Methane-derived microbial biostimulant reduces greenhouse gas emission and improves rice yield

• Sarma Rajeev Kumar[1, 2] †, Einstein Mariya David[3,4]†, Gangigere Jagadish Pavithra[1], Gopalakrishnan Sajith Kumar [3,4], Kuppan Lesharadevi[3,4], Selvaraj Akshaya[1,2],Chavadi Bassavaraddi[1], Gopal Navyashree[1], Panakanahalli Shivaramu Arpitha[1], Padmanabhan Sreedevi[1,2], Khan Zainuddin[1], Saiyyeda Firdous [3], Bondalakunta Ravindra Babu[1], Muralidhar Udagatti Prashanth[1], Ganesan Ravikumar[1], Palabhanvi Basavaraj[1], Sandeep Kumar Chavana [1], Vinod Munisanjeeviah Lakshmi Devi Kumar[1,2], Theivasigamani Parthasarathi[3] and Ezhilkani Subbian[1,2]*

†- These authors contributed equally to this work

1. String Bio Private Limited, Vinayaka Nagar, Nagasandra Bangalore-560073, Karnataka, India
2. String Bio Private Limited, Centre for Cellular and Molecular Platforms, Bangalore- 560065, Karnataka, India
3. VIT School of Agricultural Innovations and Advanced Learning (VAIAL), Vellore Institute of Technology, Vellore- 632014, Tamil Nadu, India
4. School of Biosciences and Technology (SBST), Vellore Institute of Technology, Vellore-632014, Tamil Nadu, India

- *Dr. Ezhilkani Subbian- Corresponding Author
- Dr. Theivasigamani Parthasarathi- Co-corresponding Author:
- **Email:** subbiane@stringbio.com; parthasarathi.t@vit.ac.in

Fig. S1A

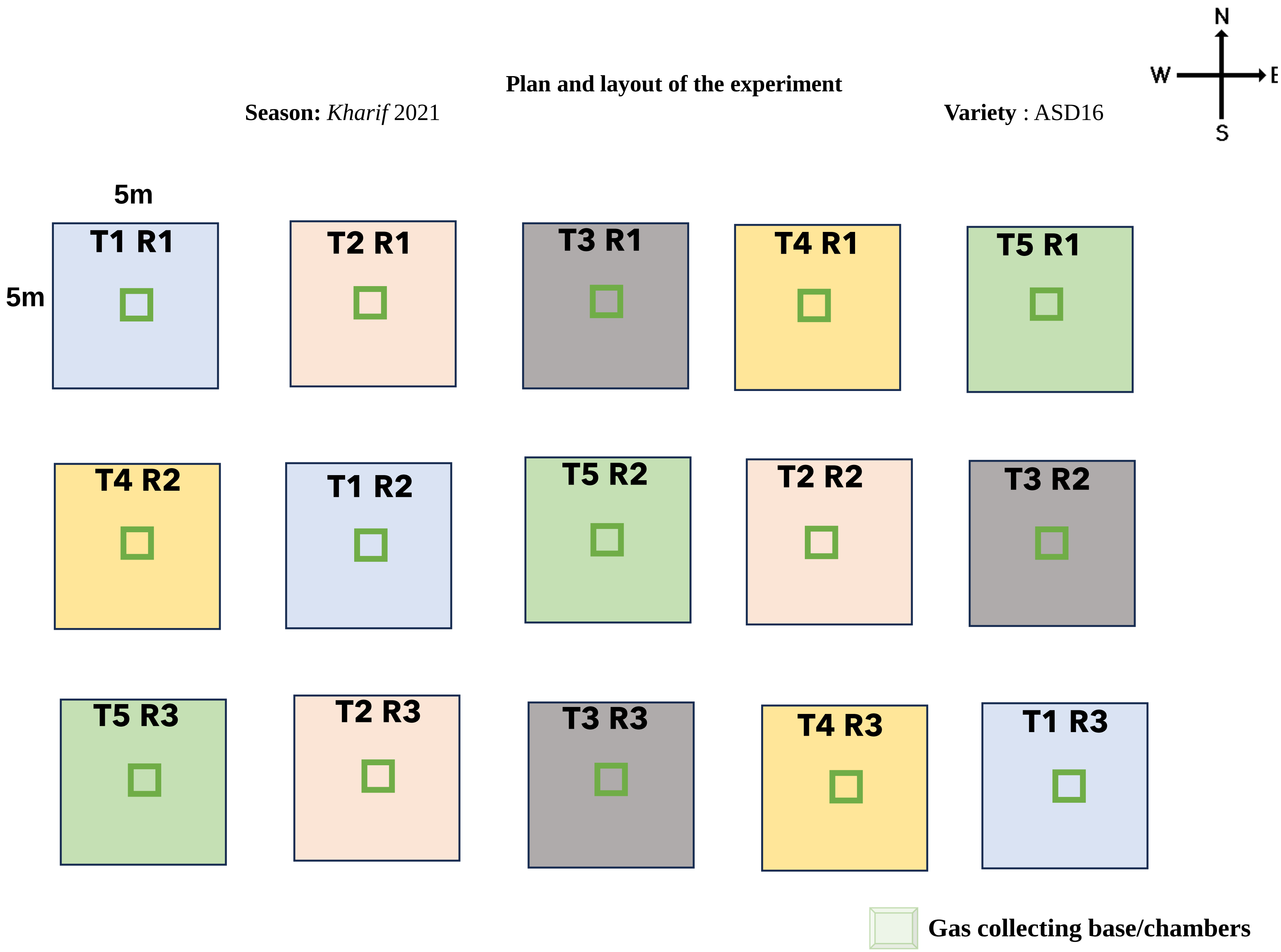


Fig S1A-Experimental field layout for season I testing- Experimental field layout for season I testing- Treatment details are below: Control (T1); 10 mlL/L dose of methane- derived microbial biostimulant under two different conditions (T2 & T3), T4 is 75% N control and T5 is 75%N+ 10 mL/L dose of methane- derived microbial biostimulant. First application was seedling root dipping for 20 min for T2, T3 and T5. For T3, second application was given as soil spray whereas in T3 & T5 it was foliar spray. T4 is 75% N control and T5 is 75%N+ 10 ml/L dose of methane derived microbial biostimulant. R1, R2 and R3 respectively corresponds to replication 1, 2 and 3. Small green box indicate position of gas collection base & chambers.

