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RESEARCH ARTICLE

Mitigation of yield-scaled greenhouse gas emissions from irrigated rice through *Azolla*, Blue-green algae, and plant growth–promoting bacteria

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Abstract

Irrigated transplanted flooded rice is a major source of methane (CH_4) emission. We carried out experiments for 2 years in irrigated flooded rice to study if interventions like methane-utilizing bacteria, Blue-green algae (BGA), and *Azolla* could mitigate the emission of CH_4 and nitrous oxide (N_2O) and lower the yield-scaled global warming potential (GWP). The experiment included nine treatments: T_1 (120 kg N ha^{-1} urea), T_2 (90 kg N ha^{-1} urea + 30 kg N ha^{-1} fresh *Azolla*), T_3 (90 kg N ha^{-1} urea + 30 kg N ha^{-1} Blue-green algae (BGA), T_4 (60 kg N ha^{-1} urea + 30 kg N ha^{-1} BGA + 30 kg N ha^{-1} *Azolla*, T_5 (120 kg N ha^{-1} urea + *Hyphomicrobium facile* MaAL69), T_6 (120 kg N ha^{-1} by urea + *Burkholderia vietnamiensis* AAAr40), T_7 (120 kg N ha^{-1} by urea + *Methylobacteruim oryzae* MNL7), T_8 (120 kg N ha^{-1} urea + combination of *Burkholderia* AAAr40, *Hyphomicrobium facile* MaAL69, *Methylobacteruim oryzae* MNL7), and T_9 (no N fertilizer). Maximum decrease in cumulative CH_4 emission was observed with the application of *Methylobacteruim oryzae* MNL7 in T_7 (19.9%), followed by *Azolla* + BGA in T_4 (13.2%) as compared to T_1 control. N_2O emissions were not significantly affected by the application of CH_4-oxidizing bacteria. However, significantly lower ($P<0.01$) cumulative N_2O emissions was observed in T_4 (40.7%) among the fertilized treatments. Highest yields were observed in *Azolla* treatment T_2 with 25% less urea N application. The reduction in yield-scaled GWP was at par in T_4 (*Azolla* and BGA) and T_7 (*Methylobacteruim oryzae* MNL7) treatments and reduced by 27.4% and 15.2% in T_4 and T_7, respectively, as compared to the T_1 (control). K-means clustering analysis showed that the application of *Methylobacteruim oryzae* MNL7, *Azolla*, and *Azolla* + BGA can be an effective mitigation option to reduce the global warming potential while increasing the yield.

Keywords Rice · Plant growth–promoting bacteria · Yield-scaled GWP emission · Methane · Nitrous oxide · Mitigation

Introduction

Climate change is undoubtedly a result of the enhanced greenhouse effect. IPCC (2014) reported that anthropogenic greenhouse gas (GHG) emission reached 49 Gigatons of CO_2 equivalent in 2010 at the global level. Agriculture is a source of anthropogenic emission of two of the major GHGs methane (CH_4) and nitrous oxide (N_2O) to the atmosphere. Emission from agriculture has been increasing with time due to increased requirement for feeding more than 7.6 billion global population leading to an intensification of farming practices. Anthropogenic emission from agricultural soil occupy over 13% of the total global GHG emission (Zhao et al. 2019) and play a significant role in global warming and climate change. Emission of CH_4 and N_2O from agriculture are about 47.5% and 72.3%, respectively, of the total emission (Ritchie and Roser 2018). Rice (*Oryza sativa* L.) is a stable food for more than 50% of the world's population and rice fields are a major source of CH_4 emission from agricultural soils (Shin et al. 2020; Bhatia et al. 2013). Generally, water

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management and nitrogen fertilizer application govern CH_4 and N_2O emission from rice. Standing water in lowland and irrigated rice provides suitable anaerobic environment which facilitates the process of methanogenesis by methanogenic bacteria (strictly anaerobic) consuming soil organic matter and liberating CH_4 as an end product (Malyan et al. 2016). Higher soil redox (Eh) potential existing in upland rice or aerobic condition during the cropping period favor higher N_2O emission (Kumar et al. 2020; Yu et al. 2001). Emission of CH_4 and N_2O from rice soil are effected by several factors such as soil Eh, pH, temperature, organic matter, fertilizer application, and water management (Bhattacharyya et al. 2019; Yao et al. 2019; Tan et al. 2018; Jain et al. 2016; Hussain et al. 2015).

Mid-season drainage for mitigation of CH_4 from submerged rice (Tariq et al. 2017) and N fertilizer management for lowering N_2O emission has been widely recommended for rice soils (Aliyu et al. 2021; Malla et al. 2005). This practice though has a limitation, as water management is a difficult task in lowland rice conditions. The farmers growing rice cannot risk draining their flooded fields, as there is no certainty of the next rainfall event and thus this practice is not followed by the farmers. Some chemical and fertilizer interventions such as application of sulfate and nitrate fertilizers for CH_4 mitigation have been reported from rice soil (Hussain et al. 2015; Ali et al. 2015), but a few of them may have environmental concerns. The application of nitrate-based fertilizer may be a source of N_2O emission through the denitrification pathway and may result in increased leaching of nitrate in frequently irrigated rice. Leaching and runoff of nitrate into ground and surface water, respectively, may impact human health. The application of ammonium sulfate may lead to a potential increase in ammonia volatilization (Choudhaury and Kenneday 2005). Ammonia volatilization is also an indirect potential source of N_2O emission (Inubushi et al. 1996) which contributes to global warming.

The application of *Azolla*-based biofertilizers may have beneficial effects on growth and yield of rice (Kollah et al. 2016; Dubey 2005). *Azolla* is a floating pteridophyte, occurs in symbiotic association with a nitrogen-fixing cyanobacterium *Anabaena azollae* (Nostocaceae family), and has been reported to reduce plant nitrogen requirement, but their impact on CH_4 and N_2O emission has been less reported. Some studies conducted in eastern India (Bharati et al. 2000) and in Southern China (Xu et al. 2017) have shown that the application of *Azolla* may reduce CH_4 emission, whereas opposite results were reported in the studies conducted in Northeastern China (Chen et al. 1997). In addition, *Azolla* may reduce NH_3 volatilization by lowering the pH of floodwater when urea is applied (Liu et al. 2017). Blue green algae (BGA) are photosynthetic nitrogen fixers and are free living. *Azolla* and BGA cyanobacteria both are oxygen-liberating biofertilizers and can reduce CH_4 emission by directly stimulating CH_4 oxidation at the soil–water interface and indirectly by promoting CH_4 oxidation in flooded paddy soils by increasing soil Eh and thereby inhibiting CH_4 production. Their impact on N_2O emission has not been reported in literature.

In addition, methane-oxidizing/utilizing bacteria (MOB) prevailing in aerobic zone of paddy ecosystem may utilize CH_4 produced by the methanogenic archaea. Methane emission from soil is the net balance of CH_4 production by methanogens in anaerobic layer followed by CH_4 oxidation by methanotrophs or MOB under aerobic conditions (Conrad 2007). MOB inhabits flooded rice soil due to the presence of CH_4 at the soil–water interface and near root hairs as a result of CH_4 leakage from root hairs (Aulakh et al. 2000; Dubey and Singh 2001). Methanotrophs have generally been considered to be obligate in nature, i.e., growing only on CH_4 as their sole source of carbon and energy. However, facultative methylotrophic organisms also have been found in major clades of microbial life such as gram-negative methylotrophs, belonging to the α, β, and γ subgroups of the proteobacteria, firmicutes, archaea, and yeasts which utilize C1 compounds including CH_4 to generate energy (Rani et al. 2021a, b; Iguchi et al. 2015). Methanotrophs and methylotrophs oxidize CH_4 to form formaldehyde, which is at the diverging point for further oxidation to CO_2 for energy source and assimilation for biosynthesis. The facultative methane- and/or methanol-utilizing bacteria can play a significant role in reducing the net methane flux by utilization of emitted methane at the source level (Rani et al. 2021a, b; Davamani et al. 2020). Previously, we isolated and characterized a large number of facultative methane-utilizing bacteria having plant growth–promoting traits from different rice-growing regions of India (Rani et al. 2021a, b). Among these isolates, the three isolates which showed significant methane utilization potential, i.e., *Methylobacterium oryzae* MNL7, *Hyphomicrobium facile* MaAL69, and *Burkholderia vietnamiensis* AAAr40, were used in the present study as bio-inoculant. *Hyphomicrobium facile*, an aerobic chemoorganotroph, has been used for denitrification of nitrate in drinking water treatment facilities (Liessens et al. 1993). Fewer reports on utilization of methane-utilizing bacteria as bio-inoculant for reducing methane emission through its oxidation at the source level are available in the literature; however, no significant findings have been reported on the role of different algal and bacterial based interventions in reducing the emissions of CH_4 and N_2O from the rice ecosystem.

