olsen P (mg kg^{-1})	AK (mg kg^{-1})
JH	5.14c[a]	30.71a	1.55a	75.25a	12.25c	0.92b	42.63a	48.49c
JT	6.85a	29.72a	1.47a	8.29c	63.13b	0.66c	10.38c	62.23b
QHD	6.17b	19.86b	0.86b	17.03b	105.33a	1.34a	13.18b	232.10a

JH Jinhua, *JT* Jintan, *QHD* Qinhuangdao

SOM soil organic matter, *TN* total N, *TP* total P, *AK* available K

[a]Data followed by different letters indicate significant differences among the soils ($P < 0.05$)

procyanidin (Macklin, Shanghai, China) with a composition of 99.06% procyanidin ($C_{30}H_{26}O_{12}$) and 0.87% water. The solution with or without procyanidin was added evenly over the soil surface, and finally we adjusted soil moisture at 80% WFPS to provide anaerobic conditions favorable for denitrification (Bateman and Baggs 2005; Hu et al. 2015; Huang et al. 2014). Nitrogen was added at a rate of 50 mg $(NH_4)_2SO_4$-N kg^{-1} and 50 mg KNO_3-N kg^{-1} soil. The procyanidin-amended treatment received 20 mg procyanidin, amounting to 1 mg g^{-1} of dry soil (Bardon et al. 2017). All glass bottles were sealed with plastic bottle caps fitted with butyl rubber stoppers, and then incubated at 25 °C in the dark for 28 days. The water loss during the incubation was determined by periodically weighing the bottles and deionized water was added to replenish the lost soil moisture.

Gas samples (10 mL) were taken from the headspace by a gas-tight syringe at 1, 2, 4, 7, 10, 13, 19, and 28 days after incubation. The concentration of N_2O was determined by gas chromatograph (GC-2010 Plus Shimadzu, Japan). After each sampling, all bottles remaining were ventilated for 20 min and then sealed again. Destructive soil samples were collected on days 0, 4, 7, 13, and 28 and monitored for exchangeable NH_4^+-N and NO_3^--N. Molecular analysis and enzyme activity assays were conducted on samples collected on days 0, 4, 13, and 28. The soil samples for molecular analysis were stored at − 80 °C until DNA extraction.

Denitrification enzyme activity

Denitrification enzyme activity (DEA) was measured using a method modified from Dassonville et al. (2011). Briefly, fresh soil (5 g oven-dry weight equivalent) was placed in 125-mL glass bottles and sealed with rubber stoppers. Then the air in each bottle was removed and replaced by a He/C_2H_2 mixture (90:10, v:v) to provide anoxic conditions and inhibit the N_2O-reductase. A nutritive solution (5 mL) containing KNO_3 (50 µg N g^{-1} dry soil), glucose (0.5 mg C g^{-1} dry soil) and glutamic acid (0.5 mg C g^{-1} dry soil) was added to the soil, followed by 5 h incubation at 25 °C with agitation (180 rpm). During the incubation, gas samples were taken at 1, 3, and 5 h and analyzed for N_2O using a gas chromatograph (GC-2010 Plus Shimadzu, Japan) equipped with an ECD detector. The slope of the linear regression was used to estimate DEA as the N_2O produced (g^{-1} h^{-1}).

Enzyme activity assays

The NaR activity was determined with α-naphthylamine-sulfanilic acid using KNO_3 as a substrate, and incubating the soil slurry for 25 °C in a gyratory shaker (180 rpm) (Abdelmagid and Tabatabai 1987). The NiR activity was measured with α-naphthylamine-sulfanilic acid using $NaNO_2$ as a substrate, and incubating the soil slurry anaerobically for 24 h at 30 °C in a gyratory shaker (180 rpm) (Pu et al. 2019). After incubation, the produced or reduced nitrite was determined at $\lambda = 520$ nm using an ultraviolet spectrophotometer (ThermoFisher Scientific, Massachusetts, USA) to estimate NaR or NiR activity. The NaR and NiR activities were expressed as mg $NO_2^- - N$ g^{-1} dry soil 24 h.

DNA extraction and quantitative PCR of *nirS* and *nirK* genes

Total DNA was extracted from a 0.5 g of soil sample, collected at 0, 4, 13, and 28 days, using the FastDNA SPIN Kit for soils (MP Biomedicals, Cleveland, OH, USA) according to the manufacturer's protocol. The DNA concentration and quality were checked by a Colibri Microvolume Spectrometer (Titertek Berthold, Germany).

Abundances of functional marker genes for microbial denitrification (*nirS* clade I, *nirS* clade II, and *nirK* clade I, *nirK* clade II, *nirK* clade III) were determined by quantitative real-time PCR in a LightCycler® 480II System (Roche Diagnostics, Switzerland). Particularly, the newly updated primers were adopted to achieve a more precise determination of *nirS*- and *nirK*-type denitrifiers (Wei et al. 2015). Dilutions (10-fold) of the soil DNA sample were used as the template for qPCR. Standard curves were generated using 10-fold serial dilutions of plasmids with insertions of target gene fragments. More details of

gene-specific primers, reaction mixture compositions, and thermal cycling conditions are listed in Table S1.

Statistical analyses

Data were analyzed by one-way ANOVA followed by an LSD post hoc test to determine the significance of treatments at each incubation time (SPSS version 20). Statistically significant differences were tested at $P < 0.05$. Linear and polynomial regressions were used to explore the relationship between cumulative N_2O emissions and exchangeable NH_4^+-N and NO_3^--N concentrations, DEA, or NaR activity, as well as the abundance of functional genes within each soil. Data analyses were performed using the R software (R Core Team 2021). Only correlations with a significance of $P < 0.05$ were presented on the scatter-plot diagrams.

