Plant microbial interaction under the influence of methane consumption in the rhizosphere of legumes

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Rajmata Vijayaraje Scindia Krishi Vishwa Vidyalaya

In partial fulfilment of the requirement for the Degree of

MASTER OF SCIENCE

In

AGRICULTURE

SOIL SCIENCE AND AGRICULTURAL CHEMISTRY

by

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2021

CERTIFICATE – I

This is to certify that the thesis entitled "**Plant Microbial Interaction Under the Influence of Methane Consumption in the Rhizosphere of Legumes"** submitted in partial fulfilment of the requirement for the degree of **MASTER OF SCIENCE in AGRICULTURE (Soil Science and Agricultural Chemistry)** of the Rajmata Vijayaraje Scindia Krishi Vishwa Vidyalaya, Gwalior is a record of the bonafide research work carried out by **Ms. Seema Choudhary, ID No. 19111310,** under my guidance and supervision. The subject of the thesis has been approved by the Student's Advisory Committee and the Director of Instruction.

No part of the thesis has been submitted for any other degree or diploma or has been published. All the assistance and help received during the course of the investigation has been acknowledged by the scholar.

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CERTIFICATE

It is certified that the thesis entitled **"Plant Microbial Interaction Under the Influence of Methane Consumption in the Rhizosphere of Legumes"** which is being submitted by **Ms. Seema Choudhary** in partial fulfilment of the requirement for the award of the Degree of **Master of Science in Agriculture** in the Department of Soil Science and Agricultural Chemistry of **Rajmata Vijayaraje Scindia Krishi Vishwavidyalaya, Gwalior** is a record of candidate's own work carried out by her under my supervision and guidance from October 2020 – August 2021. The matter embodied in this thesis has not been submitted for award for any other degree.

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Place: Gwalior

Date: **SEEMA CHOUDHARY**

LIST OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Abbreviations	Full name
CH_4	Methane
CO_2	Carbon dioxide
g	Gram
mg	Milligram
cm	Centimetre
ha	Hectare
ml	Millilitre
µg	Microgram
µl	Microlitre
Ng	Nanogram
Ppm	Parts per million
Ppb	Parts per billion
EC	Electrical conductivity
%	Percentage
ANOVA	Analysis of variance
HSD	Honestly significance different
PCA	Principal component analysis
PC	Principal component
Ds	Desi Siemens
\Tg	Tera gram
N	Nitrogen
P	Phosphorus
K	Potassium
C	Carbon
Lux	Light intensity
NifH	Nitrogen fixers
DAS	Day after sowing

CHAPTER- I

INTRODUCTION

In terms of global warming potential, methane (CH_4) is the second most important greenhouse gas. CH_4 can absorb 20–30 times the amount of infrared radiation compared to that of CO_2. CH_4 is mostly biogenic and is generated by anaerobic methanogenic bacteria in terrestrial ecosystems. In agricultural soil ecosystem, CH_4 is produced in anaerobic compartments, for example in flooded soil, it is the subsurface anaerobic zones and in aerobic soil, it is the anaerobic sites within aggregates.

CH_4 concentration in soil can reach up to 10000 to 20000 ppm in sediments. However, majority of CH_4 is consumed (oxidized) in soil and it accounts for up to 6 % of the worldwide CH_4 sink (Brzezińska *et al.,* 2012). CH_4 is used as a source of carbon and energy by the obligatory aerobic methanotrophs. Methanotrophs are classified as Alpha- and Gamma proteobacteria, with *Crenothrix* and *Clonothrix* filamentous bacteria among the latter (Dumont *et al.,* 2014). Acidophilic methane-oxidizing Verrucomicrobium also consumes CH_4 in very acidic conditions (Op den Camp *et al.,* 2009). Methanotrophic bacteria are found all throughout the world, even under the harshest conditions (Shelley *et al.,* 2014; Dunfield *et al.,* 2010). Particulate and soluble forms of CH_4 monooxygenase are the primary enzymes for CH_4 intake discovered in methanotrophic bacteria (MMO). These enzymes have a high level of substrate specificity (CH_4) (Vorobev *et al.,* 2011; Lieberman and Rosenzweig, 2004). In addition to CH_4, methane monooxygenase (MMO) can oxidise a variety of organic contaminants. High affinity methanotrophs oxidise CH_4 at or near atmospheric quantities in the terrestrial environment, whereas low affinity methanotrophs use CH_4 at concentrations several times higher than in the atmosphere (Knief *et al.,* 2005). In soil, methanotrophs are abundant, and their activities is substantial (Mohanty *et al.,* 2015). Methanotrophs have a high rate of growth that is dependent on availability of

CH_4-C. In addition to C (CH_4), methanotrophs are likely able to assimilate essential nutrients of assimilating essential nutrients.

A major fraction of CH_4 is oxidized in soil. However, a part of the biogenic CH_4 remains entrapped in soil and can interact with soil and plant rhizosphere influencing plant growth. The current experiment is carried out to establish the linkage between rhizospheric CH_4 and growth of legume with the following hypotheses. First, CH_4 may influence plant growth by altering plant-microbial interaction. Second, CH_4 stimulates methanotrophs which can affect the abundance of N fixing organisms and N fixation. The current research envisages to bridge the knowledge gap and to provide crucial information on the effect of CH_4 in the rhizosphere of legumes (soybean and pigeon pea) with the following objectives are:

1. To evaluate the effect of methane consumption on nodulation and nitrogen fixing potential of soybean and pigeon pea.
2. To quantify the abundance of methanotrophs and nitrogen fixers in the rhizosphere of soybean and pigeon pea plants.
3. To estimate plant growth parameters in response to methane enrichment and evaluate the relation among methane consumption, microbial population and plant growth attributes.

CHAPTER- II

REVIEW OF LITERATURE

Plant rhizosphere encompass a dynamic zone of interactions between microorganisms and their respective plant hosts. Present study entitled “Plant microbial interaction under the influence of methane consumption in the rhizosphere of legumes” focus on the effect of methane consumption on nodulation, abundance of methanotrophs and nitrogen fixers in the rhizosphere of plants. A brief review of research work done related to our investigation is scripted in this current chapter.

2.1. Microbial Dynamics in Plant Rhizosphere

Philippot *et al.,* (2013) reported that the rhizosphere, a small compartment of soil which is close to and is affected by plant roots, has long been regarded as one of the most important interfaces for life on Earth. Garcia *et al.,* (2018) reported that plants rhizosphere encompasses a dynamic zone of interactions between microorganisms and their respective plant hosts. For decades, researchers have worked to understand how these complex interactions influence different aspects of plant growth, development, and evolution. Studies of plant-microbial interactions in the root zone have typically focused on the effect of single microbial species or strains on a plant host. These studies, however, provide only a snapshot of the complex interactions that occur in the rhizosphere, leaving researchers with a limited understanding of how the complex microbiome influences the biology of the plant host. To better understand how rhizosphere interactions influence plant growth and development, novel frameworks and research methodologies could be implemented. In this perspective, we propose applying concepts in evolutionary biology to microbiome experiments for improved understanding of group-to-group and community-level microbial interactions influencing soil nutrient cycling. We also put forth simple experimental designs utilizing -omics techniques that can reveal important changes in the rhizosphere impacting the plant host. A greater focus on the components of complexity of the microbiome and

how this impact plant host biology could yield more insight into previously unexplored aspects of host-microbe biology relevant to crop production and protection.

Ding *et al.* (2019) Land plants directly contact soil through their roots. An enormous diversity of microbes dwelling in root-associated zones, including endosphere (inside root), rhizoplane (root surface) and rhizosphere (soil surrounding the root surface), play essential roles in ecosystem functioning and plant health. Rice is a staple food that feeds over 50% of the global population. Its root is a unique niche, which is often characterized by an oxic region (e.g. the rhizosphere) surrounded by anoxic bulk soil. This oxic-anoxic interface has been recognized as a pronounced hotspot that supports dynamic biogeochemical cycles mediated by various functional microbial groups. Considering the significance of rice production upon global food security and the methane budget, novel insights into how the overall microbial community (i.e. the microbiome) of the rice root system influences ecosystem functioning is the key to improving crop health and sustainable productivity of paddy ecosystems, and alleviating methane emissions. This mini-review summarizes the current understanding of microbial diversity of rice root-associated compartments to some extent, especially the rhizosphere, and makes a comparison of rhizosphere microbial community structures between rice and other crops/plants. Moreover, this paper describes the interactions between root-related microbiomes and rice plants, and further discusses the key factors shaping the rice root-related microbiomes.