Based on previous studies, we hypothesize that (1) application of biofertilizers alone and in combination may have a differential impact on CH_4 and N_2O emission and yield-scaled GWP emission and (2) microbial inoculations of methane-utilizing bacteria may result in significant reduction of CH_4 emission in submerged rice and on overall yield-scaled GWP emission. To test our hypothesis, a 2-year field study was conducted for quantifying CH_4 and N_2O emission, rice yield, and yield-scaled GWP under *Azolla*, BGA, and methane-utilizing bacteria in rice.

Materials and methods

Experimental site

The 2-year study was conducted during Kharif season of 2014 and 2015 at the research farm of Indian Agricultural Research Institute (IARI) (28°40′ N latitude and 77°12′ E longitude), New Delhi, India. The soil of experimental site had 46% sand, 32% silt, and 22% clay and bulk density of 1.39 g cm^{-3}. The initial soil had soil organic carbon of 0.59%, pH (1:2 soil/water) of 8.10, electrical conductivity of 0.43 dS m^{-1}, and CEC of 7.3 C mol (P^+) kg^{-1}. The climatic condition of the region is sub-tropical, semi-arid characterized by prolonged hot summer and rainfall occurring during late June to mid-September. Metrological data of the study site for both years are presented in Fig. 1. The average minimum and maximum temperature during the first and second growing season was 21.7 °C; 22.1 °C and 33.8 °C; 34.9 °C, respectively (Fig. 1). The rainfall was higher in the month of July during both years.

Experimental design and treatment details

Twenty-three-day-old seedlings of rice variety Pusa-1509 were transplanted at 20 × 15 cm spacing in the month of July during both cropping years. The experiment consisted of nine treatments in three replications arranged in randomized block design (Table 1). Nitrogen was applied through urea in three split of 50% (basal) and 25% each as two top dressings at tillering and panicle initiation stages. Phosphorus (60 kg P_2O_5 ha^{-1}) and potassium (40 kg K_2O ha^{-1}) was applied as basal dose in all the treatments. The *Azolla* and commercial formulation blue green algae (BGA) biofertilizer (*Anabaena torulosa*) for treatments T_2, T_3, and T_4 procured from the Centre for Conservation and Utilization of Blue Green Algae, Indian Agriculture Research Institute, New Delhi, India was applied to the standing water in puddled plots 15 days before transplanting. The methane-utilizing/plant growth–promoting bacterial cultures of *Hyphomicrobium facile*

Table 1 Treatment details

S. no.	Treatment	N source (120 kg N ha^{-1})		
		Urea	BGA	*Azolla*
T_1	Control	120	0	0
T_2	*Azolla*	90	0	30
T_3	BGA*	90	30	0
T_4	*Azolla* + BGA*	60	30	30
T_5	*Hyphomicrobium facile* (A)**	120	0	0
T_6	*Burkholderia* sp. (B)**	120	0	0
T_7	*Methylobacteruim oryzae* (C)**	120	0	0
T_8	A + B + C***	120	0	0
T_9	No fertilizer	0	0	0

*BGA—Blue-green algae

**Seedling was dipped in culture for 2 h which was later transplanted. Similar culture was sprayed two more times (2 days before second and third split of urea application)

***In this treatment, all the three cultures were uniformly mixed and applied

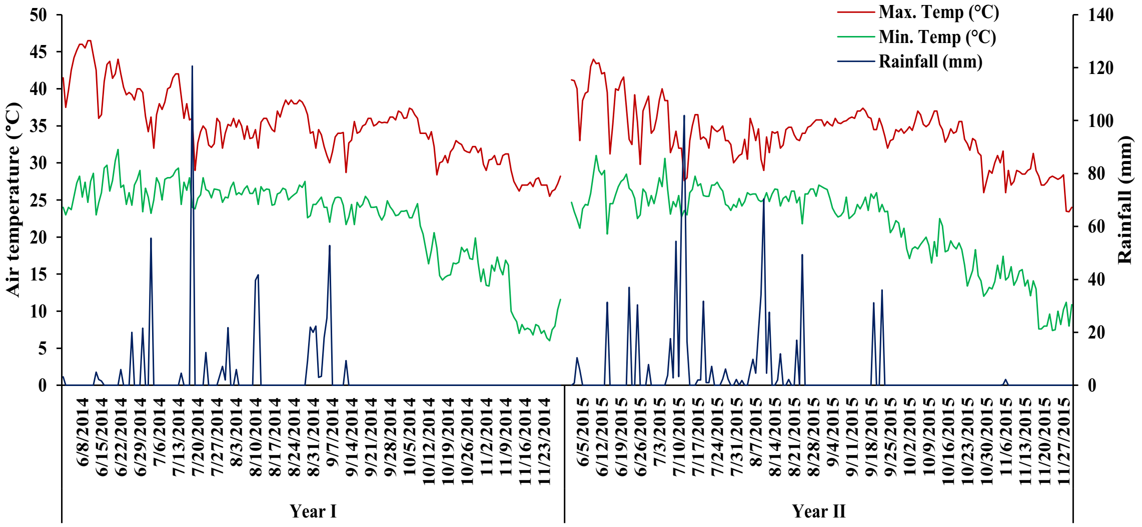


Fig. 1 Meteorological data of the study site during the two study years

MaAL69 (NCBI accession no. KY810635), *Methylobacterium oryzae* MNL7 (NCBI accession no. KY810615), and *Burkholderia vietnamiensis* AAAr40 (NCBI accession no. KY810624) were obtained from Division of Microbiology, IARI, New Delhi. These cultures were previously isolated from rice rhizosphere and phyllosphere and characterized for methane oxidation potential and plant growth–promoting attributes such as indole acetic acid production, and P, K, and Zn solubilization (Rani et al. 2021a). The liquid culture of the three bacterial isolates was raised individually in ammonium mineral salt medium (Whittenbury et al. 1970) to get a population density of 10^8 cells/mL. The liquid culture of each of the bacterial isolate was applied alone (T_5, T_6, and T_7) and in combination (T_8) (having each culture in 1:1:1 ratio) during nursery preparation through seed treatment, at the time of transplanting by root dip, and spray inoculated at the tillering and panicle initiation stage as per methodology described by Rani et al. (2021b). Then 150 mL of liquid formulation of these cultures was diluted to 1 L with irrigation water for root dip treatment for 2 h before transplanting. In the standing crop, the culture broth of these microbes was mixed with water at 20% and was sprayed two times during the crop period (2 days before second and third split of urea application) for maintaining the population of the CH_4-utilizing bacteria. The water level of 6 ± 4 cm was maintained by irrigation during rice growth period. The field was allowed to get dry naturally about 15–20 days before rice harvesting. No pesticide and herbicide was applied to avoid any additional effects. Number of panicles and leaf area index was quantified at flowering stage and the grain yield and test weight (average weight of 1000 grains of rice) were recorded at harvest.