Fig. S1B

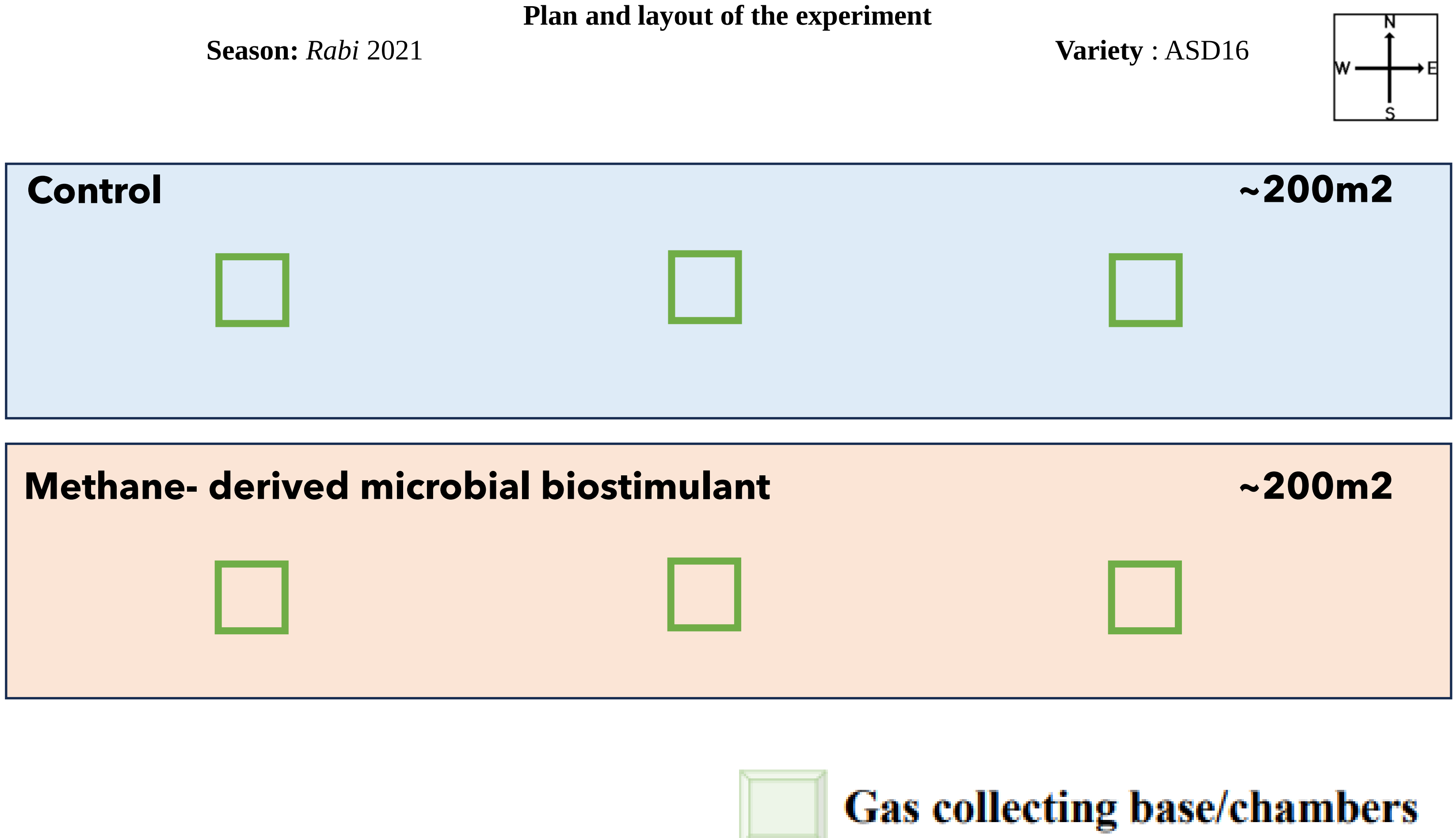


Fig S1B- Experimental field layout for season II testing- Treatment details are below: Control (T1) and methane- derived microbial biostimulant- 10mL/L (T2). Seedling root dipping during transplantation and foliar application during tillering stage and panicle development stage was followed for the application of microbial biostimulant. Control plants received water spray at the same time. Small square box indicates position of gas collection base & chambers.

Fig. S1C

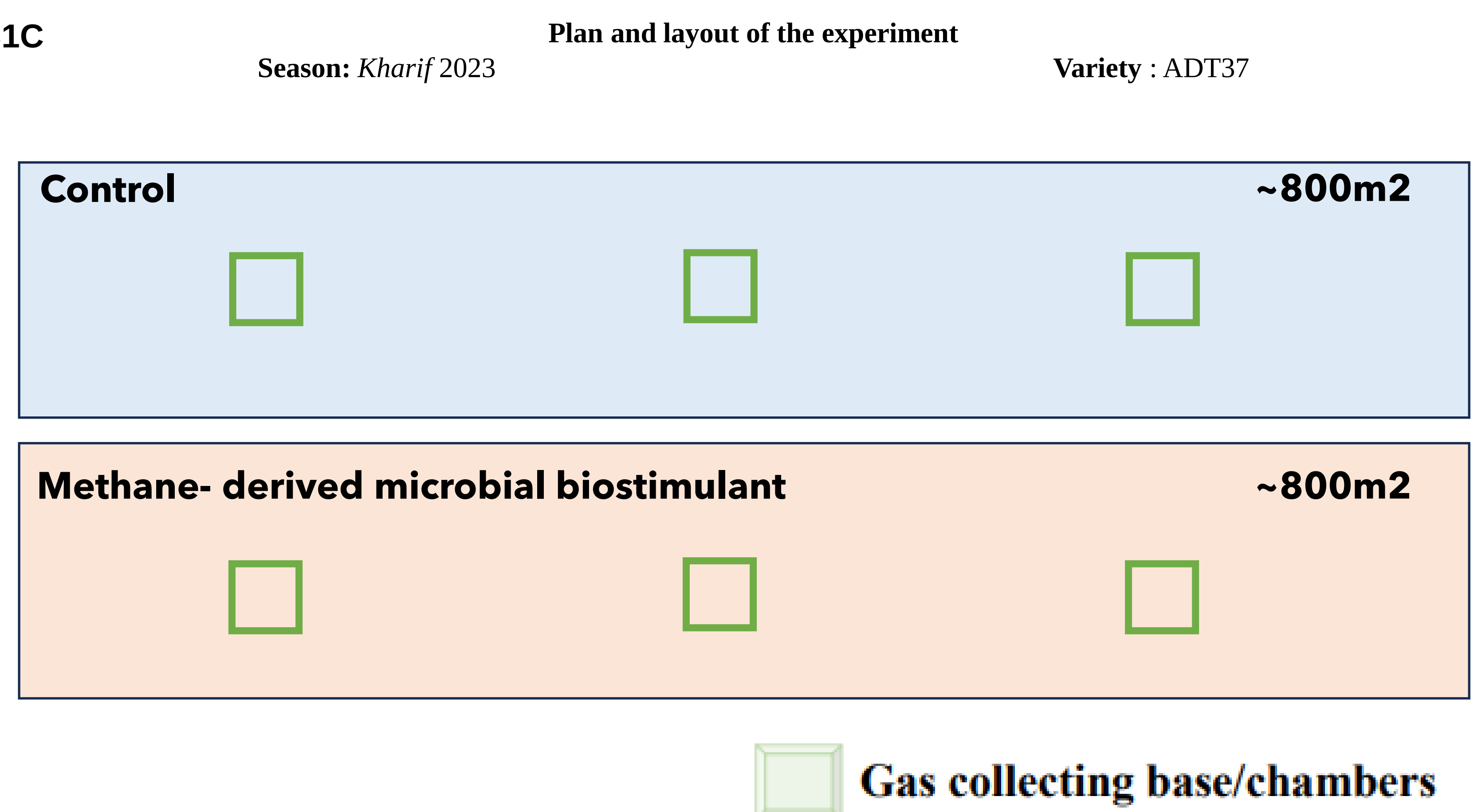


Fig S1C- Experimental field layout for season III testing - Treatment details are below: Control (T1) and methane- derived microbial biostimulant- 10mL/L (T2). Seedling root dipping during transplantation and foliar application during tillering stage and panicle development stage was followed for the application of microbial biostimulant. Control plants received water spray at the same time. Small square box indicates position of gas collection base & chambers.