2.2. Methanotrophs and nitrogen fixers in the rhizosphere of plants

2.2.1. Methanogens

Shen *et al.,* (2016) in a study reported that methanogens are strictly anaerobic *Archaea*. These microorganisms are involved in the last step of the electron transfer chain during sequential terminal electron accepting process and organic matter decomposition. Methanogens use a restricted range of substrates produced during this degradation: H_2/CO_2, CH_3COO-, and H_2/CO_2 and HCOO-, methyl compounds like CH_3OH, and $(CH_3)_3NH_2$, and some secondary alcohols like 2-propanol and 2-butanol. Methanogens metabolize at a temperature optimum between 37-40^{o}C, although they

may grow upt o 65^{o}C (Nicholson *et al.,* 2007). Depending upon 16S rRNA sequence 20 genera of CH_4 producing bacteria have been described, but only four genera *viz*., *Methanobacterium, Methanosarcina, Methanobrevibacter and Methanocarpusulum* have been isolated from submerged soil ecosystem like rice soils (Alpana *et al.,* 2017). Methanogens such as *Methanospirillum* and *Methanocarpussulum* which had been isolated from fresh water sediments may also be present in the wetlands rice soils (Zhou *et al.,* 2014).

82.2.2. Mechanism of methanogenesis

Dolfing *et al.,* (1992) reported that a number of obligate hydrogen producing acetogenic or OHPA can metabolize into acetate, H_2, and inorganic C. Homoacetogens are very versatile and can use sugars, alcohols, fatty acids, purines, aromatic compounds as well as methanol, formate, H_2, and CO_2 to produce acetate as the sole fermentation product.The processes involved in methanogenesis are solubilization, fermentation (or acidogenesis), and methanogenesis (Neue, 1993). There are generally four types of bacteria needed: (a) hydrolytic and fermentative bacteria, (b) H^+- reducing bacteria, homoacetogenic bacteria, and (d) methanogenic bacteria (Conrad, 1996). The first group hydrolyses polymers and ferments the resulting monomers to smaller molecules such as alcohols, short chain fatty acids (propionate, n-and isobutyrate, n- and isovalerate), H_2, and CO_2. Methanogens immediately convert H_2/CO_2, formate, acetate, few other simple compounds including methanol, methylated amines and dimethyl sulphide to CH_4 and CO_2. Several end product molecules in fermented step cannot serve as methanogenic substrates; these include volatile fatty acid anions with three or four carbon atoms, lactate, ethanol, aromatics, and long chain fatty acids.The other methanogenic *i.e.,* hydrogenotrophic methanogens oxidize H_2, and reduce CO_2 to form CH_4; methylotrophic methanogens use methyl compounds such as methanol, methyl amines, or dimethylsulphide and acetotrophic methanogens utilize acetate to produce CH_4 (Garcia, 1990). The hydrogenotrophic methanogens reductively fix CO_2 as C1 group on enzyme methanofuran (MFR) to produce formyl-MFR. This group is then transferred to another

coenzyme, tetrahydromethanopterin and subsequently, the formyl group is dehydrated and reduced by two 2-electron reduction to methyl level, using the coenzyme F_{420}, as electron carrier. The methyl group is then transferred from methanopterin to the coenzyme M (2-mercaptoethanosulphonate, HS-CoM) to form CH_3-S-CoM, which is the substrate for methyl coenzyme –M reductase (MR). Finally, CH_4 is reductively cleaved from CH_3-S-CoM by reaction with a methanogen specific cofactor,7-mercaptoheptanoylthreonine phosdphate (HS-HTP). A total of four reduction steps are envisaged for reduction of CO_2 to CH_4. Most of the CH_4 produced in nature originates from the methyl group of acetate (CH_3COO).

Ferry and Kastead (2007) reported that the.CO dehydrogenase is a key enzyme catalysing the decarbonylation of acetyl-CoA, the resulting methyl group is transferred to CH_3-S-CoM, followed by reduction of CH_4 using electron derived from oxidation of the carbonyl group to CO_2 by the CO dehydrogenase. Methylotrophic methanogens transfer the methyl group of CH_3OH and methylamins to CH_3-S-CoM to CH_4 are provided by the oxidation of methyl groups to CO_2.

2.2.3. Methane Oxidation

Frenzel and Bosse, (1996) reported that nearly 90% of the CH_4 produced in the flooded anaerobic soil can be oxidized in the soil-water interface and rhizosphere of plants while diffusing into the atmosphere. Studies with methyl fluoride, a specific inhibitor of CH_4 oxidation showed that CH_4 emission from the soil would be five to ten times greater if its oxidation did not occur in the surface layer (Oremland and Culbertson, 1992). Thus, the CH_4 oxidation can greatly influence the release of CH_4 to the environment. Methanotrophs are 10 times more abundant in the rhizosphere than that in the reduced soil away from the rice rhizosphere and about 1/3 times more are present in the oxic-soil-water interface (DeBont *et al.,* 1978).

2.2.4. Taxonomy and Physiology of Methanotrophic bacteria

Adamsen and King, (1993) reported that methanotrophs are strict aerobes and are divided into two groups viz Type I and Type II, based on their pathways of carbon assimilation, numerical taxonomic evaluation, DNA-DNA hybridization, phospholipid

fatty acid (PLFA) composition analysis, genomic, physicochemical properties, and phylogenetic relationships. Type I methanotrophs include three broadly homologous clusters of species and the family Methylococcaceae contains the genera, *Methylobacter, Methylomonas, Methylomicrobium,* and *Methylosinus*. Type I methanotrophs oxidize CH_4 through ribulose monophosphate pathway (RuMP) for assimilation of formaldehyde as cell carbon, while Type II methanotrophs use serine pathway. A third group, Type X is also believed to exist which possesses enzyme that catalyse both serine and RuMP pathway (Whittenbury and Dalton, 1981). They grow at higher temperature than Type I and Type II groups and are characterized by high GC content than that of Type I (Whittenbury and Krieg, 1984).

Prior and Dalton (1985) reported that the oxidation of CH_4 by aerobic methanotrophs is initiated by methane monooxygenase (MMOs), that utilize two reducing equivalents to split the O-O bond of O_2. One oxygen atom is reduced to form H_2O and the other is incorportaed into CH_4 to form CH_3OH. Two forms of MMO have been found in methanotrophs, soluble CH_4 monooxygenase (sMMO) and particulate CH_4 monooxygenase (pMMO). sMMO catalyzes the oxidation of CH_4 toCH_3OH which is subsequently oxidized to HCHO and eventually CO_2, as the end product. All methanotrophs are believed to be capable of expressing pMMO when grown in the presence of copper (Dalton, 1992). These bacteria also utilize a variety of different one carbon compounds including CH_4, methanol, methylated amins, halomethanes, and methylated compounds. Cells containing sMMO rapidly oxidize naphthalene to 1- and 2-napthols, which can be detected by addition of tetrazotized O-dianisidine resulting the formation of purple diazo dyes with large molar adsorvities (Brusseau *et al.,* 1994).

Bedard and Knowles (1989) reported that chemoautotrophic ammonium oxidizers belonging to the family Nitrobacteriaceae are also implicated in the oxidation of CH_4 in certain environments. The common habitat of methanotrophs and ammonium oxidizers and similarity between CH_4 monooxygenase and NH4 monooxygenase, probably make ammonium oxidizers capable of CH_4 oxidation. It is

apparent that methanotrophs are ubiquitously distributed and play a significant role in the global CH_4 budget and therefore in moderating the impact of CH_4 on global warming.

Chan and Parkin (2001) studied the relation between soil mineralogical composition and methane oxidation and found a negative relation between methane oxidation and soil mineral N (NO_2^- +NO_3^- + NH_4^+) concentration under ambient atmospheric condition.