Greenhouse gas sampling and analysis

Air sampling for determination soil CH_4 and N_2O fluxes was carried out using static-closed chamber technique (Bhatia et al. 2011). The air sampling was performed between 8:30 AM and 11:30 AM once a week throughout the crop season except after the three events of urea fertilization when air sampling was performed four times a week. Gas samples were collected from the top of the static closed chamber using 50 mL air-tight syringes at 0, 1/2, and 1 h. Temporal increases of the CH_4 and N_2O concentration in the air within the close chamber represented CH_4 and N_2O fluxes (Pathak et al. 2002, 2003). Concentration of CH_4 and N_2O gases in the collected gas samples were measured by using gas chromatograph equipped with a flame ionization detector and electron capture detector, respectively. Nitrogen was used as carrier gas and hydrogen and air were used for igniting the flame for analysis. Emission of CH_4 and N_2O from soil was calculated from the increase in CH_4/N_2O concentrations per unit surface area of the chamber within a specific time interval by the following equation:

$$F = \rho \times (V/A) \times (\Delta c/\Delta t) \times (273/T)$$

where F is the CH_4/N_2O flux (mg CH_4 m^{-2} h^{-1}/μg N_2O m^{-2} h^{-1}), ρ is the gas density, V is the volume of the close chamber (m^3), “A” is the surface area of the closed chamber (m^2), $\Delta c/\Delta t$ is the rate of increase of CH_4/N_2O gas concentration in the chamber (mg/μg m^{-3} h^{-1}), and T (absolute temperature) is calculated as 273 + mean temperature in (°C) of the chamber. Total CH_4/N_2O flux for the entire cultivation period was computed by linear interpolation (Bhatia et al. 2012) using the following equation:

$$\text{Total gas flux} = \Sigma_i^n\ (R_i \times D_i)$$

where R_i was the CH_4/N_2O emission flux (g m^{-2} day^{-1}) on the i^{th} sampling interval, D_i is the number of days in the i^{th} sampling interval, and n is the number of sampling intervals.

Global warming potential (GWP) and yield-scaled GWP

Global warming potential (GWP) is the quantification of warming potential of a mole of trace gas released into the atmosphere relative to a mole of CO_2 as a standard gas. GWP of CH_4 is 21 and that of N_2O oxide is 310 on a 100-years time horizon (Gupta et al. 2016; Bhatia et al. 2005). The GWP and the yield-scaled GWP that is carbon emitted per unit of grain yield of rice was estimated using the following equations:

$$\begin{aligned}
&\text{GWP}\left(\text{kg CO}_2\text{ equivalent ha}^{-1}\right)\\
&\quad = \text{seasonal CH}_4\text{ emission}\left(\text{kg CH}_4\text{ ha}^{-1}\right) \times 21\\
&\qquad + \text{seasonal N}_2\text{O emission}\left(\text{kg N}_2\text{O ha}^{-1}\right) \times 310\\
&\text{Yield−scaled GWP}\left(\text{kg CO}_2\text{ eq.ha}^{-1}\text{ grain yield}\right)\\
&\quad = \text{GWP}\left(\text{kg CO}_2\text{ eq.ha}^{-1}\right)/\ \text{grain yield}\left(\text{kg ha}^{-1}\right)
\end{aligned}$$

Soil redox potential and dissolved oxygen

Soil redox potential (Eh) and dissolved oxygen (DO) were measured weekly at regular intervals by a multi-parameter portable ORP meter (CONTECH-Cor-1) during the cropping period.

Statistical analysis

Statistical analysis of the experimental data was performed using SPSS (16.0, USA). ANOVA was carried out to check if the variations between the means were statistically significant. When the ANOVA was found significant at 5% level of significance and the error variances were homogeneous, we followed it up with Tukey's post hoc test to compare which treatment means were significantly different.

We carried out non-hierarchical cluster analysis using the *k*-means clustering algorithm using the data. For determining the optimal number of clusters, average silhouette method was used which determines how well each object lies within its cluster. A high average silhouette width indicates a good clustering. Average silhouette method computes the average silhouette of observations for different values of *k*. The optimal number of clusters is the one that maximizes the average silhouette over a range of possible values for *k*.

Results

Nitrous oxide emission

The daily N_2O flux ranged from 136 to 1850 μg m^{-2} day^{-1} during the rice growth period (Fig. 2a). Three main peaks of N_2O emission were observed during the rice growth period after each split fertilizer application. The peak N_2O flux was observed 2 to 3 days after each N fertilizer application. The magnitude of cumulative N_2O emission was highest in T_1 (120 kg N ha^{-1}) treatment. The presence of plant growth–promoting, methane-utilizing bacteria (T_5 to T_8) did not have any significant impact on the N_2O emission. Lowest cumulative N_2O emission was observed in the T_4 (*Azolla* + BGA) treatment. The substitution of 30 kg N ha^{-1} with *Azolla* and BGA biofertilizers significantly ($P < 0.01$) reduced the mean cumulative N_2O emission by 8.7% and 12.0%, respectively, over the T_1 treatment in the 2 years, respectively. The substitution of 60 kg N ha^{-1} by *Azolla* and BGA in T_4 treatments resulted in 40.7% less cumulative N_2O emission (significant at $P < 0.01$) as compared to T_1 treatment over the 2 years (Table 2). The daily average N_2O flux during the whole rice growth period varied from 275 to 868 μg N_2O day^{-1} (Fig. 3b) under the different treatments. The mean cumulative N_2O emission varied from 0.245 kg N_2O ha^{-1} to 0.785 kg N_2O ha^{-1} (Table 2).

Methane emission

Methane (CH_4) emission from rice soil varied considerably among the treatments and the dynamics of CH_4 flux during both cropping years is presented in Fig. 2b. The CH_4 flux increased significantly with plant growth in all the treatments. Irrespective of the treatments, the highest CH_4 fluxes were observed around 35 days after transplanting (DAT) in both years. The highest peak (79.1 mg CH_4 m^{-2} day^{-1}) was observed in T_8 (*Hyphomicrobium facile* MaAL69, *Methylobacterium oryzae* MNL7, and *Burkholderia vietnamiensis* AAAr40) treatments and lowest peak (41.0 mg CH_4 m^{-2} day^{-1}) was recorded in T_2 (*Azolla*) treatments, respectively, in the first year (Fig. 2b). The second highest CH_4 peak was recorded around 63 DAT in both years (Fig. 2b). The CH_4 flux rates decreased sharply at rice maturity in all the plots.

The daily average CH_4 flux during the entire crop growth period (seasonal daily average) varied from 29.0 to 39.98 mg CH_4 m^{-2} day^{-1} (Fig. 3a) in the different treatments. The highest daily average CH_4 flux was recorded in T_8 treatment and the lowest in T_7 treatment (Fig. 3a). Among the treatments, application of *Azolla*–BGA and *Methylobacterium oryzae* MLN7 significantly reduced the rate of CH_4 flux during the study period (Fig. 2b).