Results

N_2O emissions

The cumulative N_2O emissions substantially increased over the incubation, but varied significantly among the three paddy soils ($P < 0.05$), with an order of JT > JH > QHD (Fig. 1). The addition of procyanidin greatly reduced cumulative N_2O emissions in JH and JT soils by 47.3% and 57.1%, respectively (Fig. 1a and b) ($P < 0.05$). However, no significant inhibition was observed between the control and procyanidin-treated soils with respect to cumulative N_2O emissions in the QHD soil (Fig. 1c).

Exchangeable $NH_4^+ - N$ and $NO_3^- - N$ dynamics during incubation

The change over time of exchangeable $NH_4^+ - N$ concentrations differed between paddy soils. The exchangeable $NH_4^+ - N$ concentration increased gradually in the JH soil, while it declined rapidly in the JT and QHD soils over the incubation (Fig. 2a–c). When compared with the control treatment, procyanidin application did not affect exchangeable $NH_4^+ - N$ concentrations throughout the incubation in the three paddy soils tested.

The dynamics of $NO_3^- - N$ concentrations in the incubation varied among the three paddy soils. The $NO_3^- - N$ concentration peaked on day 4 in the JH soil, and then declined gradually over time (Fig. 2d), but increased throughout the incubations in the JT and QHD soils (Fig. 2e and f). Procyanidin amendment significantly decreased the $NO_3^- - N$ concentrations in the JH and QHD soils after 7 days of incubation, compared with the control treatment (Fig. 2d and f) ($P < 0.05$). However, in the JT soil, procyanidin did not affect $NO_3^- - N$ concentration (Fig. 2e).

Denitrification enzyme activity

The denitrification enzyme activity (DEA) was higher in the JH and JT soils than in the QHD soil (Fig. 3) ($P < 0.05$). When compared with the control, the addition of procyanidin decreased the DEA in all three paddy soils; particularly in the JH and JT soils ($P < 0.05$), which had high denitrification activities.

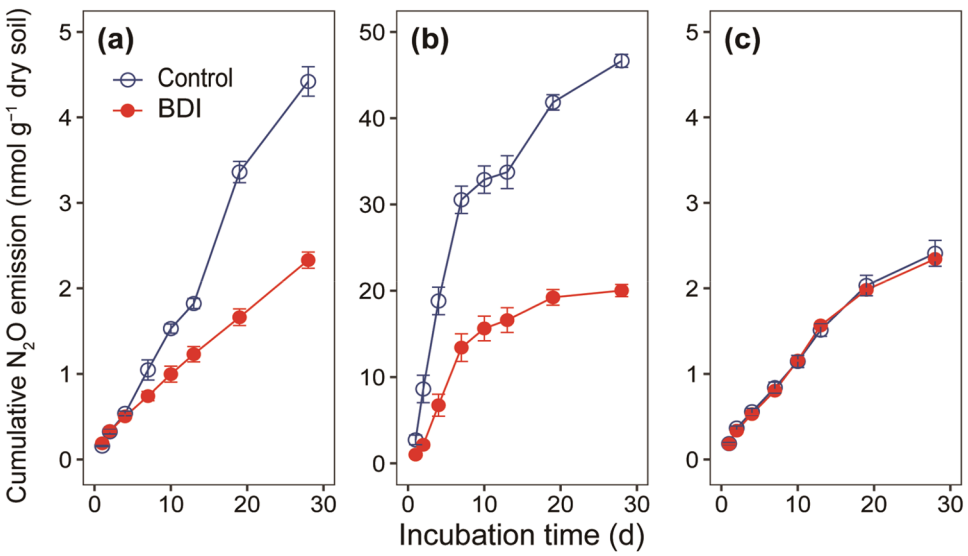

Fig. 1 Cumulative N_2O emissions in JH (**a**), JT (**b**), and QHD (**c**) paddy soils with N fertilizer addition (Control) and with N fertilizer plus procyanidin addition (BDI). Data are presented as mean values with standard errors ($n = 3$)