Pathak *et al.* (2011) in a study on soil submergence in paddy field suggested that direct-drill seeded rice does not require continuous soil submergence, and hence it can either reduce or eliminate CH_4 emissions for lowland rice when it is grown as an aerobic crop. Yamulki *et al.,* (2006) highlighted that addition of crop residues affects CH_4 oxidation in upland soils and emission patterns in flooded soils distinctly depending on their C/N ratio, residues with a high C/N ratio have little effect on oxidation while residues with a narrow C/N ratio seem to inhibit oxidation. Nisbet *et al.* (2019) studied the growth of methane in environment in recent years and found that growth of methane was so rapid in recent 4 years and such growth rate was not since observed since the 1980s. It was 12.7 ± 0.5 ppb/year (in 2014), 10.1 ± 0.7 ppb/year (in 2015), 7.0 ± 0.7 ppb/year (in 2016), and 7.7 ± 0.7 ppb/year (in 2017), abrupt increase in methane concentration started in 2007 and it resulted from 1,775 ppb in 2006 to 1,850 ppb in 2017. Kirschke *et al.* (2013) indicated that there are several sources of methane gas emission like coal mining, oil and gas industry, combustion of fossil fuels, biomass burning, agriculture (paddy fields, livestock etc.), solid waste disposal and landfills, waste water treatment and natural sources like Wetlands and Hydrates.

2.3. Methane consumption, microbial population and plant growth attributes

Chan and Parkin, (2001) reported that two microbial processes, methanogenesis and CH_4 oxidation play crucial role in methane (CH_4) flux from soil to the atmosphere and activities of three different microbial populations: the

methanotrophs, the ammonia oxidizing nitrifiers, and the methanogens reflects its dynamics.

Bharati *et al.* (2000) studied the role of azolla on CH_4 flux in paddy field and found that, use of Azolla in conjunction with urea considerably reduced CH_4 efflux without affecting the rice yields and suggested azolla as practical mitigation option for minimizing CH_4 flux from flooded paddy fields. Mohanty *et al.* (2007) explored the effect of temperature on CH_4 consumption and methanotrophs population by terminal restriction fragment length polymorphism (T-RFLP) targeting particulate methane monooxygenase (*pmoA*) genes, individual T-RFs were tentatively assigned to different methanotrophic populations (e.g. *Methylococcus/ Methylocaldum, Methylomicrobium, Methylobacter, Methylocystis/ Methylosinus*) results clearly represented that temperature affected the relative abundance of most T-RFs so temperature can be an important factor regulating the community composition of methanotrophs in soil.

Alam and Jia (2012) studied the effect of ammonium on methane oxidation in a paddy field and concluded that the methane oxidation activity in paddy soils might be inhibited when the concentration of ammonium fertilizers is high, strong nitrification might lead to a pH decline, which may affect the niche differentiation of MOB.

Kollah *et al.* (2015) studied the effectiveness of biochar (BC) on influencing methane (CH_4) consumption in a tropical clayey vertisol and through the findings revealed that BC enhanced CH_4 consumption potential in agricultural land on a tropical vertisol, particularly using the smaller size (<0.25 mm), and could be an effective strategy to mitigate atmospheric CH_4.

Hofmann *et al.* (2016) reported the abundance of Methanogens and methanotrophs along an altitudinal gradient (2700–3500 m) in the Austrian Alps and found that methanogens seem to be capable of persisting despite a highly oxic low-temperature environment. Methanogenic and methanotrophic activities and abundances of methanotrophs, Methanococcales and *Methanocella* spp. declined with altitude.

Xu *et al.* (2003) studied the impact of nitrogen (N) and biochar amendment on CH_4 concentrations and diffusive effluxes and revealed that the top 7-cm soil layers were the primary CH_4 production sites during the rice-growing seasons acts as the source of CH_4 generation and diffusion, and the deeper soil layers and the wheat season soil acted as the sink. Also, N fertilization significantly increased the CH_4 concentration and diffusive effluxes in the top 7cm layers and biochar amendment is a good strategy for reducing the soil profile CH_4 concentrations and diffusive effluxes induced by N in paddy fields. Kollah *et al.,* (2017) estimated methane (CH_4) consumption in different soil (vertisol) aggregates under elevated carbon dioxide (eCO_2) and temperature and found that mesoaggregates of 0·25–1·00 mm are hotspots for CH_4 consumption and that rising atmospheric CO_2 and temperature may inhibit CH_4 consumption significantly in a tropical vertisol. Mohanty *et al.* (2015) carried out an experiment to understand how iron (Fe) reduction oxidation (IRO) influences CH_4 oxidation in soil. Soil samples (alluvial and vertisol) were induced to undergo microbial Fe reduction and aerobic oxidation consecutively for three cycles simulating natural wetting-drying soil cycle. After each IRO cycle, soils were incubated to determine CH_4 oxidation rate, Fe mineral and methanotrophs abundance. Potential iron reduction rate k (mM Fe2þ produced g 1 soil d 1) increased from 1.26 to 2.16 in vertisol and 1.95 to 3.05 in alluvial soil. Potential iron oxidation (mM Fe2þ oxidized g 1 soil d 1) increased from 2.33 to 5.70 in vertisol and 2.43 to 9.58 in alluvial soil. The iron reduction-oxidation significantly ($p < 0.05$) stimulated CH_4 oxidation rate k. The high affinity CH_4 oxidation rate increased from 0.03 to 0.19. Low affinity CH_4 oxidation rate increased from 0.05 to 0.47 in vertisol. A similar effect of IRO on k was observed in alluvial soil. X ray diffraction (XRD) revealed that diffraction intensity of magnetite and goethite decreased over IRO cycle. Real time PCR quantification of methanotrophs (*pmoA* gene) confirmed that IRO cycle stimulated ($p < 0.05$) methanotrophs abundance. The study highlights that iron reduction-oxidation cycles can significantly enhance CH_4 oxidation in tropical soils.

Turmel *et al*. (2015) indicated that conservation agriculture (CA) is a system of agronomic practices that include reduced tillage (RT) or no-till (NT), permanent organic soil cover by retaining crop residues, and crop rotations, including cover crops. The extent of Conservation agriculture (CA) to contribute to climate regulation and serve as a global warming mitigation strategy depends on the direction and magnitude of modifications in soil Carbon, nitrous oxide (N_2O) and methane (CH_4) emissions associated with its execution compared to conventional practices. Assessment global warming potential of the farming practices depends on soil, climate and management.

Paustian *et al.* (2000) in a study on the cultivation practices and emission of CO_2 revealed that practices like ploughing, breaking of soil aggregates and exposure avail soil organic carbon to microbial attack and subsequently enhance rate of Barros *et al.* (2010). Decomposition and CO_2emissiontoatmosphere. Implication of CA on GHG emission has been studied and found that an average of 29.3 Mg ha^{-1} of GHGs is emitted over 20 years in conventional rice-wheat systems across the IGP; this decreased by only 3% with the widespread implementation of CA. Mangalassery *et al.* (2015) defined relation between different tillage practices and greenhouse gases emission and found that soil tillage practices have a fundamental influence on the physical properties of soil and the greenhouse gas (GHG) balance also recorded significantly higher net global warming potential under conventional tillage systems (26–31% higher than zero tillage systems). Mohanty *et al.* (2015) reported methane (CH_4) consumption in the rhizosphere of soybean-wheat cropping system under different fertilizer management practice in a tropical vertisol and brought out the fact that organic farming can significantly decrease global atmospheric CH_4 budget in addition to improving soil physical and biological properties.

CHAPTER- III

MATERIALS AND METHODS

3.1. Site characterization and soil sampling

Soil samples were collected from an experimental field located at the Indian institute of soil science, Bhopal, Madhya Pradesh, India (23°18'N / 77°24'E, 485 m above sea level). The field was continuously planted with rice (*Oryza sativa*) and wheat (*Triticum aestivum* L.) since 2018 during the summer and winter seasons, respectively. The fertilizer dose for wheat was 120 kg N ha^{-1}, 60 kg P ha^{-1}, and 40 kg K ha^{-1}. The fertilizer dose for rice was 80 kg N ha^{-1}, 40 kg P ha^{-1}, and 40 kg K ha^{-1}. Inorganic source of N, P, and K were urea $(NH_2)_2CO$, single super phosphate Ca $(H_2PO_4)_2$. H_2O and muriate of potash KCl, respectively.

The location has a humid subtropical climate, with a hot summer and a humid monsoon season. It experiences south western monsoon rains between July and September. Mean annual temperature was about 25 °C. Highest temperature was near 45 °C during the mid-summer (May-June) and lowest about 5°C in January. The average precipitation was 1200 mm and humidity 65%.