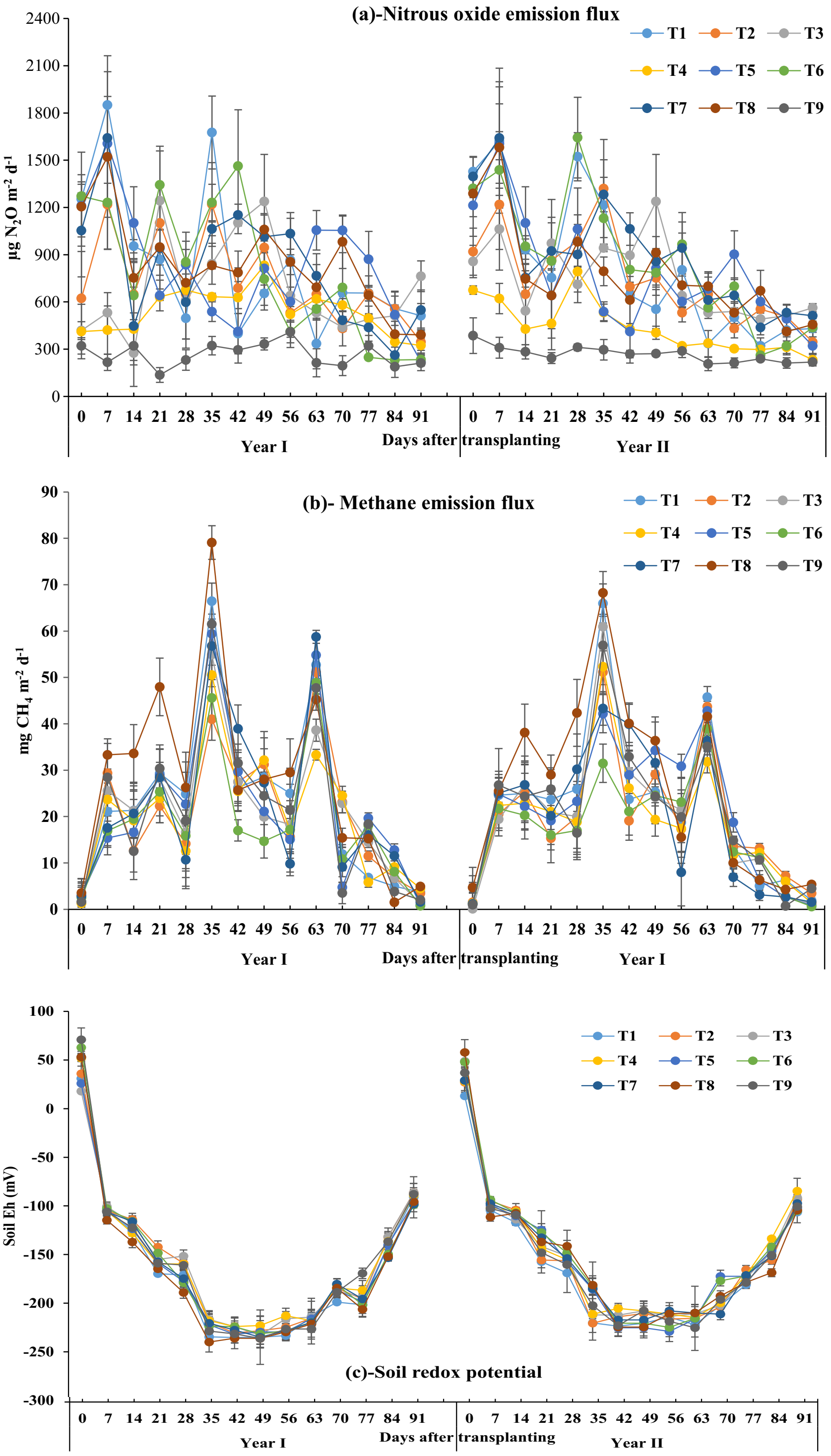
In Pusa Basmati-1509, being a short cycle variety, the total crop cycle varied from 106 to 110 days after transplanting in the two cropping years. We partitioned the CH_4 emission in three agronomic phases (Moldenhauer and Slaton 2001): vegetative phase (transplanting to panicle initiation), reproductive phase (panicle initiation to heading), and ripening phase (heading to maturity). Vegetative phase was observed up to 42–46 DAT; subsequently, reproductive phase was observed up to 72–76 DAT and the ripening phase was observed up to harvest. Among the different growth phases of rice, CH_4 emission was the highest during the vegetative growth phase and lowest during the ripening phase (Fig. 4). During the vegetative phase, the average CH_4 emission in the 2 years ranged from 55% (T_2) to 67% (T_8) of the total emission (Fig. 4), while in the reproductive phase, CH_4 emission ranged from 26% (T_9) to 33% (T_2) of the total emission and varied from 7% to 13% during the ripening phase in the different treatments.

There was a significant impact of different treatments on CH_4 emission in both years and is presented in Table 2. Among the treatments, T_7 (*Methylobacterium oryzae* MNL7), T_4 (*Azolla* + BGA), and T_2 (*Azolla*) significantly ($P < 0.05$) reduced total seasonal CH_4 emission by 19.9%, 13.3%, and 9.7%, respectively (Table 2) as compared to the T_1 control averaging over the 2 years. Treatment T_3, T_5, and T_6, reduced CH_4 emission by 7.1%, 4.9%, and 4.1%, respectively, as compared to T_1 (Table 2) over the 2 years. The cumulative CH_4 emission under T_8 treatment (*Hyphomicrobium facile* MaAL69 + *Burkholderia vietnamiensis* AAAr40 +*Methylobacterium oryzae* MNL7) was 10.3% higher than T_1 (Table 2).

Soil redox potential, dissolved oxygen, and soil carbon and nitrogen

Soil Eh decreased sharply 2 weeks after transplanting in all the treatments (Fig. 2c). Eh declined sharply under flooding

Fig. 2 **a** Nitrous oxide flux, **b** methane flux, **c** soil redox potential under different treatments during the crop growth period. T_1—control, T_2—*Azolla*, T_3—BGA, T_4—*Azolla* + BGA, T_5—*Hyphomicrobuim facile*, T_6—*Burkholderia,* T_7—*Methylobacteruim oryzae*, T_8—all methanotrophs, T_9—no fertilizer



condition and lowest Eh was observed at 35 DAT irrespective of the treatments (Fig. 2c). Lowest Eh was observed in T_8 (−240 mV) at 35 DAT (Fig. 2c). Eh showed less fluctuation between 35 and 63 DAT, and it sharply rose after 63 DAT (Fig. 2c). The DO values ranged from 3.06 to 0.85 mg L^{-1} in the first year while it was slightly lower in the second year and ranged from 2.90 to 0.94 mg L^{-1} during the crop growth period. Average DO levels were observed to be the highest in T_2 (1.74 mg L^{-1}) and lowest in T_1 (1.65 mg L^{-1}) (Fig. 3a).

We measured the change in soil organic carbon, total N, and pH after rice harvest in both years. There was a slight increase in soil organic carbon and total N in the T_2 and T_4 treatments; however, the increase was not statistically significant (results not shown).

Table 2 Effect of different treatment on global warming potential (GWP) and yield-scaled GWP

Treatment	CH_4 (kg CH_4 ha^{-1})*			N_2O (kg N_2O ha^{-1})*			GWP (kg CO_2 eq. ha^{-1})			Yield-scaled GWP (kg CO_2 equivalent kg^{-1} grain yield)		
	Y I	Y II	Mean	Y I	Y II	Mean	Y I	Y II	Mean	Y I	Y II	Mean
T_1	33.83	32.07	32.95^{B}	0.780	0.731	0.756^{AB}	952	900	926^{AB}	0.245	0.229	0.237^{BC}
T_2	30.37	29.20	29.78^{BC}	0.714	0.666	0.690^{BC}	859	820	839^{BCD}	0.192	0.182	0.187^{DE}
T_3	31.23	30.01	30.62^{BC}	0.670	0.660	0.665^{C}	863	835	849^{BC}	0.208	0.201	0.204^{BCDE}
T_4	29.44	27.73	28.58^{C}	0.469	0.428	0.448^{D}	763	715	739^{DE}	0.175	0.169	0.172^{E}
T_5	31.21	31.44	31.32^{BC}	0.764	0.721	0.742^{ABC}	892	884	888^{BC}	0.226	0.208	0.217^{BCD}
T_6	31.83	31.37	31.60^{BC}	0.755	0.782	0.768^{AB}	903	901	902^{ABC}	0.226	0.240	0.233^{BC}
T_7	27.32	25.46	26.39^{C}	0.799	0.772	0.785^{A}	821	774	798^{CDE}	0.209	0.193	0.201^{CDE}
T_8	35.83	36.93	36.38^{A}	0.763	0.732	0.748^{ABC}	989	1002	996^{A}	0.242	0.245	0.244^{B}
T_9	29.96	30.60	30.28^{BC}	0.265	0.225	0.245^{E}	711	712	712^{E}	0.339	0.321	0.330^{A}
P value of significance	0.02	0.009	0.0008	0.032	0.006	0.002	0.001	0.002	0.001	0.002	0.001	0.0008
Tukey's HSD at 5%	3.52	4.01	3.26	0.158	0.108	0.089	148.4	168.9	105	0.051	0.070	0.040

Means with at least one letter common are not statistically significant using Tukey's honest significant difference (HSD)

Y I—year 1, Y II—year 2

*Cumulative seasonal emission

Growth and yield attributes

The highest leaf area index (LAI) was observed in T_2 (4.2) with *Azolla* substitution and was significantly higher ($P < 0.05$) than all the other treatments followed by T_4 (4.1) treatment. No significant difference was observed in plant height and tillers/hill among the treatments. The test weight did not change significantly, but the number of productive panicles was significantly higher under *Azolla* treatment (T_2) as compared to control. The number of panicles varied under the different treatments from 186 to 240 panicles m^{-2}. Number of tillers was observed to be the highest in T_4 with *Azolla* and BGA application (12.2 per hill) and was the lowest in unfertilized control T_9 (10.3 per hill) (result not shown); however, the differences were not significant. Highest grain yield was observed in T_2 treatment with substitution of 30 kg N ha^{-1} with *Azolla*. The application of plant growth–promoting bacteria with CH_4-utilizing ability did not have any significant effect on rice yield (Table 2). The rice yield was higher in T_2 (14.8%) followed by T_4 (9.9%) as compared to T_1. In T_9 treatment which was having no fertilizer, the rice yield was significantly (44.8 %) lower than T_1 (control).