S2A

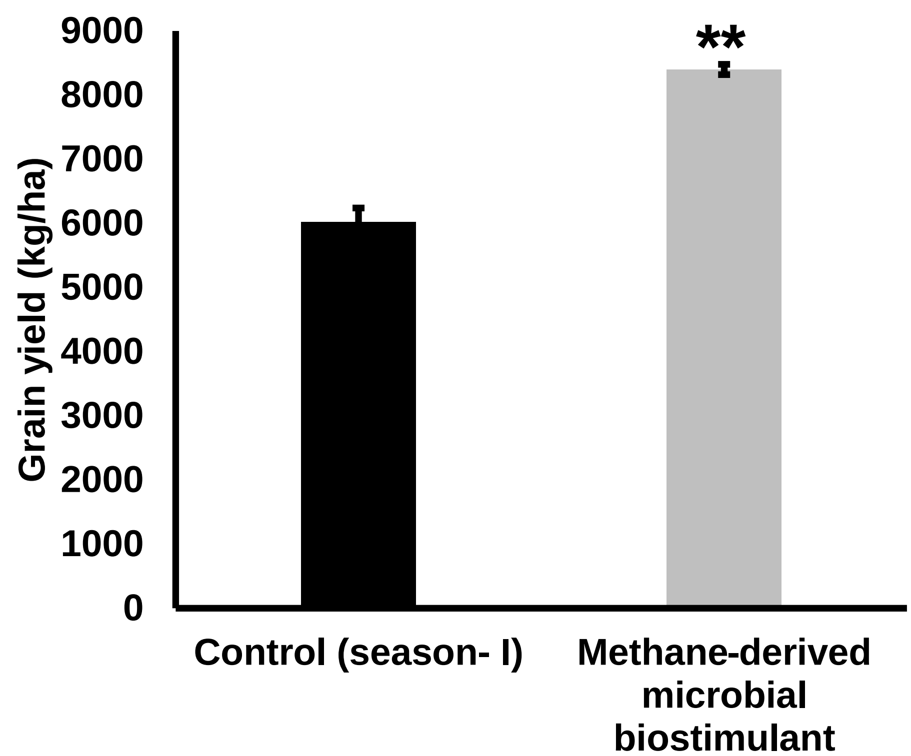


Figure S2A- Influence of methane- derived microbial biostimulant on grain yield- Second application was given as soil spray instead of foliar spray. Differences were evaluated using the two-tailed Student's *t* test and significant differences at *P* < 0.01 is represented by "**".

S2B

S2C

S2D

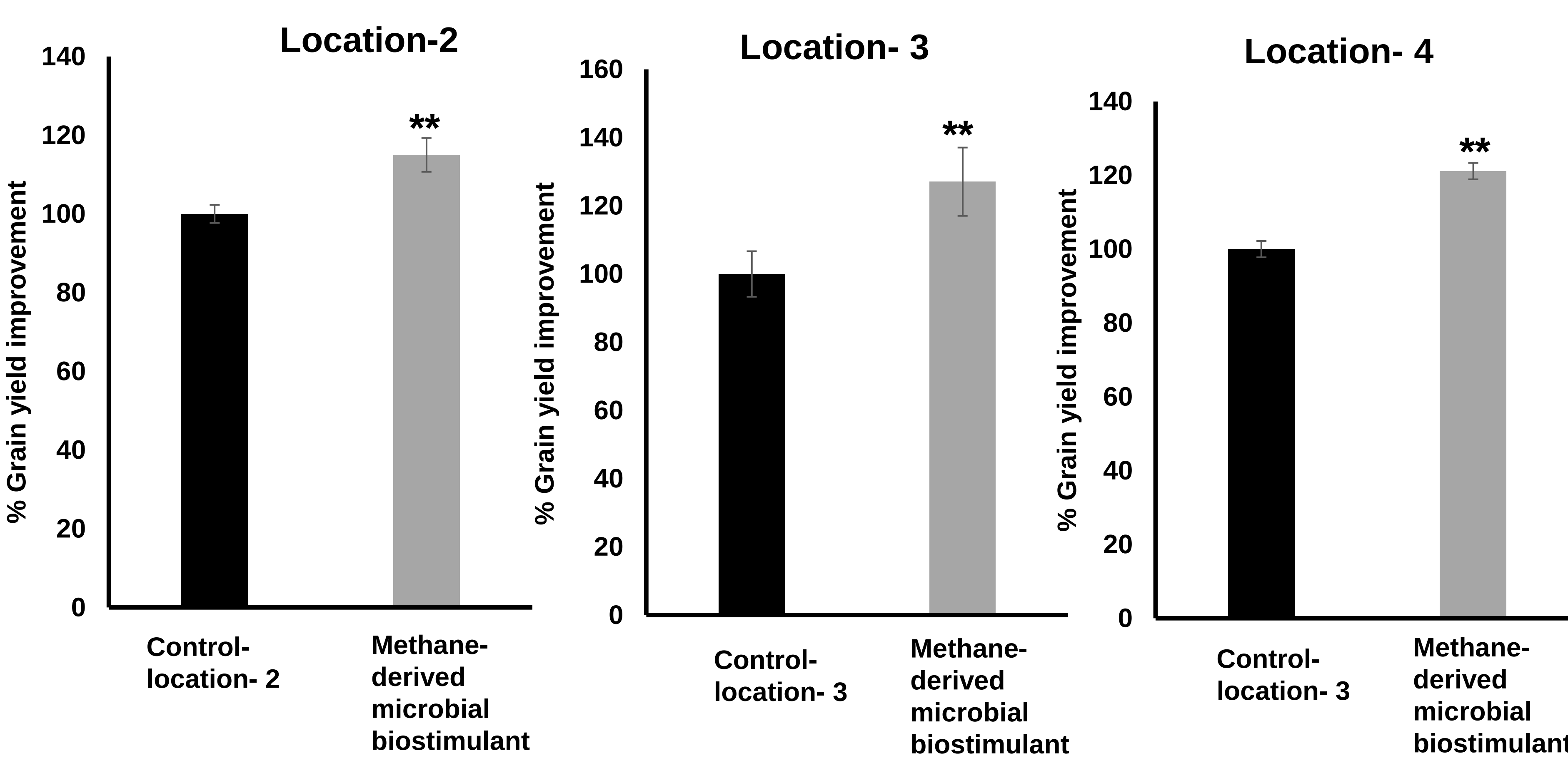


Figure S2B-D- Multilocation microbial biostimulant validation data- Grain yield improvement mediated by methane- derived microbial biostimulant under different agro-ecological locations in India. Differences were evaluated using the two-tailed Student's *t* test and significant differences *P* < 0.01 is represented by "**" .

