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Microbial ureolysis and ammonium oxidation in rice (*Oryza sativa*) rhizosphere: Impact of different fertilizers

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ABSTRACT

Nitrogen is a crucial nutrient for rice (*Oryza sativa* L.) productivity, and chemical N fertilizers are often applied to enhance rice production. However, the response of soil microbial activity and corresponding functional genes to chemical fertilization remains unclear. The present study was carried out during rainy (*kharif*) seasons of 2019 and 2020 at the research farms of ICAR-Indian Agricultural Research Institute, New Delhi to study in the microbial responses to different concentration of nitrogen and fertilizers applied to the soil of rice fields. Study included a microcosmic experiment with 3 N concentrations (0, 10, and 100 mM), and treatment included were T_1, RDF; T_2, 50% N as urea and KNO_3 at 75:25 with PK; T_3, 50% N as urea and KNO_3 at 75:25 with PK and ammonium oxidizing microbial consortium. Nitrogen addition at 10 and 100 mM increased urease activity by 19–26%, potential ammonium oxidation (PAO) by 16–49%, and *ureC* gene copies by 10–22%. Indeed, treated soils possessed 1.2 to 6.5 folds' higher copies of archaeal- and bacterial *amoA*. In the field experiments, the rhizosphere of T_1 showed the highest urease and PAO activities while having the lowest activity of ammonification. The abundance of *ureC,* archaeal-, and bacterial *amoA* genes ranged from 2.9×10^6 to 2.0×10^7, 4.6×10^3 to 2.4×10^4, and 2.3 to 9.4×10^6 copies/g soil, respectively. The *ureC* gene copies were more abundant in T_1, while archaeal and bacterial *amoA* genes exhibited the highest copies in T_3. Urease activity and *ureC* copies were highest during the vegetative stage, while PAO, and archaeal- and bacterial *amoA* gene copies were enriched during the flowering stage. The gene abundance and associated enzymatic activities showed a strong correlation, implying that structural changes in the microbial community due to different combinations of fertilizers might alter the nutrient turnover in soil. Our results showed that N-fertilizers could significantly alter the structure and activities of microbial communities, and appropriate N fertilization is necessary for improving the sustainability of rice cultivation.

Keywords: Ammonia-oxidizing archaea, Ammonia-oxidizing bacteria, N-losses, qPCR, Rice rhizosphere, Urea hydrolysis

Nitrogen (N) is a vital nutrient for crop growth and productivity. Rice (*Oryza sativa* L.) acquires N in the form of ammonium, nitrate, or organic-N from soil. Nearly 9–10 million tonnes of N fertilizer are applied annually to paddy fields (Gruber and Galloway 2008). Since the agricultural soils in India are generally deficient in N, the application of urea has become an essential agronomic practice in rice cultivation. Owing to low nitrogen use efficiency (NUE) in rice, around 60–70% of applied N is lost *via* ammonia volatilization (NH_3), nitrification (NO_3^-), or denitrification (N_2O or N_2) with detrimental effects on the environment. Therefore, the challenge of managing the N inputs judiciously in rice cultivation is of greater importance (Kaur *et al.* 2022, Bahuguna *et al.* 2023).

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The N-fertilization practices not only influence plant productivity but also the diversity and functioning of the soil microbiome. The structural and functional traits of soil microbiome are sensitive indicators of soil fertility and quality (Song *et al.* 2020). For example, the urea hydrolytic- and ammonium oxidation activities of soil microorganisms serve as common indicators for estimating the rates of ureolysis and ammonium oxidation in soil. Though many researchers have investigated the microbial hydrolysis of urea and ammonium oxidation, the abundance of key genes that encode urease (*ureC*) and ammonium monooxygenases (archaeal- and bacterial *amoA*) are poorly examined in rice soils of India. The response of ureolytic and ammonia-oxidizing microorganisms to the type and combinations of fertilizers applied to soils can differ. Information on the key genes that encode urease and ammonium monooxygenase in Indian rice soils is hardly available. Therefore, the present study was carried out to examine the microbial responses

to different concentrations of N applied to the soil in the microcosms, and different combinations of fertilizers in rice fields. Precise information on the genetic potential of microorganisms associated with N-cycling can help to develop and adopt new agricultural practices for better retention of the applied N and to reduce N losses.