A composite sample representing soybean field was collected at the vegetative stage of crop durin Aug 2019. Three randomly collected individual samples were mixed to make a composite sample. Soil samples were then hand processed by removing plant material, stones, and large organisms and subsequently stored in plastic bags at 4°C to prevent moisture loss and were used within 2 days of collection.

3.2. Soil Physico-Chemical properties

Soil samples were analyzed to estimate physical and chemical properties. The experimental soil was a heavy clayey Vertisol (TypicHaplustert). The electrical conductivity (EC) was 0.43 dS m^{-1} and the pH was 7.5 (1:2.5 of soil and water in w:v) (Smith and Doran, 1996). Soil organic C was determined by wet digestion method (Walkley and Black, 1934) and its concentration was 5.2 g kg^{-1}. Available N was determined by alkaline $KMnO_4$ method (Subbiah and Asija, 1956). Available N content

was 206 mg kg^{-1}. Available P was extracted by 0.5 N $NaHCO_3$ solution buffer at pH 8.5 (Olsen, 1954) and P in the extract was determined by ascorbic acid method (Watanabe and Olsen, 1965). Available P content was 2.6 mg kg^{-1}. Available K was extracted by shaking with neutral normal ammonium acetate for 5 minutes (Hanway and Heidel, 1952) and then K in the extract was determined by flame photometer (Lindsay and Norvell, 1978). Available k was 230 mg kg^{-1}. Bulk density was 1.36 g cm^{-3}. The textural composition of soil was: sand 15.2%, silt 30.3%, and clay 54.5%.

3.3. Microcosms set up exploring the role of CH_4 on soybean and pigeon pea

To evaluate the effect of CH_4 on legumes, experiment was carried out in completely randomized design with the following treatments: two leguminous crops, three levels of CH_4, and four replicates.

The legumes were soybean and pigeon pea, and the three levels of CH_4 were 0 ppm, 50 ppm and 200 ppm. Total 24 pots were used, where 12 were for soybean and 12 were for pigeon pea as shown in Fig 1. About 1 kg soil weighed into each plastic pot and seeds were sown in pots. There were ports for injecting CH_4 and collecting gas samples for analysis. To inject CH_4 into the rhizosphere, a set up referred as flow injection system was developed (Fig. 1). Pots were placed in a plant growth chamber (Genesis, Mumbai, India). CO_2 concentration was maintained by flushing CO_2 from an external cylinder. Temperature was maintained at 28±2 C. Humidity was maintained at ambient level. Fluorescent light intensity maintained at 25000 lux for 18 hours in a day. All parameters were maintained automatically using CO_2, temperature and humidity sensors. Each pot was fitted with silicon tubing to inject CH_4.

The CH_4 injection system was based on water displacement method. Water from bottle A was passed to bottle B by gravity at a rate of 1L d^{-1}. A micro-filter of 0.25 um was fitted to bottle A for keeping the bottles free of microorganisms and aerosols. To enrich the rhizosphere with CH_4, the gas (CH_4) of required volume was injected into the headspace of bottle B through a three-way valve. To maintain 50 ppm of CH_4 in the pots, 0.1 ml of CH_4 was injected into bottle B. Similarly, for 200 ppm of CH_4,

about 0.4 ml of CH_4 was injected into the bottle B. Pure CH_4 of 99.9 % was used in the study. Headspace gas of bottle B was passed to the rhizosphere as the water from bottle A entered to bottle B. Gas flow to pots from bottle B was regulated by a two-way valve.

The control pots received ambient air (without CH_4) from bottle B. After each day, bottle A was refilled with water from bottle B and headspace of bottle B was re-injected with the gas to continue the injection process. CH_4 concentration in the gas phase of soil in each pot was 0, 50 and 200 ppm. Experiment was conducted in 4 replicates. CH_4 enrichment was conducted till harvest of plants.

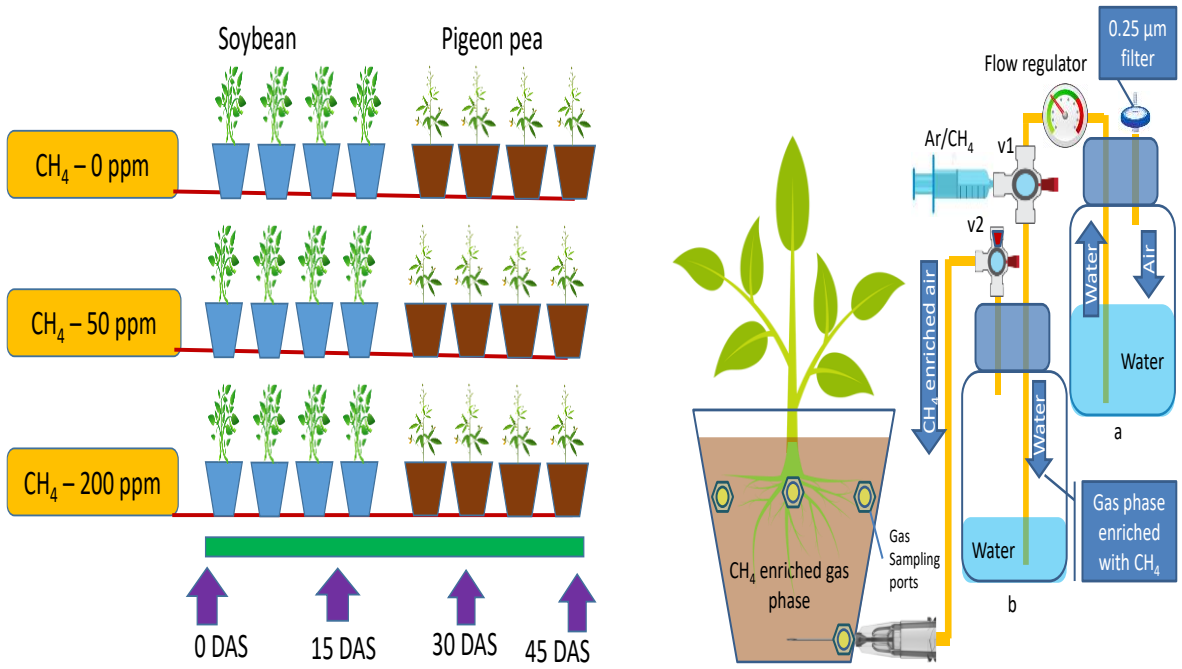


Fig 1. Left- Experimental layout to determine the role of CH4 in the rhizosphere on growth of soybean and pigeon pea. CH_4 was injected into the rhizosphere to maintain 0ppm, 50 ppm and 200 ppm. Soil sampling was carried out at 0 days after sowing (DAS), 15DAS, 30 DAS, 45 DAS. Experiment conducted in 4 replicates. Right - Set up to inject CH_4 into the rhizosphere of plants. Each pot was fitted with a constructed water displacing flow injection system as shown above to continuously inject CH_4 into the rhizosphere. Gas samples were collected from the sampling ports of the pots to determine CH_4 concentration in the soil.

3.4. Plant parameters measurement

Plants were harvested on 45 DAS for estimation of growth parameters. Soils were used to estimate CH_4 consumption potential and microbial abundance. To evaluate growth parameters, plants were carefully removed from pots. Roots were washed thoroughly in running tap water and air dried. Shoot length and shoot weight, root length, root weight, number of nodules, nodules weight, and acetylene reduction assay were estimated.

3.5. Soil sampling and estimation of CH_4 consumption

Soil samples were collected from each pot at different days after sowing (DAS) comprising 0, 15, 30, and 45 DAS for different analysis. CH_4 consumption potential of soil samples was estimated following methodology as described elsewhere (Mohanty *et al.,* 2015). Briefly, a portion of 20g soil placed into 130 ml sterilized serum bottle. The contents of the vials were mixed thoroughly, capped with rubber septa and sealed using aluminum crimp seal. Pure CH_4 was injected into the headspace of the vials for a final concentration of 1000 ppm. Vials were incubated at 28±2ºC in a biological oxygen demand (BOD) incubator (Metrex scientific instruments pvt ltd, N Delhi, India). At regular intervals (~1day), 0.1 ml of headspace gas was analyzed for CH_4. After each sampling, the headspace was replaced with an equivalent amount of high purity helium (He) to maintain atmospheric pressure.