S3A

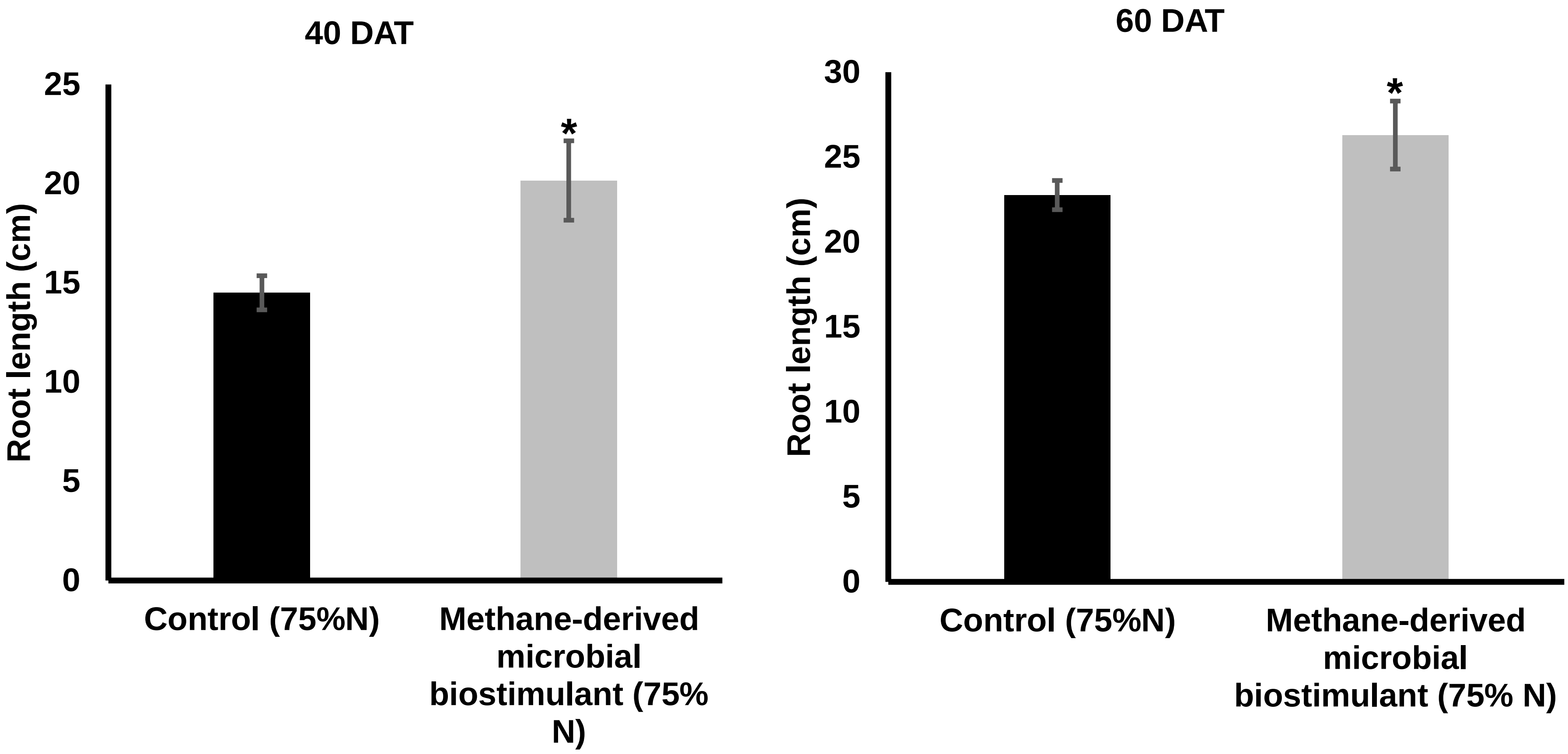


Figure S3A- Effect of microbial biostimulant on root length at 75% N fertilizer- Seedling root dipping was performed in paddy roots with microbial biostimulant. Five independent plants were uprooted from each treatment and root length was measured at 40 DAT and 60 DAT. Student's t-test Significant differences at *P* <0.05 is represented by "*".

S3B

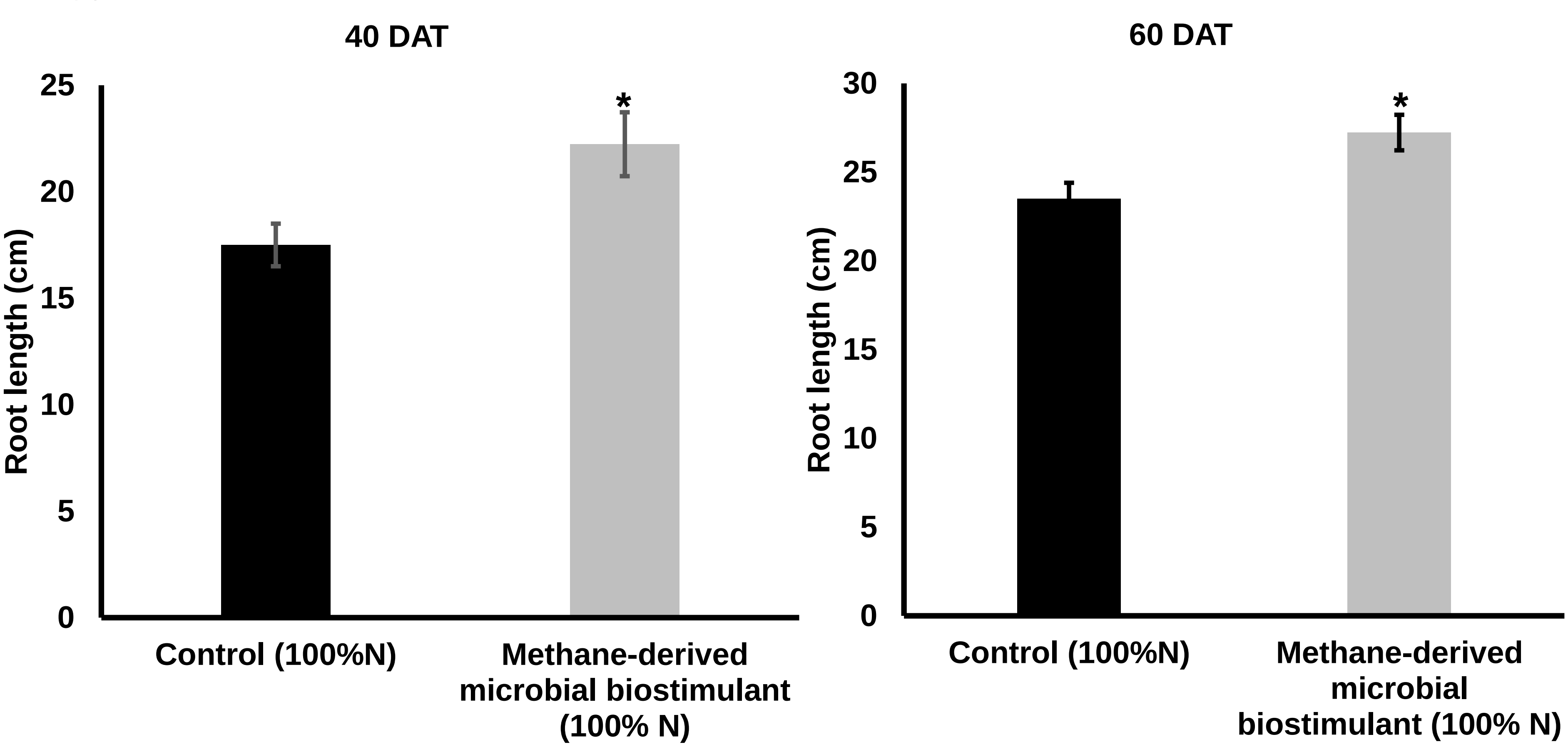
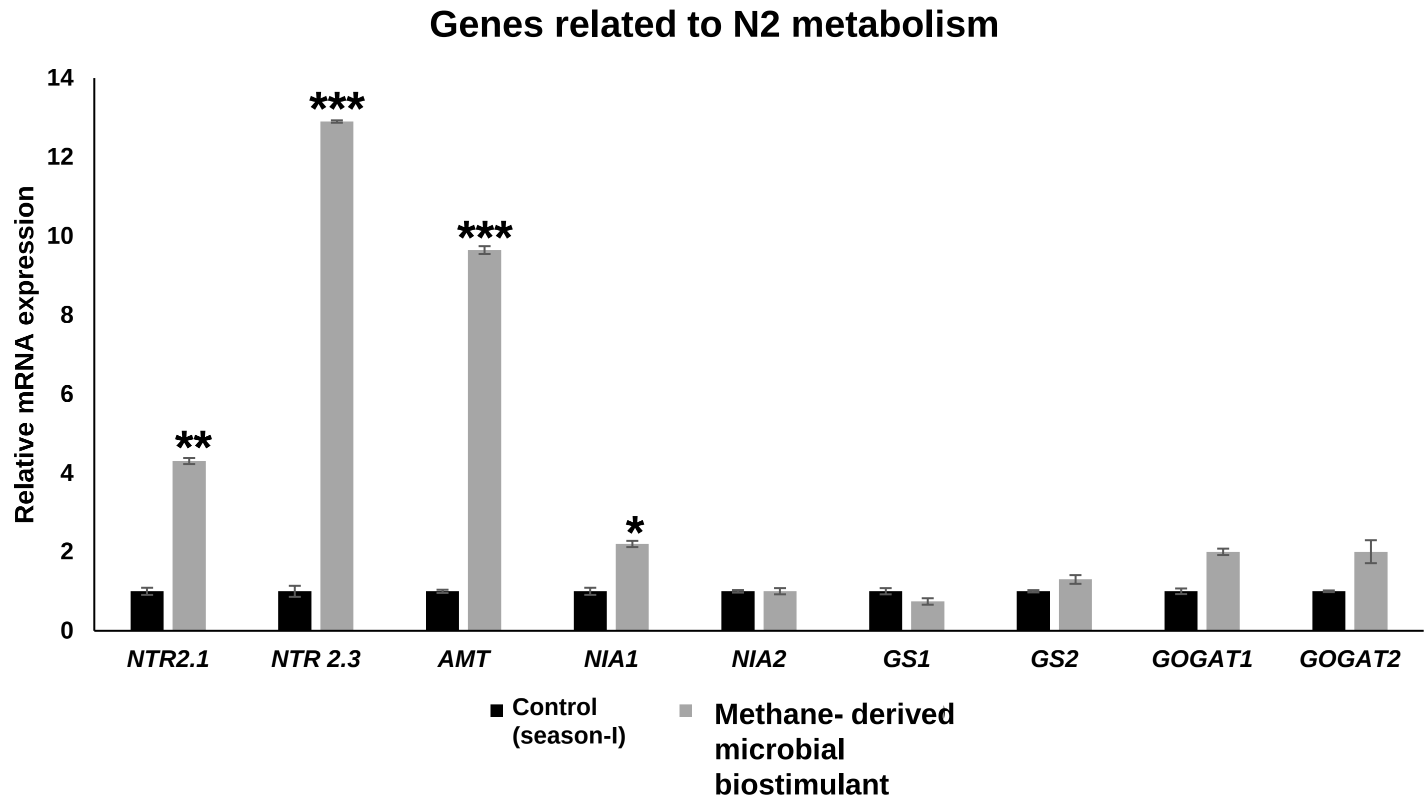