MATERIALS AND METHODS

Microcosm preparation and incubation: Rhizosphere soil samples were collected from the experimental fields of ICAR-Indian Agricultural Research Institute, New Delhi (28°40′ N, 77°12′ E). Soil is alluvial in origin, sandy clay loam (Typic Ustochrept) in texture and had *p*H 7.7, electrical conductivity 0.64 ds/m, organic carbon 6.8 g/kg, available N, and P were 163.7, and 14.7 kg/ha, respectively. The microcosms were prepared in 160 ml serum bottles using a 1.25:1 volumetric ratio of soil enrichment medium to soil samples (10 g) in triplicates. The soil enrichment medium contained: NaCl (1 g/l), $MgCl_2 \cdot 6H_2O$ (0.4 g/l), $CaCl_2 \cdot 2H_2O$ (0.1 g/l), KCl (0.5 g/l), $MgSO_4 \cdot 7H_2O$ (5 g/l), $NaNO_2$ (0.1 mM), and KH_2PO_4 (0.2 g/l), non-chelated trace elements solution (1 ml) and the vitamin solution (1 ml). The stock solutions of urea were prepared at 0, 1.308 g, and 13.08 g urea/litre to obtain 0, 10, and 100 mM N and were then used in the soil enrichment medium to have the microcosms with 3 different nitrogen (N) concentrations (0, 10 and 100 mM). The microcosms were incubated for 14 days at room temperature and then the soil samples were drawn.

Field site and sampling of rhizosphere soils: The field experiments were conducted during the rainy (*kharif)* seasons of 2019 and 2020 at the research farm of the ICAR-Indian Agricultural Research Institute, New Delhi. The location's climate is of the sub-tropical and semi-arid type with hot and dry summer and cold winter in the agro-climatic zone of "Trans-Gangetic Plains". The experiment was laid down in a randomized block design using cv. Pusa Basmati 1509 under conventional flooding method with following treatments: T_1-recommended dose of fertilizers (110, 60, 50 NPK kg/ha, as prilled urea, single super phosphate, and muriate of potash, respectively); T_2, 50% N with urea and potassium nitrate (KNO_3) at a ratio of 75:25 with full doses of PK; and T_3, 50% N with urea and KNO_3 at a ratio of 75:25 with full doses of PK and a consortium of ammonium oxidizing microbial isolates (AOM's). The AOM-consortium was applied at the time of transplanting while urea and/or KNO_3 was given in two splits; the first application of 55 kg N/ha for the T_1 and 27.5 kg N/ha for all other treatments as basal dose along with PK, and the second application was given at tillering stage. The rhizosphere soil samples were collected at the vegetative stage, 3-days after the second split application of urea, and the flowering stage. Soil samples were analyzed immediately for soil enzymatic activities, and another set of soil samples were stored at -20ºC for extracting total soil DNA.

Measurements of soil enzymatic activities: Urease activity (µg NH_4-N/g soil/ha) was assayed using the Kandeler and Gerber (1988) method, while arginine ammonification (NH_4^+-N released µg/g soil) was estimated as described by Bonde *et al.* (2001). The potential ammonium oxidation activity (PAO) was calculated as µg NO_3-N released/g soil/ha following the procedure of Olsson and Flakengren-Grerup (2000).

Soil DNA extraction and quantitative PCR: Soil DNA was extracted using a Nucleopore gDNA soil kit (Genetix, New Delhi, India). The concentrations of extracted DNA were determined using Nanodrop 3300 spectro-fluorometry (Waltham, Massachusetts, USA). The abundance of *ureC,* ammonia-oxidizing archaea (*amoA*), and ammonia-oxidizing bacteria (*amoA*) were determined using quantitative PCR (qPCR) on a LightCycler® 96 Real-Time PCR System. The reaction volume of 20 µL contained: 10 µl of KAPA SYBR® FAST, 0.5 µl of each primer (10 µM) for each gene (Table 1), bovine serum albumin (1 µl of 10 mg/ml), 4 µl of DNA template (20 ng) and 4 µl nuclease-free water. The melt-curve analysis and agarose gel electrophoresis were used to confirm the specificity of amplification.