The gas He was used because of its inert chemical nature. Vials were incubated till most of the headspace CH_4 got consumed. The rate (k) of CH_4 consumption was determined from the slope of the values of CH_4 versus incubation time during the rapid decline phase.

Soil sampling

for estimation of Methane consumption

Preparation of soil sample

3.6. Acetylene reduction assay (ARA)

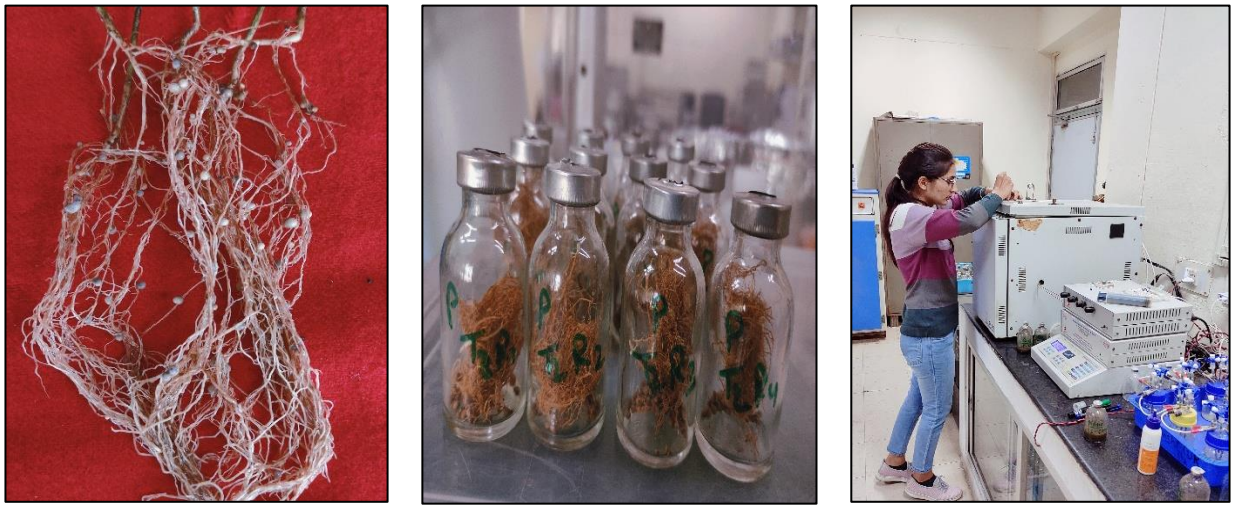
Acetylene reduction assay was evaluated using standard as described elsewhere (Itoh *et al.,* 2019). Root samples from each pot were washed with tap water for evaluating ARA activity. The root samples were placed into a 20 mL autoclaved serum vials. The vials were sealed with a butyl-rubber septum and aluminum crimp seal. Pure acetylene (C_2H_2) (Sigma gasses, N Delhi, India) injected into the headspace of vials representing 10% volume. The bottles were incubated at 28 °C for 1 day, and 0.1 mL of the headspace gas was injected into a gas chromatograph equipped with FID for ethylene measurement. Control samples without acetylene were set under the same conditions, and no detectable ethylene (C_2H_4) production was observed. The ARA was expressed as the production of ethylene (µmol) g^{-1} root dry weight day^{-1}.

3.7. Gas analysis

CH_4 and ethylene gas samples from the headspaces of serum bottles were analyzed using a gas chromatograph (CIC, India) equipped with an FID and a Porapak ssQ column (3-m lsength, diameter 2/8”, 80/100 mesh, stainless steel column) as described elsewhere (Mohanty *et al.,* 2017). The injector, column and detector were

maintained at 120^{o}C, 60^{o}C and 300^{o}C, respectively. Under these conditions, the retention time of CH_4 was 1.3 min. Ethylene was detected at 3.5 min.

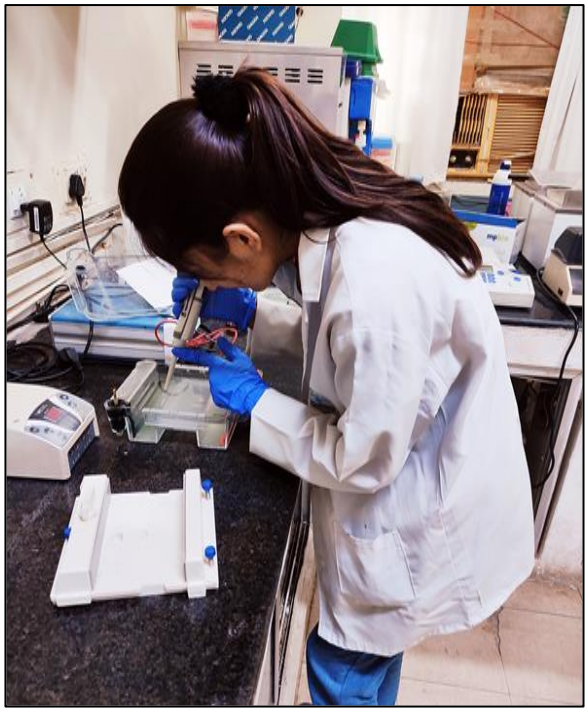
The GC was calibrated before and after each set of measurements using standard gasses. CH_4 in N_2 (Sigma Gases, New Delhi, India) was used as primary standard for (CH_4 100 ppm). Ethylene (C_2H_2) (100%) (Sigma Gases, New Delhi, India) was used as standard for ethylene.

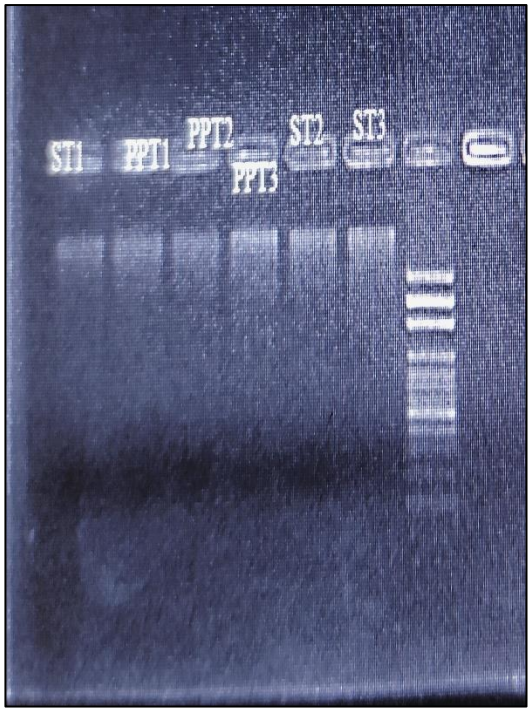


Sample preparation for ARA and Gas analysis

3.8. DNA extraction

After CH_4 consumption, about 0.5 g soil samples were taken out from bottles to extract DNA using the ultraclean DNA extraction kit (MoBio, USA) according to the manufacturer's instructions. The DNA concentrations were determined in a biophotometer (Eppendorf, Germany) by measuring absorbance at 260 nm (A260), assuming that 1 A260 unit corresponds to 50 ng of DNA per µl. DNA extraction was further confirmed by electrophoresis on a 1% agarose gel. The extracted DNA was dissolved in 50 µl TE buffer and stored at −20 °C until further analysis.





DNA extraction

3.9. Real time PCR quantification of nitrogen fixers (*nifH* genes) and methanotrophs (*pmoA* genes)

Real time PCR was performed on a Step one plus real time PCR (ABI, USA) to quantify the representative microbial species. Reaction mixture prepared with 2 µl of DNA template, 10 µl of 2X SYBR green 2master mix (Affymetrix, USA), 200 nM of primer (GCC Biotech, N Delhi). Final volume of PCR reaction mixture was made to 20 µl with PCR grade water (MP Bio, USA). Primers targeting *pmoA* gene (particulate methane monooxygenase) of methanotrophs were used to quantify their abundance. The primers for *pmoA* were A189F (5- GGN GAC TGG GAC TTCT GG-3) and mb661R (5- CCG GMG CAA CGT CYT TAC C-3)(Mohanty *et al.,* 2017). This primer set targets methanotrophs covering both type I and II including *Methylobacter or Methylosarcina, Methylococcus, Methylosinus* group, *Methylocapsa, Nitrosococcus.*

The primers for nitrogen fixing bacteria were *NifHI* (AGC ATG TCY TCS AGY TCN TCCA) and *nifHF* (TAC GGN AAR GGS GGN ATC GGC AA)(Cao *et al.,* 2021). Thermal cycling was carried out by an initial denaturizing step at 94 °C for 4 min, 40

cycles of 94 °C for 1 min, target specific annealing temperature for 30 sec, 72 °C for 45 sec; final extension carried out at 72 °C for 5 min. Annealing temperature for *nifH* was 52 ºC, and *pmoA* was 50 ºC. Fluorescence was measured during elongation step. Data analysis was carried out with Step one plus software (ABI, USA) as described in user's manual.