Figure S3B- Effect of microbial biostimulant on root length at 100% N fertilizer- Seedling root dipping was performed in paddy roots with microbial biostimulant. Five independent plants were uprooted from each treatment and root length was measured at 40 DAT and 60 DAT. Student's t-test Significant differences at *P* <0.05 is represented by "*".

S4A



S4B

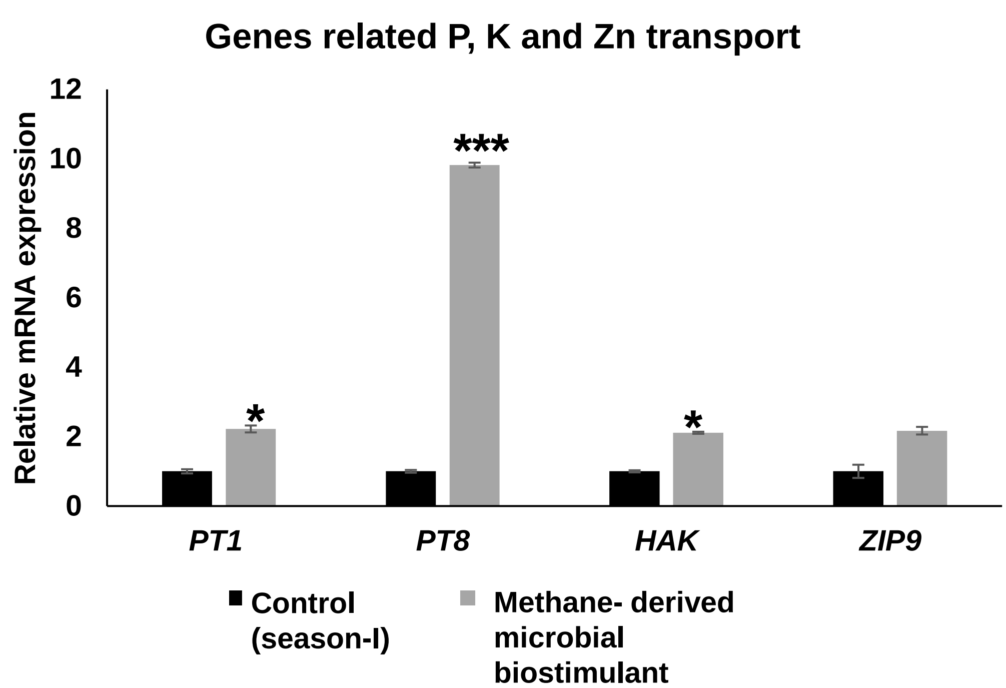


Figure S4- Influence of methane- derived microbial biostimulant on expression of root nutrient uptake and transporter genes- qRT-PCR analysis showing the expression of genes related to macronutrient transport and metabolism in roots of microbial biostimulant treated plants. Expression levels of genes were normalized to the endogenous reference gene actin and are represented relative to respective control roots, which was set to 1. Pooled root samples from control and microbial biostimulant treated roots used for RNA extraction. The results shown are from three independent experiments. Error bars indicate mean ± SE. Student's t-test: significant differences at $P < 0.05$, $P < 0.01$ and $P < 0.001$ are represented by *, ** and ***, respectively.

Nitrate transporter (*NRT*); Ammonium transporter (*AMT*); nitrate reductase (*NIA*); Glutamine synthetase (*GS*); glutamate synthase (*GOGAT*); Phosphate transporter (*PT*); High affinity potassium transporter (*HAK*); Zinc transporter (*ZIP*).