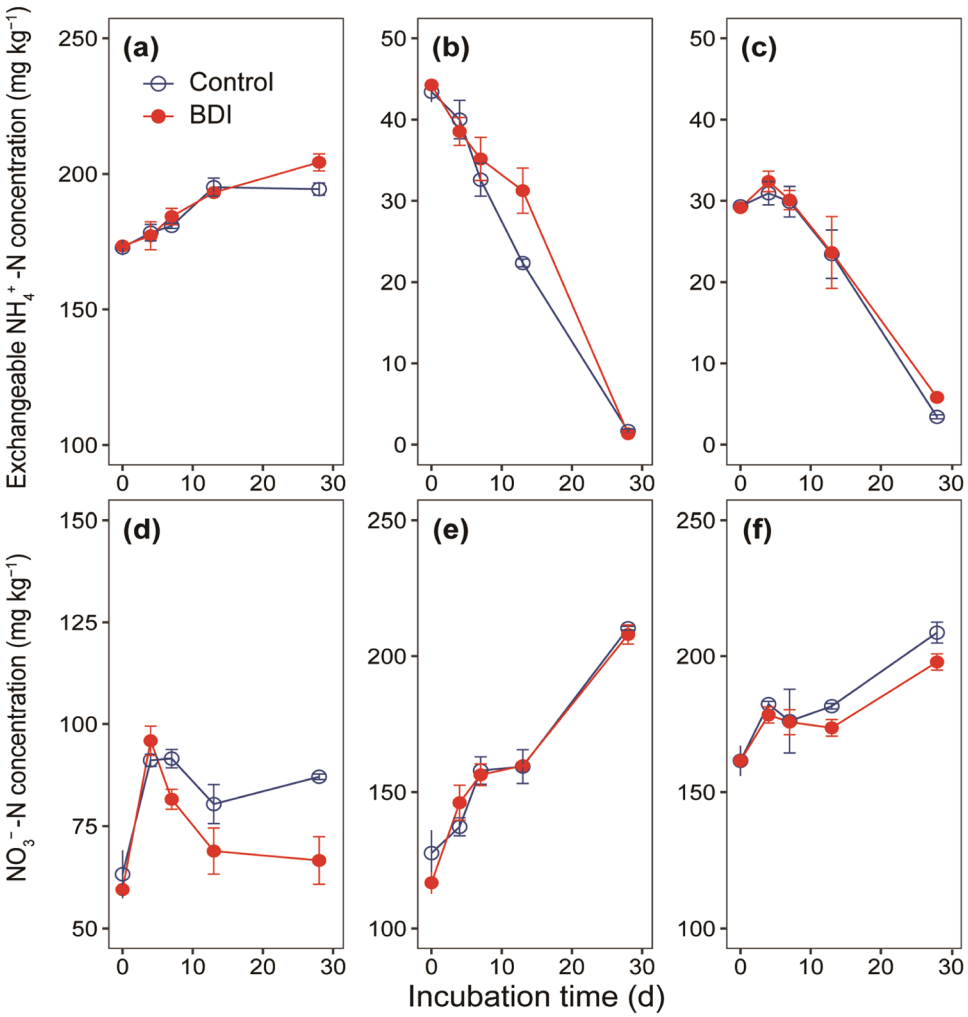

Fig. 2 The dynamics of exchangeable ammonium and nitrate concentrations in JH (**a** and **d**), JT (**b** and **e**), and QHD (**c** and **f**) paddy soils with N fertilizer addition (Control) and with N fertilizer plus procyanidin addition (BDI). Data are presented as mean values with standard errors ($n = 3$)

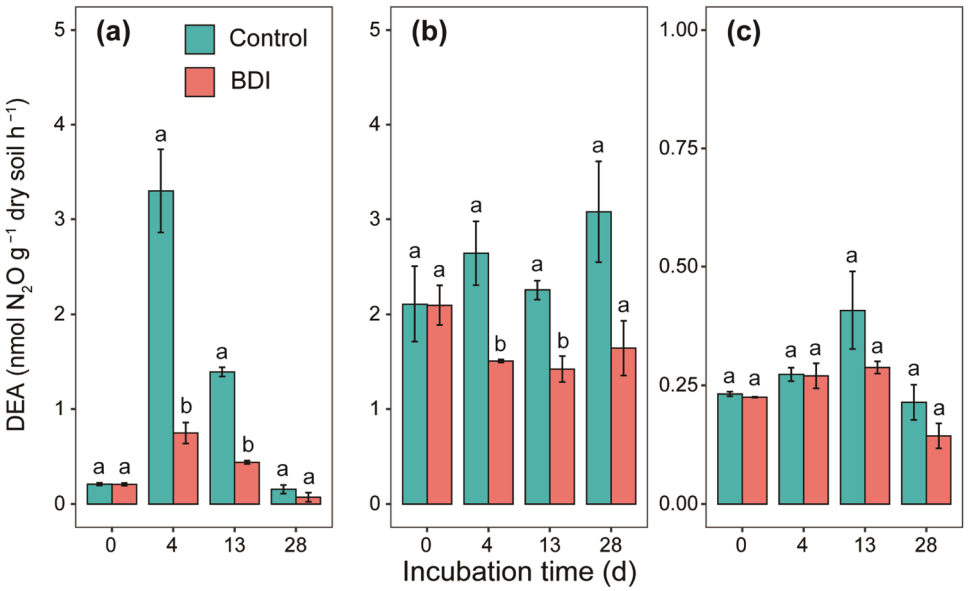

Fig. 3 The changes of denitrification enzyme activity (DEA) in JH (**a**), JT (**b**), and QHD (**c**) paddy soils with N fertilizer addition (Control) and with N fertilizer plus procyanidin addition (BDI). Data are presented as mean values with standard errors ($n = 3$). Different letters indicate significant differences between Control and BDI treatments at $P < 0.05$ at each incubation time