The cycle at which the fluorescence of target molecule number exceeded the background fluorescence (threshold cycle [C_T]) was determined from dilution series of target DNA with defined target molecule amounts. C_T was proportional to the logarithm of the target molecule number. The quality of PCR amplification products was determined by melting curve analysis with temperature increase of 0.3 °C per cycle. Standard for the genes was made from series of 10-fold dilutions of purified amplified products and data presented as number of cells per gram of soil.

Real time PCR quantification

3.10. Statistical Analyses

All statistical analyses were carried out using the Microsoft excel and “agricolae” package of the statistical software R (2.15.1) (Ihaka and Gentleman, 1996). Results for the experiments were presented as arithmetic means and standard deviation of replicated observations. Arithmetic mean and standard deviation were calculated by Microsoft Excel. Tukey’s honestly significant difference (HSD) test was performed to define the significant difference among treatments at α 0.05. Pearson’s product moment correlation estimated to define the relation between rhizospheric CH_4 concentration and plant parameters using command cor.test (factor, variable) in R.

CHAPTER- IV

RESULTS

4.1. Effect of CH_4 on plants

CH_4 in the rhizosphere exhibited negative effect on the growth of both soybean and pigeon pea. The plant parameters were shoot length, shoot dry weight, root length, root dry weight, nodule number, nodule dry weight, nodule volume and acetylene reduction assay. All parameters declined by CH_4 enrichment and followed the trend of 0 ppm >50 ppm and 200 ppm. Yield parameters of soybean are given in Fig. 2. Negative effect of CH_4 is related to its concentration, thus higher CH_4 concentration in the rhizosphere (200 ppm) exerted maximum negative effect on the plants. Soybean shoot length varied from 29 cm to 44 cm and root length 13 cm to 27 cm. Soybean shoot dry weight varied from 0.31 g to 0.49g while root dry weight from 0.09g to 0.19 g. Nodule number per plant varied from 5 to 11 /plant, nodule dry weight from 0.22g / plant to 0.42 g/plant, nodule volume was in the range of 0.21 mL to 0.61mL. Acetylene reduction assay in soybean was in the range of 1.8 to 4.43 µmol g^{-1} dry weight d^{-1}.

There was a decline of shoot length by 32%, dry weight 35%, root length 54%, root dry weight 55%, nodule number 54%, nodule dry weight 48%, nodule volume 65% and ARA by 59% than no CH_4 control. The impact of CH_4 on growth parameters of pigeon pea was similar to soybean (Fig 3). Shoot length varied from 14 cm to 29 cm, root length 15 cm to 28 cm, shoot dry weight 0.28 g to 0.47 g, root dry weight 0.13g to 0.25 g. Nodule number per plant varied from 18 to 32, nodule dry weight 0.01 g to 0.02g, nodule volume 0.17 ml to 0.36 mL, and acetylene reduction assay ranged from 0.05 to 056 µmol g^{-1} dry weight d^{-1}. Enrichment of CH_4 inhibited pigeon pea growth by declining shoot length by 54%, root length by 48%, shoot dry weight by 40%, root biomass dry weight by 49%, nodule number by 45%, nodule dry weight by 67%, nodule volume by 52%, and acetylene reduction potential by 90% than the no CH_4 control.

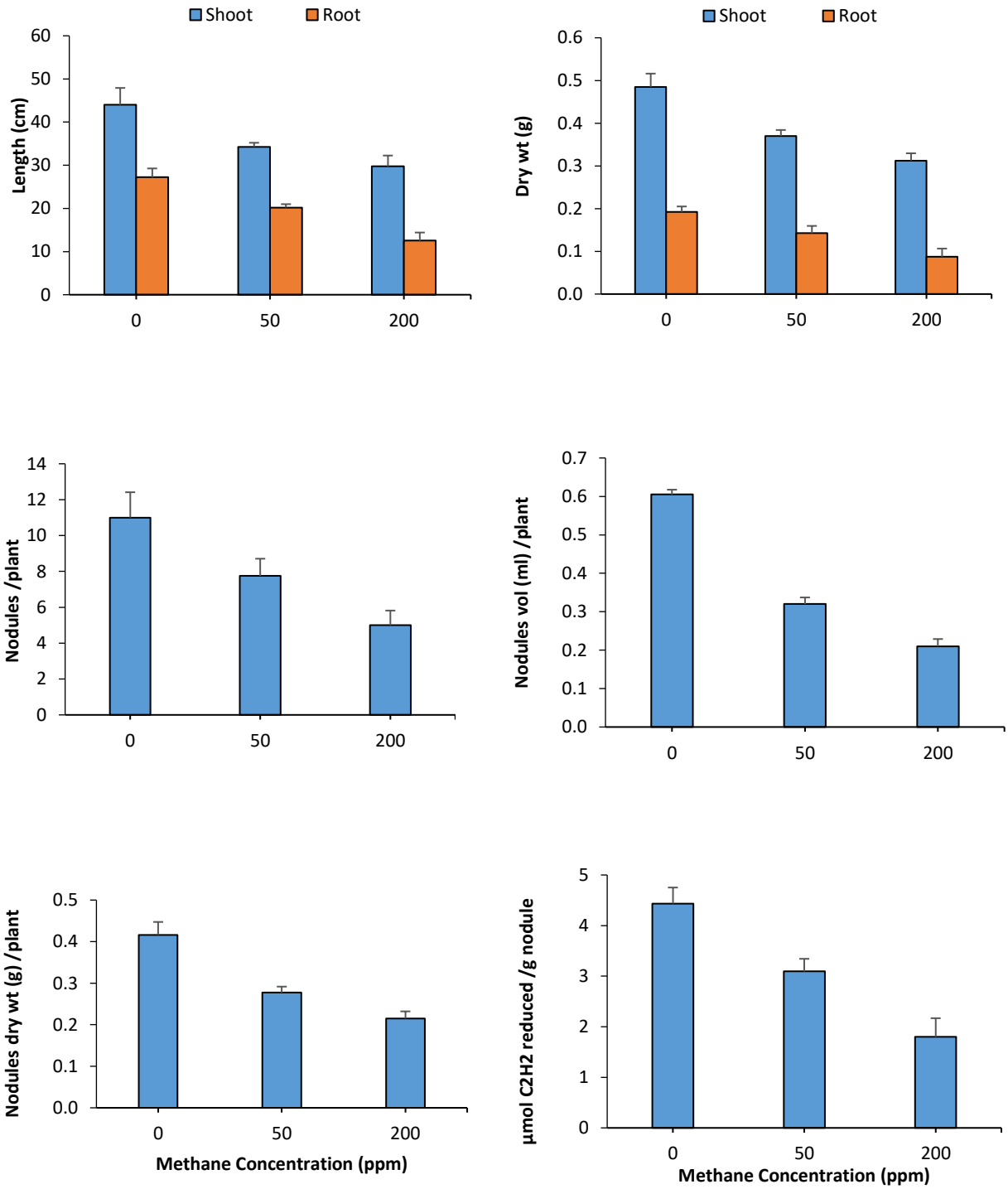


Fig 2. Effect of rhizospheric CH_4 concentration on growth of soybean. Plants were grown under controlled environment with rhizospheric soil enriched with different CH_4 concentrations (0 ppm, 50 ppm, 200 ppm). Plants were harvested and parameters were determined following standard agronomic measures. Each value is represented as arithmetic mean ± standard deviation as error bar of 4 replicated observations. X axis represented CH_4 concentration and Y axis represented different plant parameters. Top left – shoot and root length, top right – dry weight of shoot and root, middle left – nodule number, middle right- nodule volume, bottom left – nodule dry weight, and bottom right- acetylene reduction activity.