Global warming potential (GWP) and yield-scaled GWP

In the present study, the GWP in the two rice-growing years was the highest in the combined methane-utilizing bacteria treatment T_8 (996 kg CO_2 eq. ha^{-1}) and lowest in combined BGA and *Azolla* treatment T_4 (739 kg CO_2 ha^{-1}) (Table 2). The mean GWP during the 2 years was significantly higher ($P < 0.001$) in T_8 by 7.5% as compared to the T_1 treatment. The share of CH_4 in the total GWP ranged from 554 (kg CO_2 eq. ha^{-1}) to 764 (kg CO_2 eq. ha^{-1}) in the different treatments (Fig. 5). N_2O share in total GWP was the highest in T_6 (238 kg CO_2 eq. ha^{-1}) treatment. The share of CH_4 to the total GWP ranged from 74 to 89% among the different treatments (Fig. 5), and for N_2O it was the lowest in T1 (11%) and the highest in T_7 treatment (31%).

The yield scaled GWP was the least in T_4 (0.172 kg CO_2 equivalent kg^{-1} grain yield) and the highest in T_9 (0.329 kg CO_2 equivalent kg^{-1} grain yield) (Table 2). In T_1, yield-scaled emission was 0.237 kg CO_2 eq. kg^{-1} grain yield and was significantly ($P<0.001$) reduced by 21.1%, 13.8%, and 27.4%, respectively, with the application of T_2 (*Azolla*), T_3 (BGA), and T_4 (*Azolla* + BGA). The application of plant growth–promoting, methane-utilizing bacteria reduced the yield-scaled GWP by 8.6%, 1.8%, and 15.1% in T_5, T_6, and T_7 treatments, respectively, over T_1. The application of combination of bacteria (T_8), however, did not reduce the yield-scaled emission and were statistically at par with the control (T_1).

Non-hierarchical *k*-means clustering

We carried out non-hierarchical *k*-means clustering to analyze data and for finding subgroups (clusters) within treatments and for identifying the outliers. The *k*-means clustering was done on the mean data of the 2-year experiment and the cluster plot obtained is shown in Fig. 6. The clustering segregated the

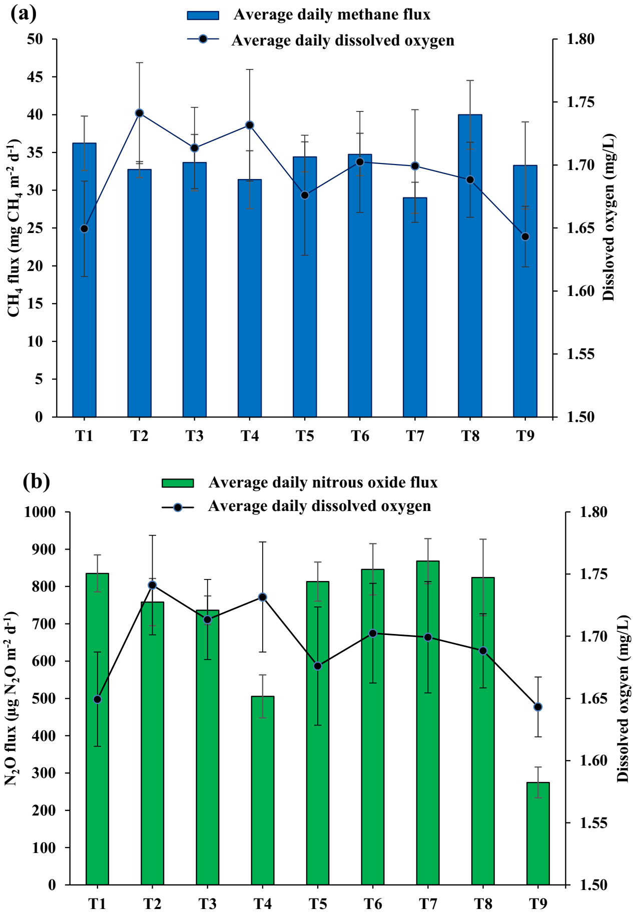


Fig. 3 Average daily emission **a** methane and **b** nitrous oxide and dissolved oxygen under different treatments during the crop growth period. *Pooled data for 2 years

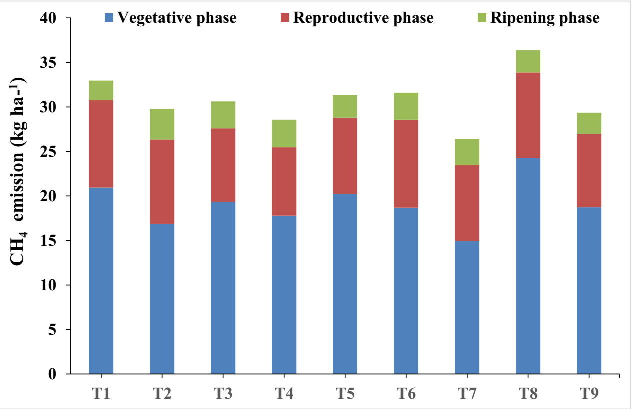


Fig. 4 Contribution of different growth rice phases to seasonal methane emissions. *Pooled data for 2 years. T_1—control, T_2—*Azolla*, T_3—BGA, T_4—*Azolla* + BGA, T_5—*Hyphomicrobuim facile*, T_6—*Burkholderia*, T_7—*Methylobacteruim oryzae*, T_8—all methanotrophs, T_9—no fertilizer

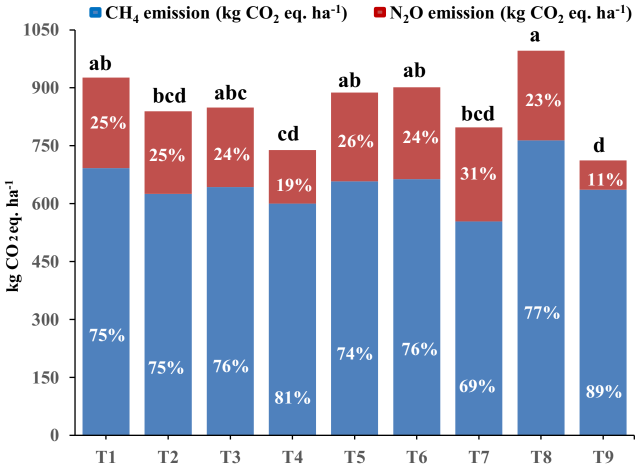


Fig. 5 Share of methane and nitrous oxide to the total global warming potential. *Pooled data for 2 years

treatments into four subgroups. Out of these, there were two major subgroups and two outliers. The grouping showed that the treatments T_2, T_4, and T_7 having GHG mitigation potential were grouped in one cluster. The control (T_1), T_3, T_5, and T_6 treatments were clustered together. The no-fertilizer N treatment T_9 was another subgroup. The treatment T_8 (combines bacteria) formed a separate subgroup and was an outlier among the treatments.