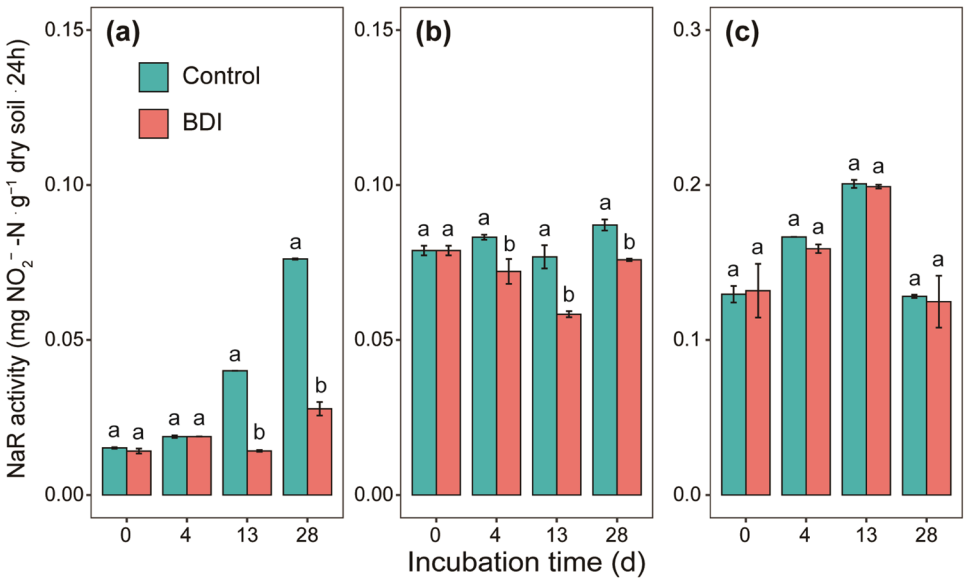

Fig. 4 The changes of nitrate reductase (NaR) activity in JH (**a**), JT (**b**), and QHD (**c**) paddy soils with N fertilizer addition (Control) and with N fertilizer plus procyanidin addition (BDI). Data are presented as mean values with standard errors ($n=3$). Different letters indicate significant differences between Control and BDI treatments at $P<0.05$ at each incubation time

Soil denitrifying enzyme activities

The addition of procyanidin significantly inhibited the NaR activity in the JH and JT soils (Fig. 4a and b) ($P<0.05$), but not in the QHD soil (Fig. 4c). Furthermore, procyanidin did not affect the NiR activity compared with the control (Fig. S1).

Abundances of *nirS* and *nirK* genes

The abundance of the clades of *nirS*- and *nirK*-harboring denitrifiers varied among the soils (Table 2), and showed contrasting responses to the procyanidin amendment (Figs. 5 and 6). The abundance of *nirS*- and *nirK*-denitrifiers was higher in the JT soil than in the other two soils (Table 2) ($P<0.05$). In addition, the procyanidin application significantly decreased the abundance of *nirS* clade I and II in the JH and JT soils ($P<0.05$), while no differences between treatments were found in the QHD soil (Fig. 5). Procyanidin significantly reduced the abundance of *nirK* clade I in the JH soil, but increased their abundance in the JT and QHD soils (Fig. 6a–c) ($P<0.05$). In contrast, procyanidin decreased *nirK* clade II gene copy numbers in the JH and JT soils ($P<0.05$), but not in the QHD soil (Fig. 6d–f). Finally, the procyanidin application reduced the population size of *nirK* clade III in all three soils (Fig. 6g–i) ($P<0.05$).

Relationship between cumulative N_2O emissions and soil properties and microbial dynamics

The linear and polynomial regressions were analyzed to explore potential relationships between cumulative N_2O

Table 2 The abundance of *nirS* and *nirK* genes in soil microcosms. One-way ANOVA was performed to evaluate the significance of difference between Control and BDI treatments within a soil at $P<0.05$ for each incubation time. The values are mean ± SE ($n=3$)

Site	Treatments	*nirS*[a] ($\times 10^6$ copies g^{-1} dry soil)				*nirK*[b] ($\times 10^6$ copies g^{-1} dry soil)			
		Day 0	Day 4	Day 13	Day 28	Day 0	Day 4	Day 13	Day 28
JH	Control	6.14 ± 0.01a[c]	8.28 ± 1.11a	14.90 ± 0.55a	16.20 ± 0.16a	0.99 ± 0.05a	7.57 ± 0.08a	34.85 ± 0.47a	11.54 ± 0.05a
	BDI	6.04 ± 0.19a	7.60 ± 0.03a	11.86 ± 0.10b	13.65 ± 0.13b	0.96 ± 0.07a	8.07 ± 0.56a	29.22 ± 0.54b	10.85 ± 0.05b
JT	Control	99.76 ± 0.27a	110.39 ± 1.32a	136.37 ± 0.38a	126.97 ± 1.99a	3.36 ± 0.12a	22.58 ± 0.15a	48.61 ± 0.20a	60.47 ± 2.33a
	BDI	98.30 ± 0.59a	93.17 ± 1.12b	115.01 ± 1.07b	105.92 ± 0.50b	3.11 ± 0.05a	15.95 ± 0.24b	43.22 ± 0.85b	47.17 ± 1.27b
QHD	Control	14.97 ± 0.16a	17.01 ± 0.70a	24.25 ± 0.31a	13.52 ± 0.05a	4.47 ± 0.03a	5.97 ± 0.13a	7.46 ± 0.10a	8.79 ± 0.31a
	BDI	15.48 ± 0.30a	17.51 ± 0.47a	24.33 ± 0.36a	13.95 ± 0.43a	4.20 ± 0.10a	5.74 ± 0.09a	7.28 ± 0.27a	8.32 ± 0.21a

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[a]The abundance of *nirS* is the sum of *nirS* Cluster I, and *nirS* Cluster II

[b]The abundance of *nirK* is the sum of *nirK* Cluster I, *nirK* Cluster II, and *nirK* Cluster III

[c]Different letters indicate significant differences between treatments within a soil at each incubation time ($P<0.05$)