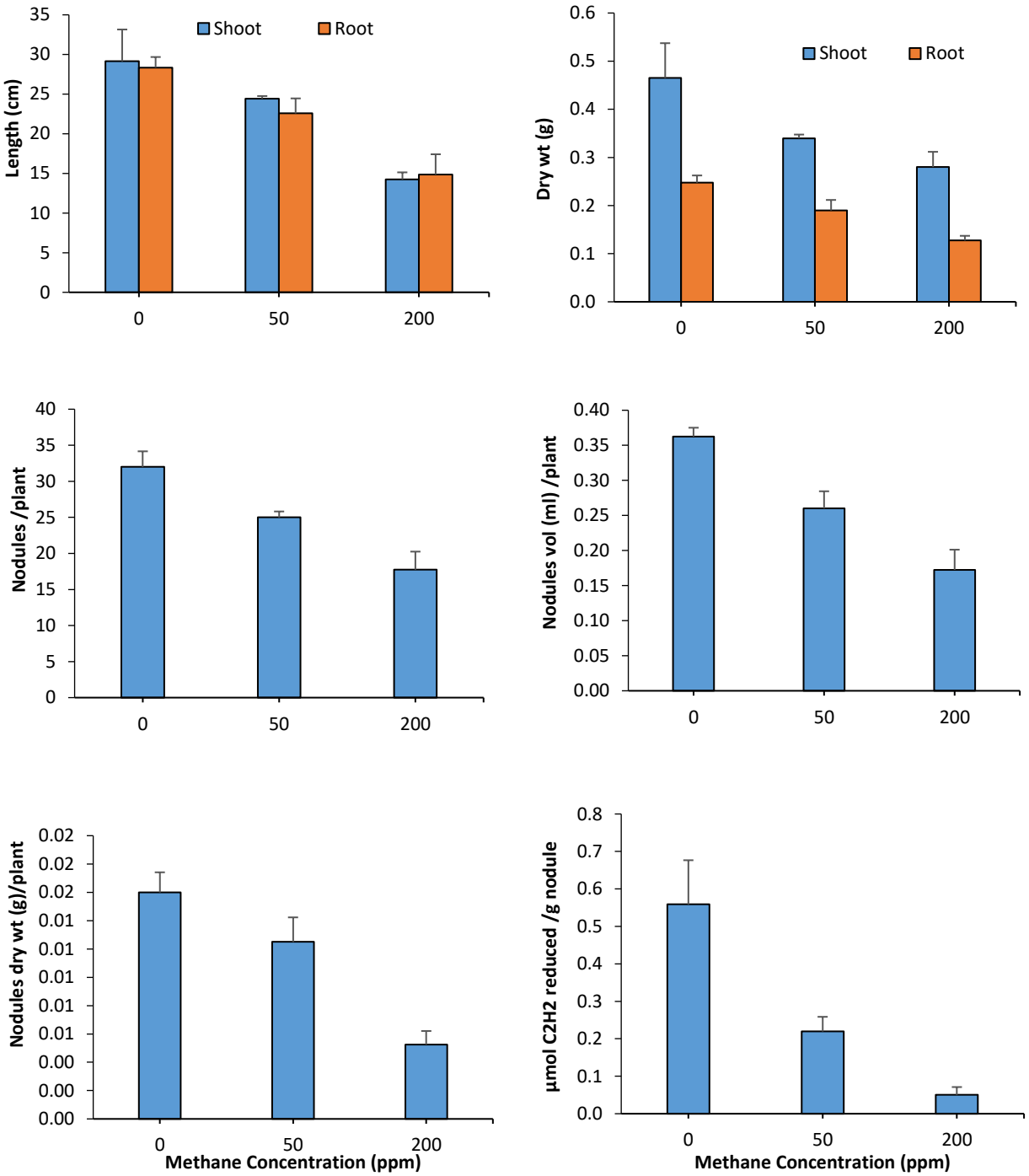


Fig 3. Effect of rhizosphere CH_4 concentration on growth of pigeon pea. Plants were grown under controlled environment with rhizospheric soil enriched with different CH_4 concentrations (0 ppm, 50 ppm, 200 ppm). Plants were harvested and parameters were determined following standard agronomic measures. Each value is represented as arithmetic mean ± standard deviation as error bar of 4 replicated observations. X axis represented CH_4 concentration and Y axis represented different plant parameters. Top left – shoot and root length, top right – dry weight of shoot and root, middle left – nodule number, middle right- nodule volume, bottom left – nodule dry weight, and bottom right- acetylene reduction activity.

4.2. CH_4 Consumption

Soil samples collected at different days after sowing were evaluated to determine how enrichment of rhizosphere with different concentration of CH_4 influence CH_4 consumption potential of soil. This was achieved by determining temporal variation of CH_4 consumption in soils. Rate of CH_4 consumption was evaluated to determine (1) CH_4 consumption potential during different growth phases of crops and (2) CH_4 consumption in the rhizosphere of legumes. Temporal variation of CH_4 consumption indicated that CH_4 consumption followed classical way of microbial metabolism comprising initial lag phase, rapidly declining log phase followed by stationary phase (Fig 4). This was typically observed in soil samples collected at 0 days of sowing. However, when the soils were exposed to CH_4, the trend of CH_4 consumption was faster. Consumption of headspace CH_4 was completed within 10-12 days of incubation. There was a lag phase of 4 days in soils of 0 DAS, while the lag phase declined in soil samples of 15, 30 and 45 DAS. Log phase was observed during 4-8 days in soils of 0 DAS and it declined to subsequently with the soils collected subsequently. Duration of complete CH_4 consumption declined with the exposure period. For example, CH_4 consumption was completed within 5 days of incubation in soils of 45 DAS and within 7-8 days in samples of 30 DAS. Temporal variation of CH_4 consumption indicated faster CH_4 consumption in soybean than pigeon pea. Higher level of CH_4 in the rhizosphere also exhibited faster CH_4 consumption. In order to determine the CH_4 consumption potential of different treatment, CH_4 consumption rates were determined. CH_4 consumption rate (k) was estimated as ng CH_4 consumed g^{-1} soil d^{-1} (Table 1). CH_4 consumption rate was higher in soybean than pigeon pea. Secondly, the CH_4 consumption rate increased with growth phase irrespective of treatments.CH_4 consumption rate in soils collected from both legumes at 0 DAS was 3.5 ng CH_4 consumed g^{-1} soil d^{-1}. CH_4 consumption rate in soybean was highest of 7.27 in samples representing 200 ppm CH_4 and 45 DAS. Similarly, highest CH_4 consumption rate of 6.10 observed in soybean representing 200 ppm CH_4 and 45

DAS. Concentration of CH_4 in the rhizosphere exhibited positive effect towards enhancing k values. For example, in soybean k increased by 15-17% at 50 ppm of CH_4 and about 40% at 200 ppm CH_4 than no CH_4 control. In case of pigeon pea, k values increased by 8-13% in 50 ppm CH_4 and 10- 26% over 0 ppm CH_4.

Table 1. CH_4 consumption rate (k) of rhizospheric soil of soybean and pigeon pea enriched with CH_4.

Crop	CH_4 (ppm)	CH_4 consumption rate k (ng CH_4 consumed g^{-1} soil d^{-1})			
		0 DAS	15 DAS	30 DAS	45 DAS
Soybean	0	3.50±0.10^{a}	4.01±0.31^{e}	4.63±0.19^{e}	5.20±0.25^{e}
	50	3.50±0.10^{a}	4.65±0.09^{b}	5.44±0.12^{b}	5.98±0.25^{c}
	200	3.50±0.10^{a}	5.25±0.07^{a}	6.51±0.25^{a}	7.27±0.30^{a}
Pigeon pea	0	3.50±0.10^{a}	3.90±0.14^{f}	4.32±0.17^{f}	4.81±0.26^{f}
	50	3.50±0.10^{a}	4.23±0.15^{d}	4.89±0.12^{d}	5.37±0.15^{d}
	200	3.50±0.10^{a}	4.30±0.16^{c}	5.15±0.13^{c}	6.10±0.16^{b}
Tukeys HSD (df error 22, P 0.05)		0.07	0.05	0.11	0.12

Plants were grown in soil enriched with 0, 50 and 200 ppm of CH_4. Soil samples were collected at 0, 15, 30 and 45 days after sowing (DAS) to determine CH_4 consumption rate k. Each value represents arithmetic mean ± standard deviation of 4 replicated observations. At $p<0.05$, values preceded by the same letters are not substantially different.