S5

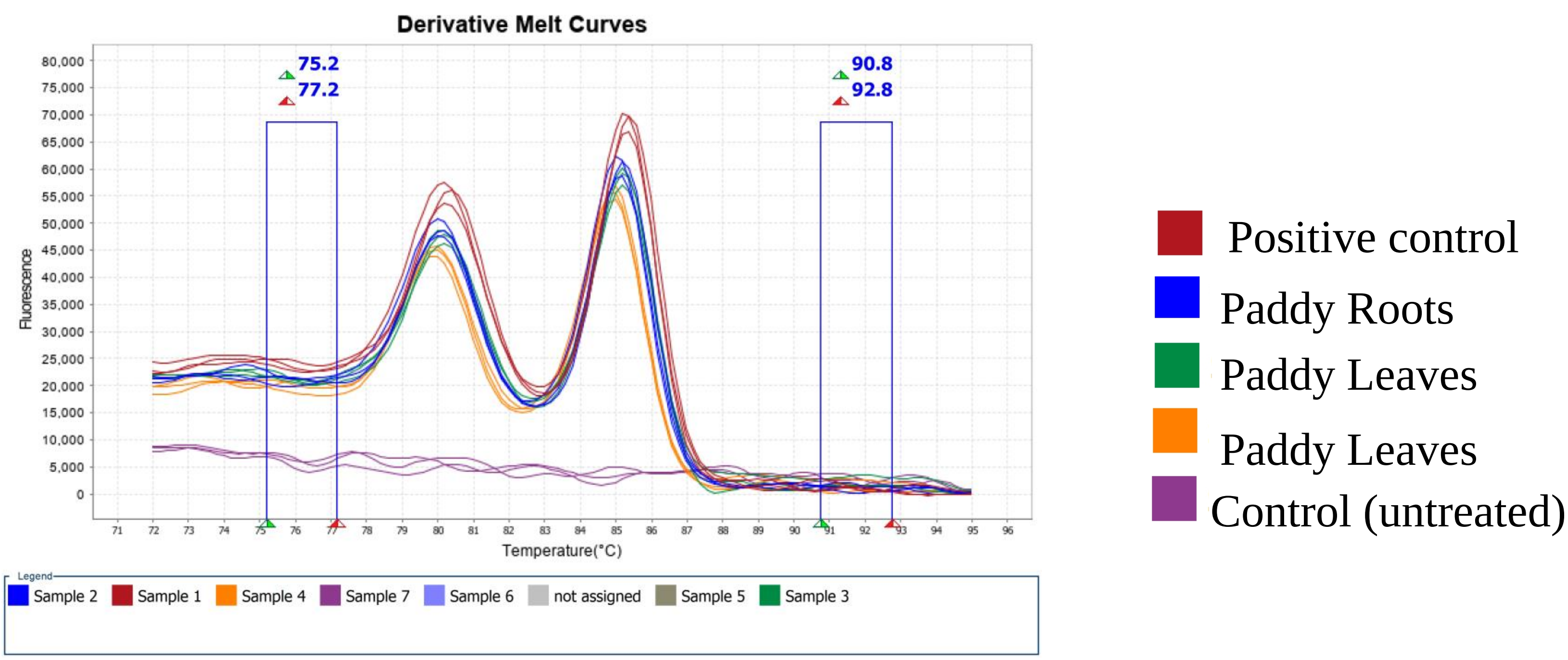


Figure S5- qRT-PCR based derivative melt curve analysis showing the presence of *M. capsulatus* in paddy roots and leaves.

Fig 6A

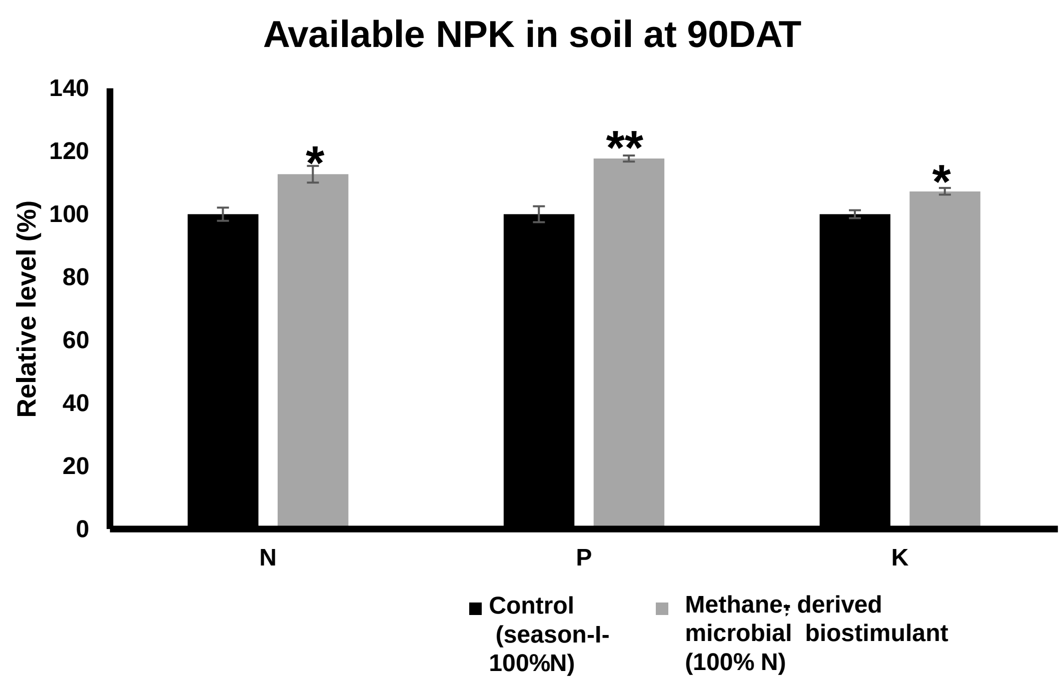


Fig 6B

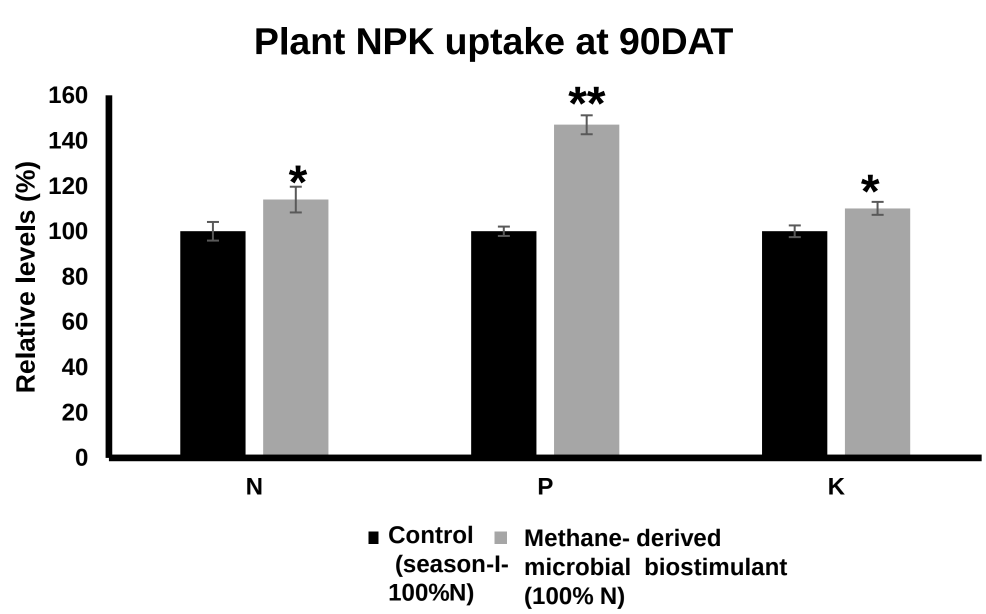


Figure S6A&B- Soil and plant nutrient analysis- Influence of microbial biostimulant on soil NPK levels (A) and plant NPK levels (B). Student's t-test: significant differences at $P < 0.05$ and $P < 0.01$ are represented by “*” and “**” respectively.