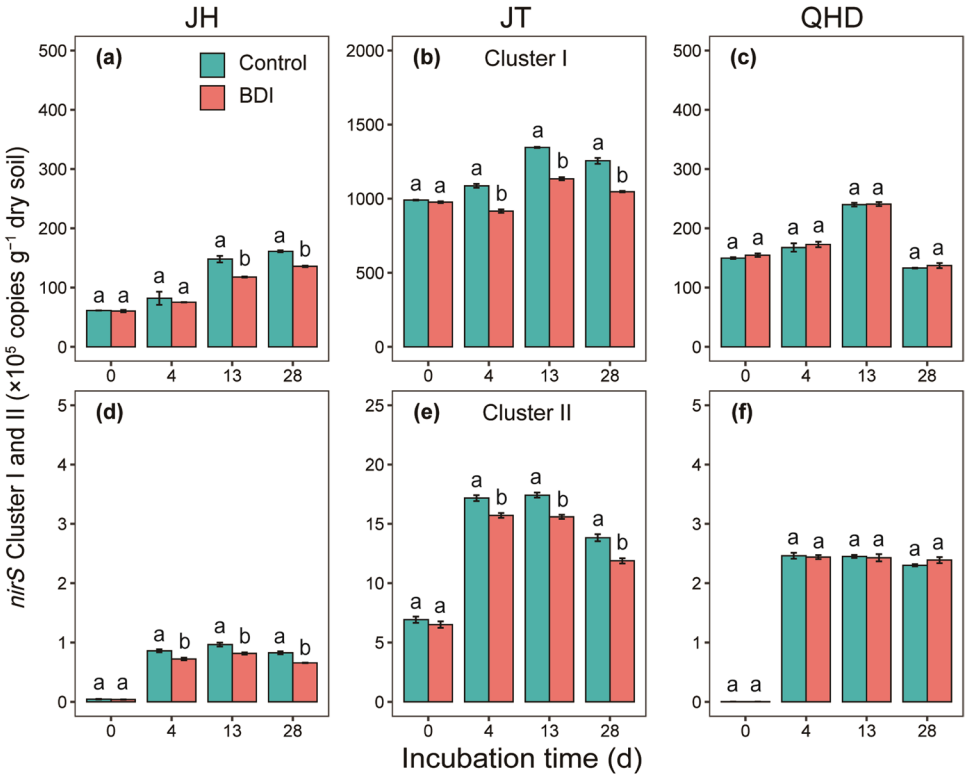

Fig. 5 The abundance of the *nirS* Cluster I and II in JH (**a** and **d**), JT (**b** and **e**), and QHD (**c** and **f**) paddy soils with N fertilizer addition (Control) and with N fertilizer plus procyanidin addition (BDI). Data are presented as mean values with standard errors ($n = 3$). Different letters indicate significant differences between Control and BDI treatments at $P < 0.05$ at each incubation time

emissions, soil properties, soil denitrifying activity, and the abundance of the functional genes (Fig. 7). The cumulative N_2O emissions (y) of the JH soil was related positively to exchangeable ammonium concentration (x) ($y = 0.081x - 13.611$), and negatively to DEA (x) ($y = 0.3233x^2 - 1.8236x + 2.9074$). The cumulative N_2O emissions (y) from the JT soil was related negatively to exchangeable ammonium concentration (x) ($y = -0.0197x^2 + 0.245x + 33.258$), and positively to nitrate concentration (x) ($y = 0.2635x - 21.067$) and DEA (x) ($y = 11.624x - 0.547$). The cumulative N_2O emissions (y) of the QHD soil was related negatively to exchangeable ammonium concentration (x) ($y = -0.0618x + 2.7192$), and positively to nitrate concentration (x) ($y = 0.0442x - 6.7734$).

The correlation analysis of cumulative N_2O emissions (y) with microbial abundance, shows that x is the $\log_{10}$-transformed gene copy number. The cumulative N_2O emissions (y) were positively related to the NaR activity (x) ($y = 58.273x - 0.0937$, JH; $y = 51607x^2 - 6771x + 235.82$, JT) and the population size of *nirS* clade I (x) ($y = 33.978x^2 - 469.21x + 1620.2$, JH; $y = 197.24x - 1564$, JT) in JH and JT soils, but negatively to the NaR activity (x) ($y = 383.19x^2 - 131.1x + 12.29$) and the population size of *nirS* clade I (x) ($y = 90.707x^2 - 1319.3x + 4797.3$) in the QHD soil. There was no significant relationship between cumulative N_2O emissions and the abundance of *nirS* clade II in any soil. Among the three soils, only the abundance of *nirK* clade I of the JH soil was positively related to cumulative N_2O emissions ($y = 5.4678x - 30.536$). However, the cumulative N_2O emissions were positively related to the population size of *nirK* clade II in the JT and QHD soils ($y = 112.33x^2 - 1628.5x + 5911.7$, JT; $y = 9.5575x - 63.978$, QHD). Furthermore, the abundance of *nirK* clade III was positively related to cumulative N_2O emissions in all three soils ($y = 4.2846x - 13.18$, JH; $y = 80.087x - 372.11$, JT; and $y = 2.2941x - 7.611$, QHD).

Discussion

Effects of procyanidin on soil mineral N

Our results consistently demonstrate that the application of procyanidin had little impact on the turnover of exchangeable $NH_4^+ - N$ in all tested soils (Fig. 2a–c), indicating that procyanidin most likely did not affect nitrification, but inhibited denitrification (Bardon et al. 2016a, 2017). The variable effect of procyanidin on soil $NO_3^- - N$ concentrations (Fig. 2d–f), as it decreased the $NO_3^- - N$ in the JH soil (Fig. 2d), was inconsistent with previous findings (Bardon et al. 2017; Galland et al. 2019, 2021). Microbial $NO_3^- - N$ immobilization and dissimilatory $NO_3^- - N$ reduction to NH_4^+-N (DNRA) compete with denitrification for NO_3^--N and can result in variable effects on soil NO_3^--N concentrations (Pandey et al. 2021; Zhang et al. 2015a). Procyanidin application might simultaneously enhance the microbial NO_3^--N immobilization and