Statistical analysis: Data from the microcosmic experiment was assessed using one-way ANOVA to investigate treatment effects on tested parameters. Field data of each year was first subjected to a two-way ANOVA to assess significant differences between the treatments and rice growth stages and their interactions as fixed effects. Further, three-way ANOVA was carried out to test differences among years. Because there were no significant differences ($P>0.05$) across years, data from two years were combined and displayed in graphs. Significant differences ($P<0.05$) were computed and a multi-comparison was done

Table 1 Molecular markers used for the analysis

Gene marker	Primers (F/R)	Sequences	Annealing temperature (ºC)	References
ureC	UreC1F	AAGMTSCACGAGGACTGGGG	56	Koper *et al.* (2004)
	UreC2R	AGRTGGTGGCASACCATSAGCAT		
Archaeal *amoA*	Crenamo23F	ATGGTCTGGCTWAGACG	58	Tourna *et al.* (2008)
	Crenamo616r	GCCATCCATCTGTATGTCCA		
Bacterial *amoA*	Bamo143F	TGGGGRATAACGCAYCGAAAG	53	Mahmood *et al.* (2006)
	Bamo1315R	AGACTCCGATCCGGACTACG		

by Tukey's HSD. Pearson's correlation coefficient matrix was generated to unravel the interrelationships among enzymatic and molecular parameters across studied treatments spanning plant growth stages. Statistical analysis of collected data was performed using the OriginPro® 2022 (OriginLab, Massachusetts, USA).

RESULTS AND DISCUSSION

Response of ureolytic- and ammonia-oxidizing microorganisms to different N concentrations in microcosms: In the microcosmic experiment that was carried out with three different N levels (0, 10, and 100 mM N as urea), urease (EC 3.5.1.5) activities ranged from 25.6 to 32.3 µg NH_4-N/g soil/ha, highest in the soil treated with 100 mM N and the lowest were at 0 mM N (Fig. 1). For example, the addition of N at 10 mM and 100 mM increased urease activity by 19 and 26%, respectively. The enrichment of substrate (urea) in treated soils probably induced transcription of urease genes (*ureA, ureB,* and *ureC*), as many bacteria, archaea, and fungi have urease as the constitutive enzyme. Our findings corroborated with a meta-analysis from 64 studies which reported that N fertilization markedly boosted urease activity by 11.6% (Chen *et al.* 2018). Similarly, Xiao *et al.* (2023) also found that urease activity accelerated by 49% after N application. In fact, we observed increased urease activities with rising urea concentration. For example, soils with 100 mM N had 6.2% higher urease activity than the soils with 10 mM N. Arginine ammonification was not substantially different among treatments, although it was found to be slightly greater in the untreated soil (0.66 µg NH_4-N/g soil/ha) compared to the soils with 10-mM (0.64 µg NH_4-N/g soil/ha) and 100-mM N (0.63 µg NH_4-N/g soil/ha) (Fig. 1). This decrease in ammonification activities implies that the mineralization of organic N was inhibited with the application of N (Chen *et al.* 2018).

Intriguingly, the potential for ammonium oxidation (PAO) was significantly higher in the soil treated with 100 mM N (0.79 µg NO_3-N/g soil/ha) followed by the soil with 10 mM N (0.62 µg NO_3-N/g soil/ha) while control soil showed least ammonium oxidation (0 mM N, 0.54 µg NO_3-N/g soil/ha) (Fig. 1). This increase in PAO can be explained by higher ammonium availability in treated soils. Our results are consistent with previous reports of enhanced PAO activity in the fertilized (NPK) paddy soils (Wang and Huang 2021). Higher PAO in the treated soils signifies more N losses (NO_3^- leaching, N_2O, or N_2) from fertilized paddy soils.

The influences of N addition at 0, 10, and 100 mM on copies of *ureC,* and archaeal- and bacterial *amoA* provide a better understanding of these processes. The gene copies of *ureC* were in the ranges of 6.0 to 8.1×10^6 copies/g soil and induced by N addition at 10 and 100 mM (Fig. 1). These observations are consistent with a recent study in which paddy soil of Hapli-Udic Cambisol nature was found to contain 10^6 copies of *ureC*/g soil (Zhao *et al.* 2022).