Discussion

Variation in CH_4 and N_2O emission during crop growth

In the present study, CH_4 flux pattern was similar in both cropping years. The cumulative CH_4 fluxes under all treatments during year I was higher as compared to year II (Fig. 2b). This was likely due to higher rainfall (Fig. 1) in the first year as compared to the second year. Rainfall enhances the methanogenic activity by maintaining optimum soil temperature and increases CH_4 flux (Hussain et al. 2015). Kim et al. (2016) reported enhanced CH_4 emission from paddy soil due to occurrence of rainfall during the crop growth period. Variations in CH_4 emission were observed in the different treatments; however, maximum fluxes of CH_4 were observed during tillering and reproductive stages in all the treatments in both years (Fig. 2b). It might be due to the combined effect of high root exudation during tillering that provided substrate for methanogenesis (Singh et al. 2009) and direct transport of generated CH_4 to the atmosphere by the rice tiller through parenchyma, reducing chances of oxidation near the surface soil (Sass and Cicerone 2002). At the beginning of the crop cycle, when rice plants were little developed, bubble formation and vertical movement in the bulk of the soil was the main transfer mechanism. After tillering, diffusion through the parenchyma becomes the dominant process, and was responsible for more than 90% of the CH_4 emission during active

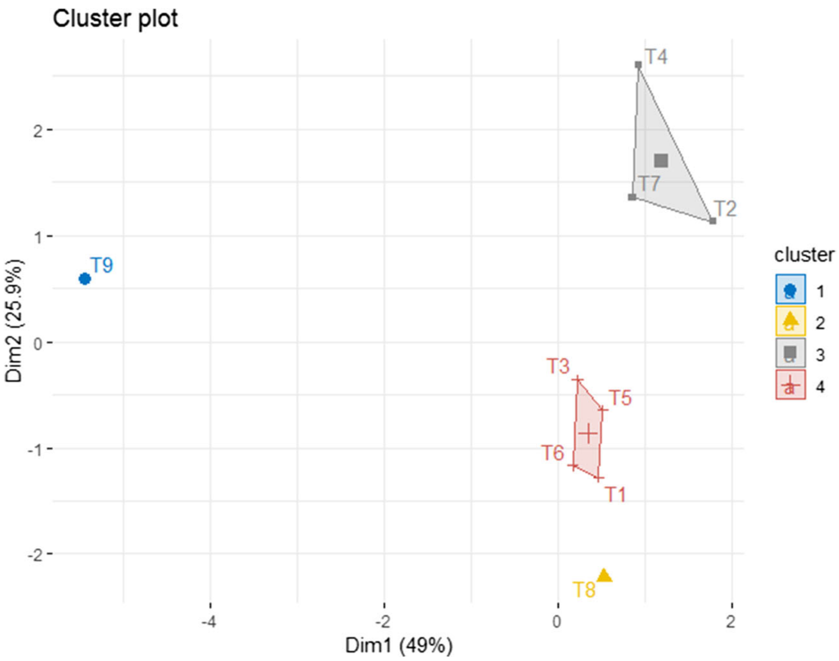


Fig. 6 *K*-means clustering plot for different treatments*. *Pooled data for 2 years

tillering and reproductive stages (Tyler et al. 1997). The N_2O fluxes were driven by the fertilizer application events. Peaks of N_2O flux were obtained after each fertilization event. Malyan et al. (2019) reported that applied urea fertilizer was hydrolyzed to ammonium and further nitrified and denitrified producing high fluxes of N_2O.

Impact of methane-utilizing bacteria on CH_4 and N_2O emission

In the present study, three plant growth–promoting bacteria capable of utilizing CH_4 as sole C source were evaluated for their ability to consume CH_4 in rice. In our previous work, a commercial liquid formulation of these cultures was developed. They were isolated from the rhizosphere and phyllosphere of different rice-growing regions of India and were evaluated for their plant growth–promoting attributes and CH_4 oxidation potential by culturing them in NMS media having different CH_4 concentration from (0.5 to 5%) as sole C source (Rani et al. 2021a, b). Facultative methylotrophy in all the three bacterial cultures have been reported earlier by various workers (Van Aken et al. 2004; Mcdonald et al. 2001); however, genetic analysis of the ability to utilize CH_4 as sole C source by bacteria belonging to these genera is a topic of further research (Dedysh and Dunfield 2011; Theisen and Murrell 2005). In order to reduce CH_4 emission in rice rhizosphere under flooded condition, it is essentially required for methanotroph population to be maintained above the threshold level not only in rhizosphere but also in phyllosphere (Iguchi et al. 2015). In the present study, while carrying out rhizosphere and phyllosphere inoculations, the populations of all the three bacteria, whether inoculated alone or in combination, were maintained above $>10^8$ cells mL^{-1}.

Inoculation of *Methylobacterium oryzae* MNL7 (T_7) caused significant reduction in CH_4 emission by ~20% as compared to un-inoculated treatment (T_1). Previously certain strains of *Methylobacterium* sp. have been reported to have the ability to utilize CH_4 as sole C source of energy. *Methylobacterium* strain, BJ001T, had been isolated from poplar tissues and has been reported to be able to use CH_4 as the sole source of carbon and energy (Van Aken et al. 2004). In contrast, inoculation of *Hyphomicrobium facile* (T_5) and *Burkholderia* sp. (T_6) did not cause any significant reduction in CH_4 emission. Difference in the ability of the isolates to act differently under field conditions can be attributed to several factors such as decline in population due to competition with native population, utilization of C sources other than CH_4 due to their facultative methylotrophy in nature, survival under anoxic conditions, etc. (Iguchi et al. 2015).

In order to avoid population decline two spray schedules were carried out as stated earlier. Dubey (2005) observed that temperature, CH_4 concentration, soil moisture, oxygen availability, nitrogenous compounds, and soil pH play a significant role in CH_4 oxidation by bacteria. Results showed that inoculation with *Methylobacterium oryzae* MNL7 (T_7) alone was capable of significantly reducing CH_4 emission and could be used for developing commercial-scale technology for use in flooded paddies. From this study, it was observed that the stage of bacterial inoculation was also important in getting the desired level of reduction in CH_4 emission. Spraying of cultures to enrich the population in phyllosphere and on water surface at the right stage of the crop may also be important for significant reduction in CH_4 emission. The growth and activity of CH_4-oxidizing microbes in the rice rhizosphere may also be stimulated by ammonium-based fertilization. Urea has been reported to enhance the activity and population size of methanotrophs in rice rhizosphere (Dong et al. 2011; Xie et al. 2010). No effect of plant growth–promoting bacteria on N_2O emission was observed in our study.

Effect of *Azolla* and BGA on CH_4 and N_2O emission

Azolla–BGA biofertilizers in rice are globally used and are known to liberate oxygen in flooded water (Kollah et al. 2016; Bharati et al. 2000). In our study, the application of *Azolla* biofertilizer in treatment T_2 and in combination with BGA in T_4 reduced cumulative CH_4 and N_2O emission from the rice soils. This was due to liberation of photosynthetic oxygen in paddy water by *Azolla* and BGA (Malyan et al. 2016) which increased the DO concentration in flooded water, and eventually decreased the CH_4 emission from paddy soil by enhancing the CH_4 oxidation (Ali et al. 2015).

Among the treatments, seasonal cumulative CH_4 emission were reduced in T_2 and T_4 by ~9.7 and ~13.3%, respectively, as compared to T_1, due to higher average DO concentration during the crop growth (Fig. 3a) and higher soil redox potential (Fig. 2c) which might have enhanced the activity of CH_4-oxidizing bacteria (Kimani et al. 2018). Similar reductions in CH_4 emission of 20.4% and 12.3% were observed by Ma et al. (2012) and Xu et al. (2017), respectively, after incorporating *Azolla* in rice. Bharati et al. (2000), however, observed up to 42.5% reduction in the cumulative CH_4 emission under *Azolla* application. Methanogenesis is a multistep process in which methanogenic bacteria uses organic carbon and produces CH_4 as an end product under anaerobic environment (Malyan et al. 2016; Ali et al. 2012). In the current study, the oxygen liberated by *Azolla*–BGA in standing water of rice increased the soil redox potential resulting in suppression of methanogenesis process leading to lower production of CH_4 as compared to the T_1 control treatment (Fig. 7). However, in some previous studies, Adhya et al. (2000) and Ying et al. (2000) reported that application of *Azolla* increased cumulative CH_4 emission from rice, probably due to decomposition of dead *Azolla*. Malyan et al. (2019) reported that *Azolla* has a potential to mitigate the cumulative CH_4 emission. Kimani et al. (2018) observed significant reduction in CH_4 emission; however, no-significant effect of *Azolla* on N_2O emission was reported in a pot experiment growing rice.