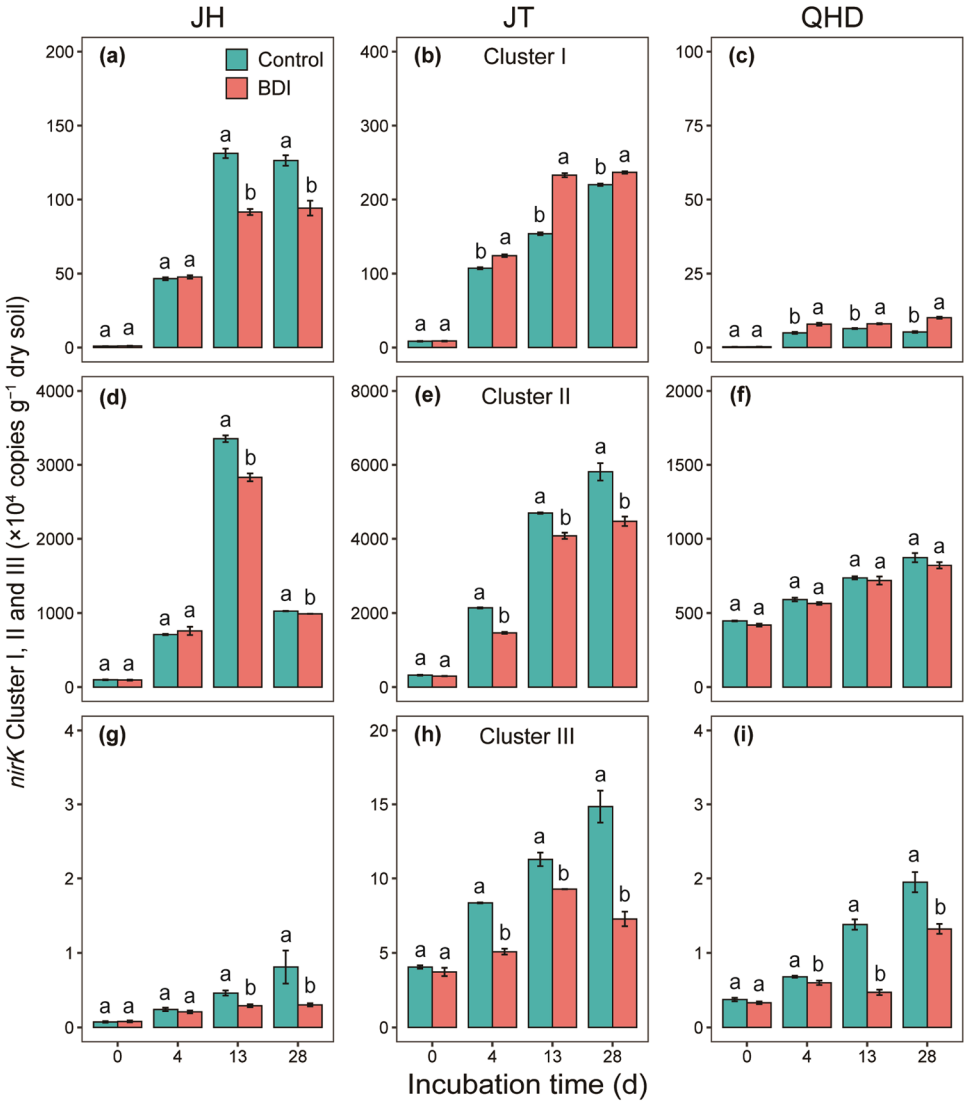

Fig. 6 The abundance of the *nirK* Cluster I, II, and III in JH (**a**, **d**, and **g**), JT (**b**, **e**, and **h**), and QHD (**c**, **f**, and **i**) paddy soils with N fertilizer addition (Control) and with N fertilizer plus procyanidin addition (BDI). Data are presented as mean values with standard errors ($n = 3$). Different letters indicate significant differences between Control and BDI treatments at $P < 0.05$ at each incubation time

DNRA processes by inhibiting denitrification, with more NO_3^--N being converted into the microbial biomass N pool (Cheng et al. 2017) and reduced to soil-retainable NH_4^+-N (Silver et al. 2001), and could therefore increase soil N retention. The JH soil in our study might possess high microbial NO_3^--N immobilization and DNRA rates due to the high SOM content in relation to NO_3^--N (Table 1) (Li et al. 2021b; Putz et al. 2018; Yoon et al. 2015). These suggest that the conservation of N might be responsible for the declined NO_3^--N concentration in the JH soil. However, further research employing stable isotope techniques is necessary to elucidate the mechanism for N conservation.

Effects of procyanidin on N_2O emissions in three paddy soils

The mitigatory effect of procyanidin on N_2O emissions varied dramatically among soils (Fig. 1), as the denitrification inhibition efficacy of procyanidin is highly dependent on soil properties (Bardon et al. 2016a). In addition, the different dominant denitrifiers among different paddy soils (Figs. 5 and 6) made it difficult for procyanidin to have consistent effects in different soils. Therefore, the contrasting effects of procyanidin on N_2O emissions were likely due to the variations in both soil properties and soil microbial community compositions.