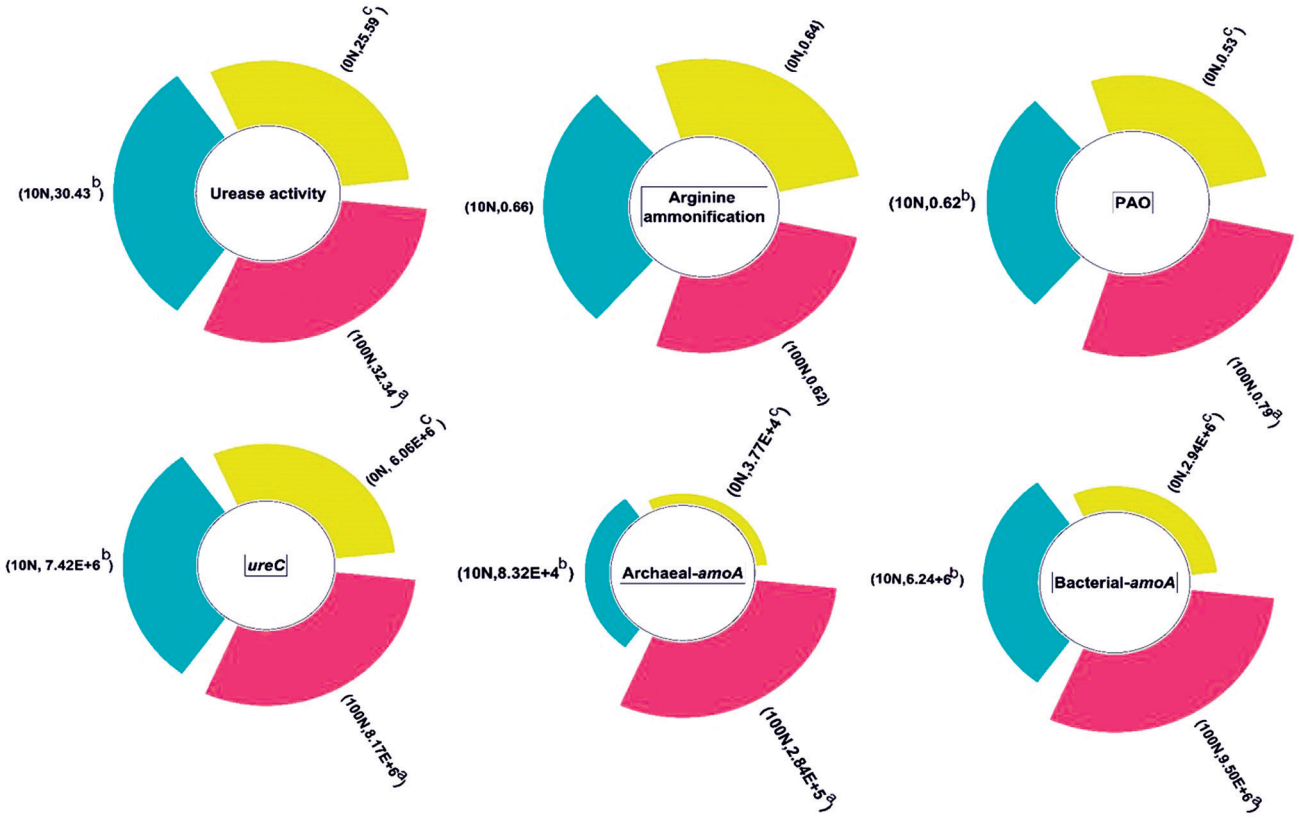

Fig. 1 Urease activity, arginine ammonification and potential ammonium oxidation (upper panel) and the abundance of *ureC,* archaeal- and bacterial *amoA* (lower panel) in the paddy soils.