Gene	Gene Accession No.	Forward Primer sequence (5' - 3')	Reverse Primer sequence (5' - 3')
OsActin	AB047313.1	ACCATTGGTGCTGAGCGTTT	CGCAGCTTCCATTCCTATGAA
OsPsaH	Os05g0560000	GAGGACATCGGCAACACCA	GCCCTTCTTGATCGGAAGCA
OsFD1	LOC_Os08g01380	AGCAACAAGCTGGGAGACAG	GAGTAAGGCAGGTCGATCCC
OsET	LOC_Os04g33630	GTGGGTGGCGGTGGCAAG	CACGACGGGCCTCAGCTC
OsPsbD	LOC_Osp1g00170	CTGCTACTGCTGTTTTCT	GATGTTATGCTCTGCCTG
OsPsbP	Os07g0141400	CACGGAGTTCATCGCCTACA	AAGCAAGAAGTCGACCTGGG
OsPsbR3	Os08g0200300	GAGGTGGCATGTCAGTCGAT	TTTGCGCCGTACTTGTCAAC
OsPsbS1	LOC_Os01g64960	GCTGTTCGGCTTCACCAAGG	ACGCCGGTCTCGATGTTGA
OsCAB1R	LOC_Os09g17740	GTTCTCCATGTTCGGCTTCT	GACGAAGTTGGTGGCGTAG
OsCAB2R	LOC_Os01g41710	TGTTCTCCATGTTCGGCTTCT	GCTACGGTCCCCACTTCACT
OsCP24	XM_015781325.2	CCTTAACCTTAACGGCCATTTCC	CCCGCAGTAAAATCCTGAGC
OsOEP3	Os07g0544800	AGCCGCTCATCGAGAAGAAG	GTACTTCTCCGCCTCTTCGG
OsTLP	LOC_Os08g39430	GGAGGGAGACGGGGTGGG	TCAGAAAGGGAGGAGAGCCGT
OsLHC2.1	LOC_Os02g52650	GGCGCGGTCGAACGAGCT	GTTTAGCCATTAGTCAAAGCAATC
OsChlI	LOC_Os03g36540	CTTCACCGTCTGCAATGTAG	GATCTTAGGGTCGATGACGTT
OsChlH	LOC_Os03g20700	GCACGGGAACTTGGCGTTTCATTA	ACATGTCCTGGAGCTGCTTCTCAT
OsChlD	LOC_Os03g59640	TAGCACAGCTGTCAGAGTGGGTTT	TTGCCAGCCACCTCAAGTATCTCA
OsHEMA	AB011416.1	GATGCAATCACTGCTGGAAAGCGT	CCATCTTGCCAGCACCAATCAACA
OsHEMC	LOC_Os02g07230	GAGCTGTCTCATTCAGATCTGT	AGGGATTACAATAACCGTGGAA
OsYGL13	LOC_Os08g06630	CTCTCCCACAAGATCAAGGATG	GTTTGTATGAGGGCTCATTTCC
OsYGL8	Loc_Os01g73450,	TGGATCTAACATGACACGCACCCA	ACTGTAACGGCATTCTTCTCCGGT
OsRCA	U74321.1	CTCTTCGTGCCCGTGTTTAC	TCGGAGTTAGCGTCACCAAG
OsRbcS2	L22155.1	AGTCTGGTGGCAACTAAGCC	GCACGGCCGGTAAAATCAAA
OsRbcS3	AK068555.1	ACCATCTCAATGGCCTCTGC	TGTGTGCATATAGCCGGAGC
OsRbcS4	AK070257.1	AACGTTAGGCAGGTGCAGTT	TGCAGCTTAACACGGACACA
OsRbcS5	AK099574.1	GGAGTCCGGCGGAAACTAAG	GGAAACCAATGCAAGGTGGC
OsHYR	Os03g02650	CCGAGGGCTTGATGATGAG	CGTAAGCCCATTTCAGGAATG
OsMOC1	AY242058	CTGCTCCGGCTGCACTAC	CCACGCTGAGACGGAGAG
OsSLR1	Os03g0707600	GAGTCGCTGCACTACTACTCC	GCGCGTGTGCCAGCCCAGCGT
OsLAX2	AB669025	TTCTTGCCTCAGATTCCAAG	CCTCTGCATGTTATCTCCAC
OsMADS57	LOC_Os02g49840	CAGATTATGTTGTCGGATGCTC	AAAGCAATAGAGAGTAAGCAGGGT
OsHSF2AD	LOC_Os03g06630	CAGCAGGCACTTGGCACC	TTCTTGTCACGCTTTAGCCTGT
OsCKX11	Os08g0460600	CAACGCAATCATTGACGCC	TTGCACCCCTCCCAAATGT
OsRGN1	LOC_Os01g49160	CGGCTACACCGACCAGGAG	CGCGATGATGGACCACCT
OsNOG1	MF687920	TCCGACTTACAATGAACAC	GGTAGCAGGACTCCACTT
OsSPL9	LOC_Os05g33810	AGATGGGCAGGTGATTAT	TGTGGGAGAGCTTTAGTC
OsIPA1	LOC_Os08g39890	CGGTCGACTAGCTGCATCTGTTGG	CATCGTGTTGCTGGTTTGGTCGAAG
OsSPL14	Os08g0509600	CAAGGGTTCCAAGCAGCGTAA	TGCACCTCATCAAGTGAGAC
OsLC2	AK101341	AGCATCAGCTTTGGACGAGGA	CAGTTGGTGGAATAGAGCCAGAAT
OsVIL2	XM_015764905.2	GGAGTATGCTTTCCGGATCA	GTGGGAAACAACATGTGCAG
OsGRF4	LC333011.1	GAAAGCCTGTGGAAACGCA	CAACGCCGAGCCAAATGAG
OsNRT2.1	NM_001401744.1	CTTCACGTCGTCGAGGTACT	CACTCGGAGCCGTAGTAGTG
OsNRT2.3A	XM_015773038.2	CGCTGCTGCCGCTCATCCG	CCGTGCCCATGGCCAGAC
OsNIA1	XM_015767224.2	TCAAGGTGTGGTACGTGGTG	CGAGGTCATAGCCCATCTTC
OsNIA2	NM_001422594.1	TGTACCAGGTCATCCAGTCG	CGATGACGTACCACACCTTG
OsAMT1	AF001505.2	GGTTTCTCTCCCTCTCCGAT	CCACCTTCACACCACACATT
OsGS1	AB180688.1	TGTTTCTCCTCATCCCTGC	TCACAGTCCTCGCTTTGC
OsGS2	X14246.1	GGAGAGGTCATGCCTGGTCAGT	ACTACACCAGCCTGCTCCGTTA
OsGOGAT1	XM_015793761.2	GTGCAGCCTGTTGCAGCATAAA	CGGCATTTCACCATGCAAATC
OsGOGAT2	NM_001402625.	CCTGTCGAAGGATGATGAAGGTGAAACC	TGCATGGCCCTACTATCTTCGCATCA
OsPT1	AF536961.1	CGCTTCCGTACGAGTGGTAGT	GGTTCTTTCAAATCCAGGGAAA
OsPT8	AF536968.1	AGAAGGCAAAAGAAATGTGTGTTAAAT	AAAATGTATTCGTGCCAAATTGCT
OsHAK1	NM_001402365.1	GTTGATGATGCTGATGTTGGAAG	CCAACACTTTCAGCTGAAAC
OsZIP9	NM_001420533.1	CATCAGTTCTTCGAAGGGATAGG	TGTGGTTAGCGAGAAGAAGATG
McMopB	AF031148.1	ACAGGCCGAAGAGACTTTCA	GGTGGTGTTGCCTTCGTA AT

Supplementary Table 1- List of oligonucleotide primers used in this study.