Wagner (1997) reported that *Azolla* having high photosynthetic ability could release copious amounts of oxygen in standing water thereby increasing the DO concentrations and improving the soil redox potential. Xu et al. (2017) and Prasanna et al. (2002) reported that application of *Azolla* with N fertilizers like urea has stronger capacity of CH_4 oxidation as compared to *Azolla* alone.

Biological decomposition of organic matter and N fertilizer application are two important sources of N_2O emission from agricultural soils (Bremner 1997). In rice, applied *Azolla* after completing its life span undergoes rapid decomposition and enhanced the N_2O emission from rice (Chen et al. 1997). Availability of oxygen is one of the major factors affecting the formation of N_2O in rice soil by denitrification pathway (Bhatia et al. 2012). The dissolved oxygen was higher in T_2 and T_4 treatments due to photosynthesis by *Azolla*, thereby leading to lower denitrification N_2O flux in these treatments. In our study, the cumulative N_2O emission under *Azolla* and BGA applied plots (T_2, T_3, and T_4) were significantly ($P < 0.01$) lower than T_1 (Fig. 2a). Another reason for lower emission was the reduced amount of fertilizer N application in these treatments (Table 2). N_2O emission from soil depends on several factors including the rate of N fertilization, type of N applied, and soil-water content (Ladha et al. 2005; Pathak et al. 2002). The urea N application was lower by 25% in T_2 and T_3, and by 50% in T_4 as compared to T_1; however, the N_2O emission were reduced by 8.7 to 12% in T_2 and T_3, and by 41% in T_4 as compared to T_1 treatment. The N fixed by *Azolla* and BGA was probably more efficiently used for plant growth as compared to 100% synthetic nitrogen applied in the control (T_1) treatment leading to reduced N_2O losses in T_2, T_3, and T_4 treatments. Kimani et al. (2018) reported that *Azolla* cover in northeastern Japan rice cultivation reduced the N_2O emission from 2.7 to 2.6 mg N m^{-2}. Malyan et al. (2019) observed that application of *Azolla* along with reduced dose of N fertilizer lowered the GHG intensity in rice by 16 to 19%. Xu et al. (2017) also observed a reduction in N application by the application of *Azolla* in double rice cropping system in southern China due to nitrogen-fixing properties of these biofertilizers. They also observed lower yield-scaled CH_4 emission on the application of *Azolla* along with nitrogenous fertilizer.

Effect of urea application on CH_4 and N_2O emission

There are contradictory reports on the effect of N fertilizers on methanotrophs in the rice soil (Hussain et al. 2015; Dubey 2005; Schimel 2000). Datta et al. (2013) reported that cumulative CH_4 emission from rice soil increased with the addition of urea fertilizer, whereas Dong et al. (2011) and Xie et al. (2010) reported the stimulation of methanotrophs with the addition of N fertilizers in rice rhizospheric soil (Dong et al. 2011; Xie et al. 2010) leading to lower CH_4 emission. In this study, there was higher emission of CH_4 in the urea alone treatment (T_1) as compared to the no-fertilizer (T_9) application. This may be due to the rapid hydrolysis of applied urea fertilizer to ammonium ion. Ammonium ion being similar in chemical structure to CH_4 may compete with CH_4 for the binding site of methane monooxygenase enzyme, a key enzyme for CH_4 oxidation (Bédard and Knowles 1989) and can

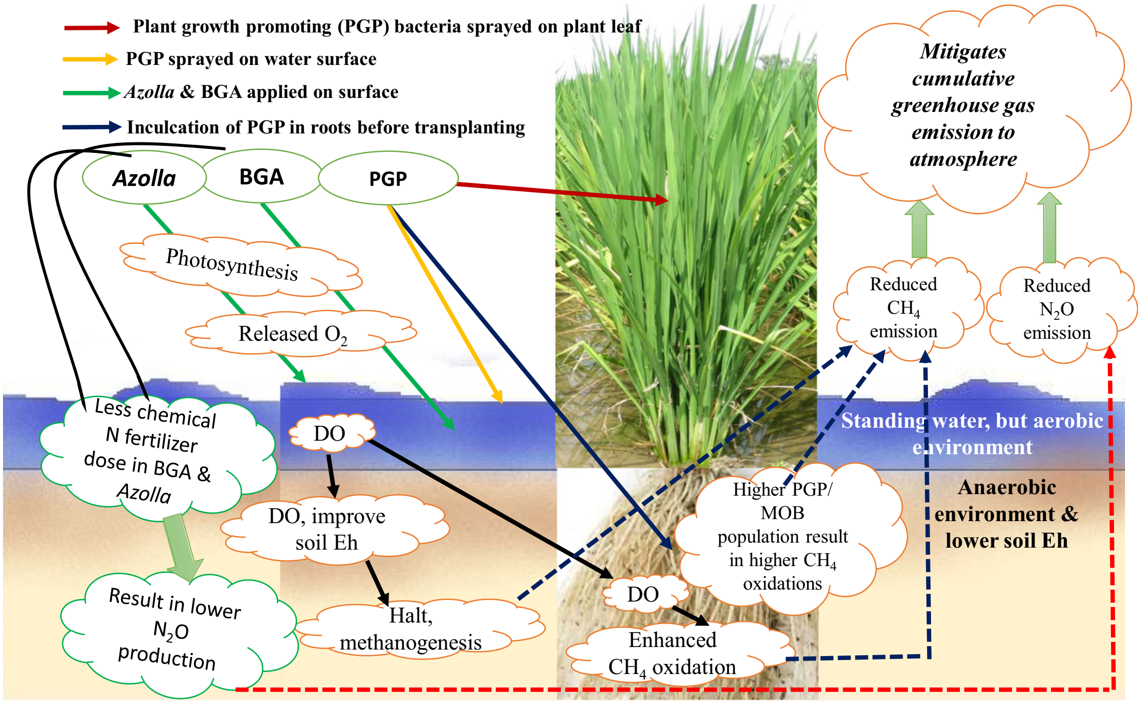


Fig. 7 Mechanism of different treatments for the mitigation of greenhouse gas emission from rice

also lead to the competition between CH_4 and ammonium oxidizers for oxygen resulting in increased CH_4 emission. N application in the form of urea resulted in 8% higher cumulative CH_4 emission as compared to no N fertilizer application. Application of nitrogen fertilizer in T_1 resulted in higher below- and above-ground biomass over treatment T_9 (no fertilizer) and may have provided higher substrates in the form of root exudates for the methanogenic bacteria to produce more CH_4. Datta et al. (2013) observed 26.9% higher CH_4 emission under 110 kg N ha^{-1} urea application over no N fertilizer application in rice fields of Cuttack, India. The high CH_4 flux from the urea applied plots could also be due to isostructural and isoelectric symmetry between CH_4 molecule and ammonium ion (Schimel 2000). Hanson and Hanson (1996) reported that, due to the presence of high concentration of ammonium ions in soil (such as in urea application conditions), methanotrophic bacteria bind with ammonium ions as a substitute of CH_4 molecule and the methanotrophic activity is reduced resulting in higher CH_4 emission.