Likewise, ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB) responded positively to N addition, with 1.2 and 6.5 folds higher abundances of archaeal- and bacterial *amoA* in soils with 10 and 100 mM N than the control (0 mM N). The abundance of archaeal *amoA* gene varied from 3.8×10^4 to 2.8×10^5 copies/g soil while the bacterial *amoA* copies were in the range of 2.9 to 9.5×10^6 copies/g soil, respectively (Fig. 1). These results contradict previous reports which stated that *amoA* of archaea was more prevalent in paddy soils than the bacterial-*amoA* (Zhang *et al.* 2016). Nevertheless, the report of Ke *et al.* (2015) on the functional domination of ammonia-oxidizing bacteria, rather than those of archaea in agricultural soils supports the findings of the present study.

Influences of different fertilizer combinations on ammonification, urea hydrolysis, and ammonia oxidation in the rhizosphere of rice under field condition: The enzyme activities related to urea hydrolysis and ammonia oxidation were characteristically dependent on the plant growth stage and fertilizer management practices. Urease activities ranging from 27.3 to 35.9 µg NH_4-N/g soil/ha, peaked directly after the fertilizer N addition and were maximum at the vegetative stage (Fig. 2). Further, the highest urease activity (µg NH_4-N/g soil/ha) was recorded in the rhizosphere of T_1 (31–36 µg) followed by the T_3 (27–33 µg) while T_2 (27–31 µg) exhibited the least urease activity. These findings corroborated with a recent study demonstrating that N fertilization in rice increased urease activity; the highest activity was observed at the vegetative stage which decreased significantly with later growth stages of rice (Zhong *et al.* 2023). Our results indicated that reduced N fertilizer inputs (50% N+RDPK) can curtail the N losses from soil and hence may improve N retention.

Arginine ammonification activities ranged from 0.62 to 0.78 µg NH_4-N/g soil/ha (Fig. 2). The ammonification activity was lowest in the rhizosphere of T_1 while it was highest in T_3. For instance, in comparison to T_3, T_1 and T_2 showed nearly 6 to 14% lower ammonification activities. Moreover, arginine ammonification improved with plant growth and was highest in the rhizosphere of T_3 (0.78 µg NH_4-N/g soil/ha) at the flowering stage (Fig. 2). Our observations of reduced ammonification activity in T_1 agree with the resource allocation model, which states that activities of nitrogen acquisition enzymes should decrease with N enrichment owing to the diminished N demand (Chen *et al.* 2018). The ammonium oxidation potential was measured in terms of µg NO_3-N released/g soil/ha which ranged from 0.47 to 0.72. Across treatments, the rhizosphere of T_3 showed the highest oxidation rates (except at the vegetative stage), followed by T_1 (Fig. 2). The ammonium oxidation potential improved as plant growth progressed, probably due to the increased capabilities of rice plants to transport molecular oxygen to the rhizosphere regions. Our observations of higher ammonium oxidation activities in T_3 imply the successful establishment and functioning of ammonia-oxidizing consortium in rice rhizosphere.

The gene copies of *ureC* were abundant, ranging from 2.90×10^6 to 2.01×10^7 copies/g soil in the rice rhizosphere, with highest copies in T_1. The results of the present study showed higher abundance of *ureC* during vegetative stage that was reduced by 46 to 55% at flowering stage (Fig. 3). The abundance of *ureC* in these soils could be due to the ubiquitous presence of ureolytic microorganisms in soils and the modern agronomic practice of urea-N application to increase rice productivity. In the soils of Wye Island, about 1–20% of bacterial communities were found to be ureolytic. Our results of higher abundance of *ureC* during the vegetative stage contradict the findings of Xu *et al.* (2023) who reported that urease activities peaked during the flowering stage of rice growth. On the other hand, the findings of Zhong *et al.* (2023) supported our results of maximum urease activities at the vegetative stage. These inconsistencies could be due to differences in the soil characteristics, microbial composition, or fertilizer application.