Sample	IAA concentration (mg/L)
Microbial biostimulant without Tryptophan	0
Microbial biostimulant with Tryptophan	1.83-3.61

Table S2- Indole acetic acid levels observed in microbial biostimulant grown in presence or absence of Tryptophan.

Season	Treatment	10 DAT	20 DAT	30 DAT	40 DAT	50 DAT	60 DAT	70 DAT	80 DAT	90 DAT	
		CH_4 flux (g/ha/h)									
Season II	Control	31.33±3.38	28.67 ±3.84	70 ± 1.53	109.66 ± 4.7	123.33 ± 6.33	64 ± 4.62	34.2 ± 0.9	22.43 ± 1.68	9.86 + 0.61	
	Methane - derived microbial biostimulant	23.66 ± 1.2	35.66 ± 1.42	53.66 + 1.76	71.33 ± 4.98	61.73 ± 1.92	49.46 ± 4.66	23.13 ± 1.73	15.73 ± 1. 07	5.80 ± 0.45	
		N_2O flux (g/ha/h)									
	Control	0.76 ± 0.02	0.83 ± 0.07	0.78 ± 0.05	2.67 ± 0.08	1.32 ± 0.05	1.82 ± 0.03	3.91 ± 0.25	3.91 ± 0.11	2.14 ± 0.15	
	Methane-derived microbial biostimulant	0.41 ± 0.07	0.55 ± 0.03	0.57 ± 0.03	0.82 ± 0.04	0.51 ±0.06	0.69 ± 0.04	2.07 ± 0.33	2.65 ± 0.13	1.47 + 0.08	
		CH_4 flux (g/ha/h)									
Season	**Treatment**	**10 DAT**	**20 DAT**	**30 DAT**	**40 DAT**	**50 DAT**	**60 DAT**	**70 DAT**	**80 DAT**	**90 DAT**	**100 DAT**
Season III	Control	59.33 ± 1.86	44 ± 1.73	86.66 ± 0.88	103 ± 2.31	128 ± 2.65	149.33 ± 1.45	92.33 ± 3.28	49 ± 1.15	36.66 ± 1.45	28.33 ± 1.45
	Methane-derived microbial biostimulant	37.33 ± 1.20	26.33 ± 0.88	54.33 ± 1.45	47.66 ± 2.96	58 ± 3.46	72.66± 2.46	47± 1.53	16.33± 1.45	20.33± 0.88	12.66±0.67
		N_2O flux (g/ha/h)									
	Control	1.28±0 .03	0.94 ± 0.02	0.76 ± 0.02	1.05 ± 0.03	1.13 ± 0.03	1.28 ± 0.07	1.79 ± 0.04	4.72 ± 0.03	2.70 ± 0.03	3.46 ± 0.26
	Methane-derived microbial biostimulant	0.58 ± 0.03	0.68 ± 0.03	0.45 ± 0.02	0.40 ± 0.02	0.62 ± 0.03	0.68 ± 0.02	0.66 ± 0.01	2.2 ± 0.2	1.06 ± 0.11	1.25 ± 0.03

Table S3A- Temporal CH_4 and N_2O emission from control and microbial biostimulant- treated paddy fields collected during season II and season III trials

Season	Treatment	Average CH_4 emission (kg/ha/season)	Average N_2O emission (kg/ha/season)
Season I Kharif 2022	Control (Season-I)	213.65	16.24
	Methane- derived microbial biostimulant	82.43	10.73
Season II Rabi 2022	Control (Season-II)	131.60	5.32
	Methane- derived microbial biostimulant	90.72	2.80
Season III Kharif 2023	Control (Season-III)	226.97	3.10
	Methane- derived microbial biostimulant	117.83	1.54

Table S3B- Average CH_4 and N_2O emission from control and microbial biostimulant- treated paddy fields.

Season	Treatment	Grain yield (kg/ha)	% improvement in grain yield over control	CH_4 emission				N_2O emission			
				Average CH_4 emission (kg/ha/season)	% reduction in CH_4 emission over control	CO_2 eq CH_4 emission at 25- year time period (kg/ha/season)	Yield-scaled CO_2-eq emission (kg CO_2-eq/t)	Average N_2O emission (kg/ha/season)	% reduction in N_2O emission over control	CO_2 eq N_2O emission at 25- year time period (kg/ha/season)	Yield-scaled CO_2-eq emission (kg CO_2-eq/t)
Season I Kharif 2022	Control (Season-I)	6024	-	213.65	-	17946.43	2979.16	16.24	-	4839.52	803.37
	Methane- derived microbial biostimulant	8004	32.87	82.43	-61.42	6924.29	865.10	10.73	-33.93	3197.54	399.49
Season II Rabi 2022	Control (Season-II)	5015	-	131.60	-	11054.40	2204.27	5.32	-	1585.36	316.12
	Methan-e derived microbial biostimulant	6997	39.52	90.72	-31.06	7620.48	1089.11	2.80	-47.37	834.40	119.25
Season III Kharif 2023	Control (Season-III)	5081	-	226.97	-	19065.60	3752.33	3.10	-	923.80	181.81
	Methane- derived microbial biostimulant	7058	38.91	117.83	-48.09	9897.41	1402.30	1.54	-50.32	458.92	65.02

Table S4- Effect of methane-derived microbial biostimulant on yield-scaled CO2-eq emission in rice- Yield-scaled CO2e-emission of CH_4 and N_2O were found to be significantly lower in methane-derived microbial biostimulant treatment compared to controls.

Conversion factor for CH_4 (1kg of CH_4= 84Kg of CO_2 equivalent) and N_2O (1Kg of N_2O= 298Kg of CO_2 equivalent) were taken from climatechangeconnection.org/emissions/co2-equivalents/

Methane emission (million metric ton)	380		
Methane emission from paddy - 10% contribution (million metric ton)	30.4		
Global land under paddy cultivation (million ha)	162		
Reduction in methane emission from methane-derived microbial biostimulant (percentage per ha)	60%		
Global targeted methane reduction per annum (2% of total - COP26 target) million metric tonnes	7.60		
Percentage of global paddy cultivation area targeted formethane-derived microbial biostimulant application	10.00%	30.00%	50.00%
Actual paddy cultivated area targeted for methane-derived microbial biostimulant application (million ha)	16.2	48.6	81
Total methane emission from target area (million metric tonnes)	3.04	9.12	15.2
Methane emission from methane-derived microbial biostimulant use in target area (million metric tonnes)	1.824	5.472	9.12
Percentage of global methane target achieved	24%	72%	120%

Table S5- CH_4 emission reduction from paddy field using methane-derived microbial biostimulant to meet COP26 target for methane reduction by 2030.