The denitrification activity is an important factor affecting N_2O emissions generated from denitrification (Forte and Fierro 2019; Meng et al. 2020; Morse et al. 2012; Šimek et al. 2004). In this study, the inhibition of procyanidin on the denitrification activity was more effective in the JH and JT soils, which is consistent with the N_2O emissions (Fig. 1) and the denitrification enzyme activity data (Fig. 3), indicating that there is great potential for procyanidin to regulate denitrification-derived N_2O emissions in these two paddy soils. Such differences of the performance of procyanidin

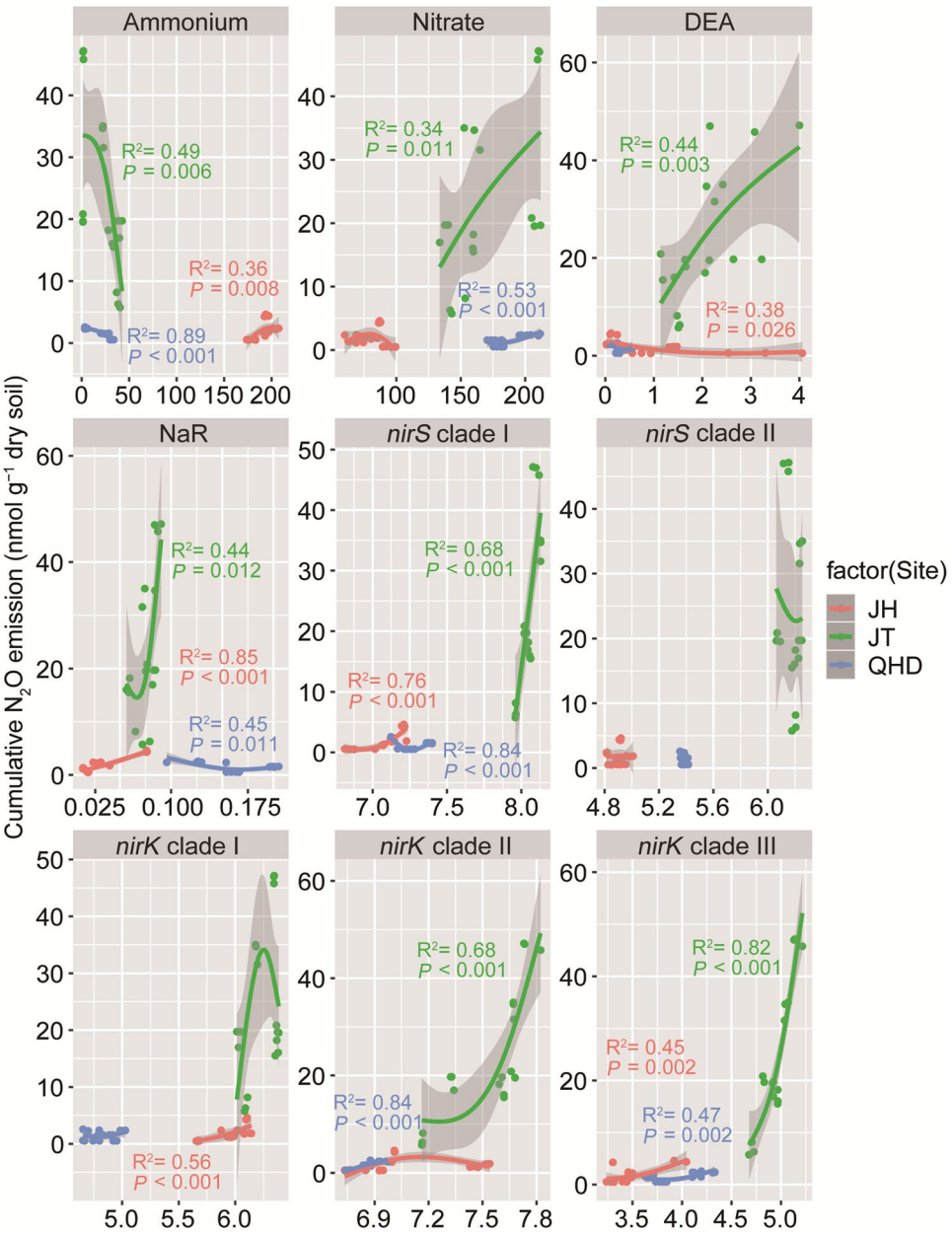

Fig. 7 Linear and polynomial regression relationships either between the cumulative N_2O emissions and ammonium, nitrate, DEA, and NaR activity or between the cumulative N_2O emissions and the abundance of N_2O-emission-related functional guilds. Only correlations that are significant at $P < 0.05$ are given. The shaded bands show 95% confidence intervals

in different soils can be attributed to differences in the SOM content among the soils, which was significantly higher in the JH and JT soils than in the QHD soil (Table 1); SOM is a major control of denitrification activity (Malique et al. 2019; Wu et al. 2017). The SOM contains readily decomposable organic C and may trigger denitrification by enhancing respiration (through the creation of anoxic microsites) and by providing energy for denitrifiers (Butterbach-bahl et al. 2013; Köster et al. 2015). Our results suggest that denitrification activity is more prevalent in SOM-rich soils, in agreement with previous findings (Chen et al. 2018; Malique et al. 2019; Yin et al. 2015). Therefore, our results imply that the use of procyanidin reduced N_2O emissions more effectively under conditions favoring denitrification, i.e., high soil moisture content (i.e., 80% WFPS) and high SOM content in the soil. The QHD soil typically had low denitrification activity (Fig. 3c), indicating that the contribution of denitrification to N_2O emissions was weak in this soil.