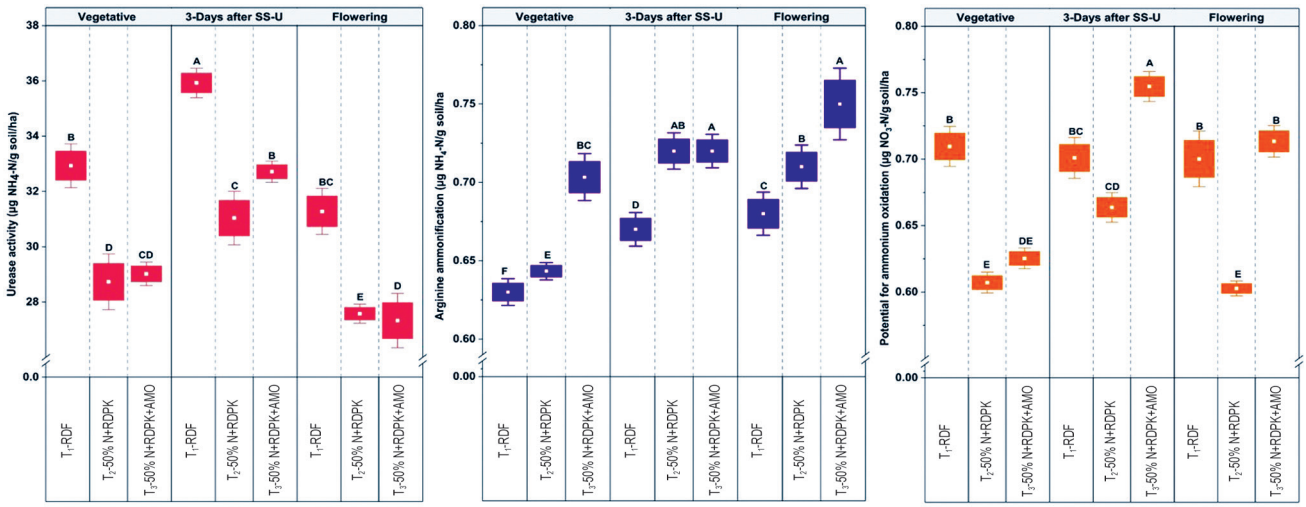

Fig. 2 Activities of urease, arginine ammonification and ammonium oxidation in response to different N fertilizer combinations in the rice rhizosphere.