Across all the treatments, the lowest cumulative N_2O emission was observed under the T_9 (without N fertilizer) treatment in our study (Table 2). In comparison to T_1 (control–120 kg N ha^{-1}), the rice cropping without N fertilizer (T_9) decreased the cumulative N_2O emission by 67.6% (Table 2). Pathak et al. (2002) also reported that total N_2O emission from no N fertilizer treatment were reduced by 56.0% as compared to urea-applied rice soils. Das and Adhya (2014) observed a 78.9% decrease in N_2O emission in non–N-fertilized soils as compared to urea-applied rice soils. The higher N_2O emission in urea treatment is due to availability of mineral N to soil microorganisms which controls the nitrification and denitrification process.

Effects of different biological interventions on CH_4 emission during rice phases

During both years, CH_4 emitted during the vegetative stage was higher as compared to the other two stages (Fig. 4). The least amount of CH_4 was emitted during ripening stage of rice (Fig. 4). The higher CH_4 emission from vegetative stage may be due to higher methanogenic activity (Ali et al. 2015) and higher labile organic carbon present in rice during this stage due to growing plant biomass and more root exudation activity. The low CH_4 emission during ripening stage was due to higher soil Eh and lower soil temperature which may have suppressed methanogenesis activity. In our study, we found that there was slightly higher CH_4 emission from T_2 and T_4 treatment as compared to other treatments during ripening stage, which may be due to the degradation of *Azolla* in the last few weeks of the cropping period.

Impact of different interventions on growth and yield attributes

The application of plant growth–promoting methanotrophs did not lead to any significant impact on growth and yield attributes; however, the application of *Azolla* led to significant increase in growth and yield attributes of rice (Table 3). Under the *Azolla* treatments T_2 and T_4, higher plant height, tillers/hill, and LAI were observed as compared to urea (T_1) alone,

Table 3 Leaf area index (LAI) and rice grain yield and test weight of rice under different treatments

Treatment symbol	Grain yield (kg ha^{-1})			LAI			Test weight (g)		
	Y I	Y II	Mean	Y I	Y II	Mean	Y I	Y II	Mean
T_1	3890	3920	3910^{BC}	3.95	4.05	4.00^{B}	21.40	21.27	21.33
T_2	4470	4500	4490^{A}	4.19	4.25	4.22^{A}	21.30	22.33	21.82
T_3	4210	4135	4160^{ABC}	3.96	4.12	4.04^{B}	21.73	21.57	21.65
T_4	4479	4210	4300^{AB}	4.08	4.06	4.07^{AB}	21.53	21.67	21.60
T_5	3800	4250	4100^{ABC}	3.94	3.86	3.90^{B}	21.20	21.33	21.27
T_6	4000	3815	3877^{C}	3.98	3.97	3.98^{B}	21.67	21.00	21.33
T_7	3920	3995	3977^{BC}	3.92	4.06	3.99^{B}	20.87	21.77	21.32
T_8	3900	4186	4090^{ABC}	4.01	3.96	3.98^{B}	20.67	22.00	21.33
T_9	2100	2190	2160^{D}	3.43	3.20	3.32^{c}	21.41	21.73	21.57
Tukey HSD at 5%	63.6	62.4	41.6	0.19	0.26	0.15	ns	ns	ns

Y I—year 1, Y II—year 2, LAI—leaf area index

and this led to an increase in grain yield by 15.2% in the T_2 treatment over the control. The increase of growth attributes in *Azolla* and *Azolla* + BGA treatments may be due to nitrogen fixation and release of some growth-promoting metabolites (5-aminolevulinic acid and exopolymeric substances) in rice soil (Kantachote et al. 2016) that may have led to an increase in yield. Similar findings were also observed by Ali et al. (2015) and Bharati et al. (2000), and they reported that *Azolla* plus BGA application increased yield in rice significantly due to its biofertilizer property (Bharati et al. 2000).

Effect of different treatments on GWP and yield-scaled GWP

In our study, maximum reduction in average GWP was observed in T_4 treatment (*Azolla* + BGA, 20.2%) as compared to T_1 (fertilized control) (Table 2 and Fig. 5). Higher average DO and higher soil Eh was observed in this treatment in both years. The application of *Methylobacterium oryzae* MNL7 in T_7 lowered the GWP by 13.8% due to reduction in CH_4 emission. The lowest yield-scaled GWP was observed in T_4 (*Azolla* + BGA) followed by T_2 (*Azolla*) and T_7 (*Methylobacterium oryzae* MNL7) treatment, and it was 27.4%, 21.1%, and 15.1% lower than control, respectively (Table 2). Lower CH_4 and N_2O emission in T_4 treatment decreased the GWP leading to lower yield-scaled GWP. Lower CH_4 and N_2O emission was observed due to higher DO concentrations in this treatment whereas lower urea N application (50% N at 60 kg N ha^{-1}) led to 41% less N_2O emission in T_4. A 25% reduction in application of N fertilizer by 30 kg N ha^{-1} in T_2 and T_3 treatment decreased the N_2O emission by 9 and 12% as compared to T_1.

The *k*-means cluster analysis grouped the treatments into subgroup having common features of reducing GHG emission and increasing the rice yield (Fig. 6). From the result of the *k*-means clustering, it was evident that the three treatments T_2, T_4, and T_7 formed one cluster and were the most effective in reducing the GWP and the yield-scaled GWP. The next cluster was of T_3, T_5, and T_6 indicating that these treatments were similar to the control (T_1) and had no impact on the GWP. The T_8 and T_9 treatments were the outliers having very different treatment effects. T_8 significantly increased the CH_4 emission and T_9 was the no-fertilizer treatment which had reduced rice yield and lower N_2O emission.

Azolla, BGA, and methane-utilizing bacteria can be used for reducing the GWP of transplanted puddled rice cultivation in the Indo-Gangetic Plains region having around 10.5 Mha of land under rice cultivation. Earlier mitigation options like intermittent irrigation and direct seeded rice have been suggested for reducing the CH_4 emissions in this region. The rice yield penalty and weed growth are the major reasons for these options not being successfully implemented and taken up by the farmers of the region. However, the application of *Azolla*, blue green algae, and plant growth–promoting bacteria can be promoted among the farmers as they not only reduce the yield-scaled GWP but also lead to saving in N (*Azolla* and BGA substitution). The use of biofertilizers for promoting the growth of different crops is already popular among farmers. Farmers will only use microbial inoculants capable of reducing methane and nitrous oxide emission if they also promote the growth of crops. Hence, it is essential to integrate microbial cultures having dual ability of plant growth promotion and methane utilization with existing package and practices of biofertilizers. A suitable delivery mechanism of such biofertilizers needs to be worked out as it is essential to maintain the population of methane-utilizing microbes in the rhizosphere as well as phyllosphere at the critical stages of crop growth. Further research can be undertaken focusing only to develop suitable delivery mechanisms by integrating popularly used algal and cyanobacteria-based paddy biofertilizers with methane-utilizing bacteria.

Conclusion

Plant growth–promoting bacteria *Methylobacterium oryzae* and biofertilizers *Azolla* and Blue-green algae can be effective interventions for reducing the global warming potential and yield-scaled GWP in flooded rice ecosystems by reducing the emission of both CH_4 and N_2O. Compared with control, *Azolla*, *Azolla* + BGA, and *Methylobacterium oryzae* decreased the yield-scaled GWP by 21.1%, 27.4%, and 15.2% from the rice fields, respectively. Thus, in irrigated flooded rice, the application of *Methylobacteruim oryzae* MNL7, *Azolla* alone, or along with BGA could be an effective option for mitigation of yield-scaled GWP, saving inorganic fertilizer and increasing rice yields for achieving the goal of sustainable agriculture. Suitable commercial formulations of the methane-utilizing plant growth–promoting bacteria have to be prepared so that their optimum populations can be maintained during the rice growth period, thus enabling its use by the farmers of the region.

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Author contribution S.K.M.: investigation, writing—original draft. A.B.: conceptualization, supervision, writing—review and editing. R.T.: sample analysis. R.C.H.: field management. A.B.: statistical analysis. N.J.: writing—initial draft. R.K.: conceptualization, supervision.

Data availability All relevant data are within the manuscript and available from the corresponding author on request.

Declarations

Ethical approval Not applicable.

Consent for participate The authors have agreed with the content and all have given consent to publish.

Competing interests The authors declare no competing interests.

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