In addition, the adsorption of procyanidin to surface-reactive soil particles has been regarded as a crucial factor governing its effectiveness as a BDI (Galland et al. 2019), as procyanidin strongly binds to soil components (e.g., SOM, and metals) and becomes less bioavailable (Kraal et al. 2009). In this study, a lower efficacy of procyanidin in the JH soil (47.3%) compared to the JT soil (57.1%) (Fig. 1a and b) can partly be attributed to a greater adsorption of procyanidin in the JH soil, which has a higher SOM content (Table 1). It has also been shown that procyanidin

can chelate metals with their *o*-diphenol groups (Dixon et al. 2005). The available K concentration in our study was significantly higher in the QHD soil than in the other two soils (Table 1). Accordingly, the bioavailability of procyanidin might be impacted by the binding of K (Longo et al. 2018), which led to a low inhibition effect on N_2O emissions in QHD soil.

The sensitivity of dominant denitrifying microorganisms in soils to procyanidin may also result in differences in the efficacy of the BDI. The sensitivity of denitrifiers can be modulated by changes in the composition of the denitrifying community (Bardon et al. 2016a). The different compositions of the *nirS*- and *nirK*-harboring denitrifiers in soils in this study (Figs. 5 and 6) provides an explanation for the differential effects of procyanidin in the three paddy soils. Additionally, procyanidin can be used by soil microbes as a C and nutrient source and become degraded, thereby influencing its bioavailability (Bardon et al. 2016a; Kraus et al. 2004). The degree of degradation depends on the composition of the soil microbial community (Bhat et al. 1998), which is different in different habitats (Martiny et al. 2006; Nelson et al. 2016). The different degradation potential of procyanidin affects its bioavailability among the soils tested. However, the fate of procyanidin in different soils needs to be further investigated.

Possible microbial mechanisms for procyanidin to reduce N_2O emissions from paddy soils

Procyanidin drastically decreased N_2O emissions in the JH and JT soils but to a smaller degree in the QHD soil by inhibiting denitrifying enzyme activities, which is consistent with procyanidin significantly inhibiting NaR activity in the JH and JT soils and the slight inhibition in the QHD soil (Fig. 4), as well as with the literature (Bardon et al. 2016b, 2017; Galland et al. 2019). This suggests that the inhibition of NaR activity by procyanidin was responsible for decreasing soil N_2O emissions. However, no obvious inhibition of NiR activity was observed in any of the paddy soils (Fig. S1), likely due to NiR being distributed in the periplasm, making it less sensitive to the inhibitory effect of procyanidin than the membrane-bound NaR (Bardon et al. 2016b). These imply that procyanidin addition reduced N_2O emissions by directly affecting NaR rather than NiR activity in our study.

Different clades of *nirS*-/*nirK*-denitrifiers played different roles in producing N_2O in the paddy soils, with the abundance of *nirS* clade I denitrifiers significantly related to N_2O emissions in all three soils (Fig. 7). This might be because *nirS* clade I shared high homology with the sequences of denitrifying isolates, which have a high capacity to produce N_2O as the end product of denitrification (Wei et al. 2015). Furthermore, N_2O emissions significantly increased with the abundance of clade I and III *nirK*-denitrifiers in the JH soil, but increased with the abundance of clade II and III *nirK*-denitrifiers in the JT and QHD soils, suggesting that N_2O emissions were associated with different denitrifier clades in different soils (Chen et al. 2019; Saito et al. 2008).

We found that the response patterns of denitrifier clades to procyanidin addition varied among soils (Figs. 5 and 6). Our data therefore suggest that the reduction in N_2O emissions in the procyanidin-treated soils likely resulted from procyanidin-induced inhibition of the different clades of *nirS*-/*nirK*-denitrifiers. The divergent response patterns of these denitrifier clades to procyanidin may be linked to the differences in SOM content in different soils, as SOM strongly influences the size, composition, and activity of *nirS*- and *nirK*-type denitrifiers (Tables 1 and 2; Cui et al. 2016; Kandeler et al. 2006). Our data suggest that procyanidin was more effective when the denitrification-derived N_2O emissions were high. It is worth noting that procyanidin decreased *nirS*- and *nirK*-type denitrifiers abundance, but not NiR activity in the paddy soils, suggesting an indirect effect of less NO_2^--N due to reduction of NaR activity. Additionally, the abundances of *nirS*- and *nirK*-harboring denitrifiers were higher than those quantified by traditional primers, which only targeted *nirS* clade I/*nirK* clade I (Figs. 5a–c and 6a–c; Table 2). We believe conventional primers targeting *nirS* and *nirK* poorly cover some potential abundant denitrifiers, such as Burkholderiales and Rhizobiales bacteria (Wei et al. 2015; Yoshida et al. 2009). These denitrifying bacteria are known to be potential N_2O emitters in rice paddy soil (Saito et al. 2008).

Conclusions

Our study demonstrates for the first time that procyanidin, a biological denitrification inhibitor, can mitigate N_2O emissions from paddy soils, most likely by inhibiting the activity of nitrate reductase and the growth of the different clades of *nirS*-/*nirK*-denitrifiers. In addition, the efficacy of procyanidin in reducing N_2O varied dramatically among soils. The use of procyanidin has great potential for mitigating N_2O fluxes, especially in soils with a high soil moisture or organic matter content that favors denitrification. Identifying the key factors that control the efficacy of procyanidin is a crucial step in understanding the effect of BDI on denitrification in soils, thus encouraging wider adoption of this BDI to achieve environmental benefits. Moreover, it is important to examine whether applying procyanidin is feasible to limit gaseous N losses and increase rice yield under field conditions. Future studies need to evaluate the different types of environmental benefits of applying procyanidin in paddy fields.

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