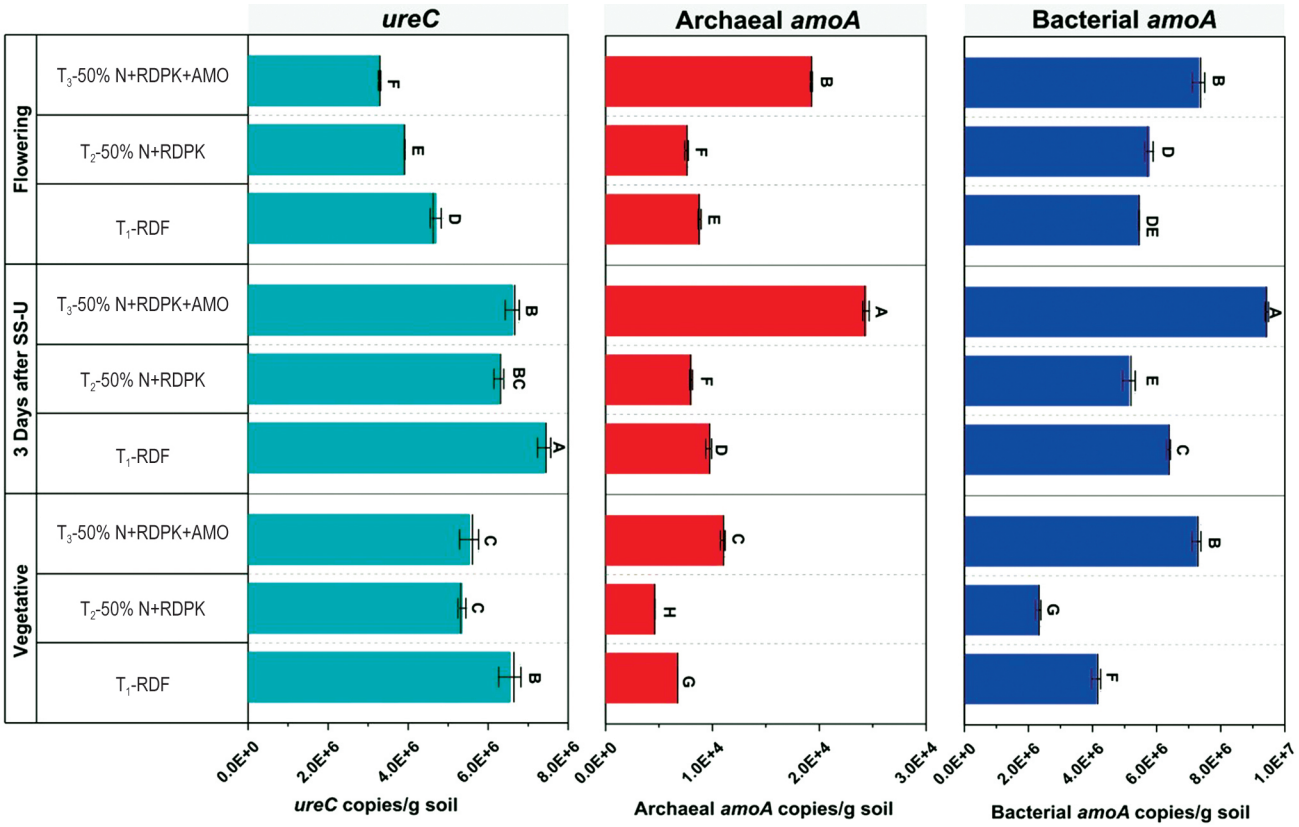

Fig. 3 The abundance of *ureC*, archaeal-, and bacterial *amoA* gene copies as influenced by different N fertilizer combinations in the rice rhizosphere.

In the rice rhizosphere, the abundance of *amoA* of AOA ranged from 4.6×10^3 to 2.4×10^4 copies/g soil while those *amoA* gene copies of AOB were about 2.30 to 9.4×10^6 copies/g soil. The addition of AOMs along with chemical fertilizers in T_3 resulted in the highest copies of archaeal- and bacterial *amoA* genes, followed by the T_1, while soils treated with T_2 possessed 75 to 81% lower copies of these genes (Fig. 3). These results suggest higher potential of AOMs to enhance ammonium oxidation under field conditions. Our findings of stimulated AOA and AOB in T_1 and T_3 corroborated with Ouyang *et al.* (2018) and Ren *et al.* (2023) who reported that the abundance of archaeal and bacterial *amoA* increased substantially after N fertilization in rice. Similar to these studies, we found that AOB dominated among the ammonia-oxidizing microbial communities in the rice rhizosphere. The significance of our results is that AOB is a more crucial target microbial group to decrease N loss and increase NUE. The abundance of archaeal and bacterial *amoA* increased with plant growth and was relatively higher during the flowering stage, while the least copies were recorded after the addition of basal N. These findings are in line with Xu *et al.* (2023) who observed the highest abundance of archaeal- and bacterial *amoA* at the flowering stage of rice.

Pearson's correlation analysis revealed a highly significant and positive correlation ($P<0.001$) among tested parameters across different N fertilization treatments and at distinct plant growth stages. The *ureC,* and archaeal- and bacterial *amoA* gene copies were significantly correlated ($P<0.001$) with their corresponding enzyme activities (arginine ammonification, urease activity, and potential ammonium oxidation). Indeed, urease activity was strongly correlated with the ammonification and ammonium oxidation activities (r=0.81 and 0.88, respectively, $P<0.001$). These results signify that molecular gene markers and enzymatic assays are sensitive indicators of N transformations and should be effectively utilized for developing N-management strategies.

Agricultural fertilization practices significantly alter the biochemical transformations of nutrients, which are essentially due to the activities of microbial communities. The present study investigated the influence of N fertilization on ureolytic- and ammonia-oxidizing microbial communities. The rhizosphere of rice, depending on the availability of essential nutrients that were modulated by chemical fertilizers or a combination of fertilizers with the AOM's consortium, could recruit a diverse array of ureolytic and ammonia-oxidizing microorganisms. Our results proved that excessive N supply stimulated microorganisms responsible for N-losses from the soil. Since the environmental impacts of fertilizer N are huge relative to the agronomic benefits, adequate N fertilization is highly warranted to make rice cultivation sustainable.

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