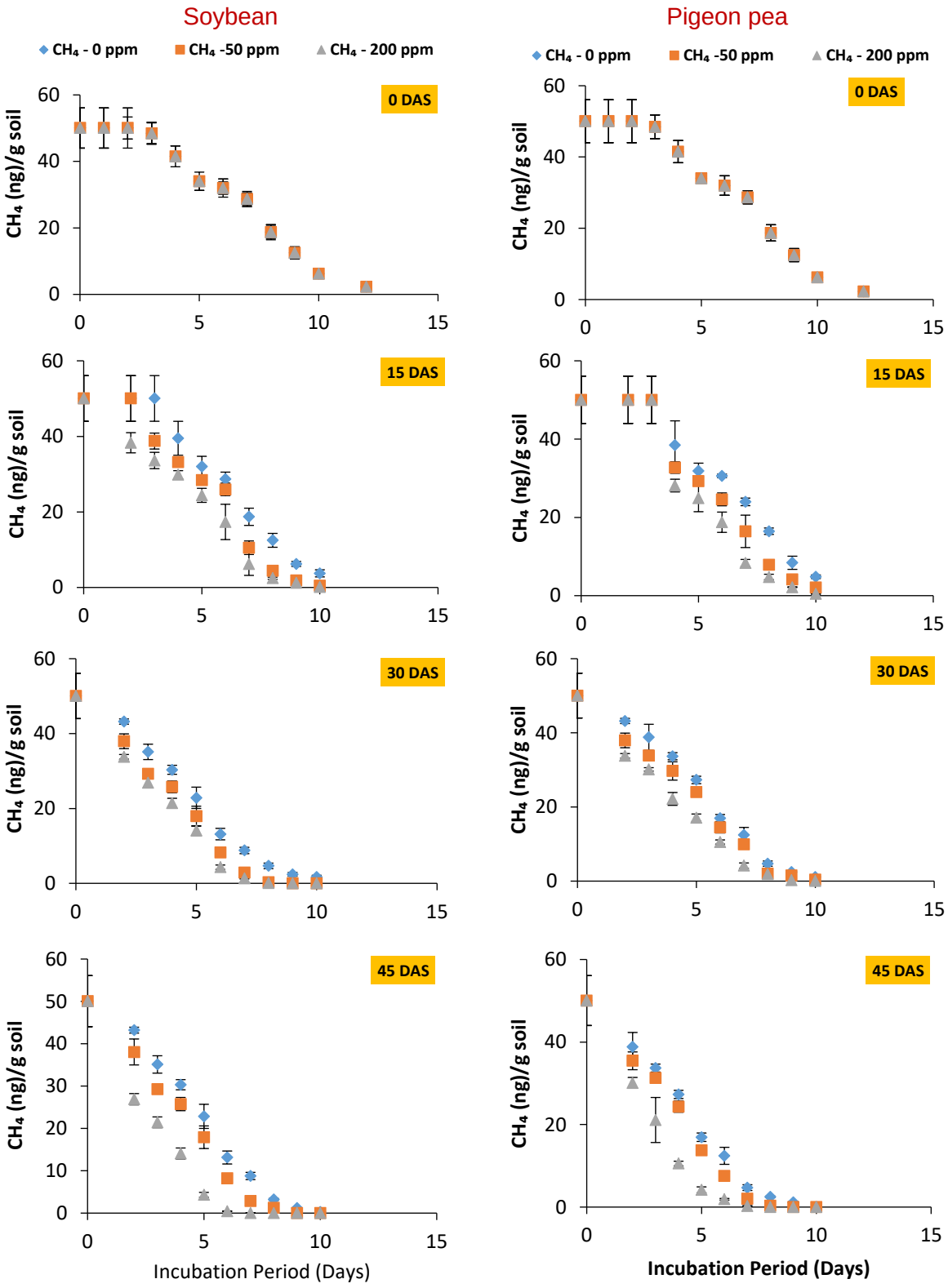


Fig. 4. CH_4 consumption in the rhizospheric soils of soybean (left panel) and pigeon pea (right panel). Plants were grown under controlled environment where rhizosphere was enriched with different CH_4 concentration (0 ppm, 50 pm, and 200 ppm). Soil samples were collected at 0, 15, 30 and 45 days after sowing (DAS). Soil samples were placed in to vials and pure CH_4 was injected into the headspace. Change in CH_4 concentration in the vials monitored at different incubation period. X axis represents incubation period in days, Y axis represent CH_4 concentration (ng g^{-1} soil). Each value represents arithmetic mean with standard deviation as error bar of 4 replicated observations.

4.3. Microbial abundance

Microbial abundance in the soils at 0 DAS and 30 DAS determined to exhibit how the microbial groups representing methanotrophs and nitrogen fixers vary in the rhizosphere of legumes under CH_4 enrichment (Table 2). Methanotrophs were expressed as 10^5 *pmoA* gene copies g^{-1} soil and nitrogen fixers as 10^5 *nifH* gene copies g^{-1} soil. Both microbial groups were lowest at the 0 DAS. Abundance of *pmoA* gene copies increased with CH_4 enrichment while the abundance of *nifH* gene copies decreased with CH_4 enrichment. In general, soybean had higher *pmoA* gene abundance than pigeon pea. Secondly, increasing CH_4 concentration stimulated *pmoA* gene abundance and inhibited *nifH* gene abundance. At 0 DAS, there was 11 ± 1.258 x 10^5 *pmoA* gene copies g^{-1} soil and increased to maximum (80 ± 5.944 x 10^5 *pmoA* gene copies g^{-1} soil) in the treatment soybean-30 DAS-200 ppm of CH_4. In case of pigeon pea, *pmoA* gene abundance peaked to 57±4.655 x 10^5 g^{-1} soil in the treatment of 30 DAS-200 ppm CH_4. Abundance of *nif H* gene in 0 DAS was 48±2.449 x 10^5 g^{-1} soil. Highest values were in control and lowest in 200 ppm CH_4. Abundance of *nifH* varied from 15±2.449 x 10^5 g^{-1} soil at 200 ppm CH_4 to 86±4.546x 10^5 g^{-1} soil at 0 ppm CH_4 in soybean. Similarly, the abundance of *nifH* varied from 26±3.873 at 200 ppm CH_4 to 67±3.594 at 50 ppm CH_4 in pigeon pea.

Table 2. Abundance of methanotrophs and nitrogen fixers in the rhizospheric soil of soybean and pigeon pea enriched with CH_4.

Crop	CH_4 (ppm)	*pmoA* (x 10^5 gene copies g^{-1} soil)		*nifH* (x 10^5 gene copies g^{-1} soil)	
		0 DAS	30 DAS	0 DAS	30 DAS
Soybean	0	11±1.258[a]	25±2.500[e]	48±2.449[a]	86±4.546[a]
	50	11±1.258[a]	40 ±3.916[c]	48±2.449[a]	31±1.732[d]
	200	11±1.258[a]	80±5.944[a]	48±2.449[a]	15±2.449[f]
Pigeon pea	0	11±1.258[a]	19±1.002[f]	48±2.449[a]	67±3.594[b]
	50	11±1.258[a]	33±3.317[d]	48±2.449[a]	46±4.796[c]
	200	11±1.258[a]	57±4.655[b]	48±2.449[a]	26±3.873[e]
Tukeys HSD *(df error 22, P 0.05)*		1.085	4.051	1.286	6.192

Plants were grown in soil enriched with 0 ppm, 50 ppm and 200 ppm of CH_4. Soil samples were collected at 0 and 30 days after sowing (DAS) to determine microbial abundance. Methanotrophs and nitrogen fixers were determined as *pmoA* and *nifH* gene copies g^{-1} soil. Each value represents arithmetic mean ± standard deviation of 4 replicated observations. At $p<0.05$, values preceded by the same letters are not substantially different.

4.4. Statistical interpretation

The effect of CH_4 concentration on various plants attributes was examined through Pearson's product moment correlation. CH_4 concentration exhibited negative effect on all plant growth attributes including shoot and root biomass ($P < 0.001$), nodule number ($P < 0.0001$), nodule dry weight ($P < 0.001$), nodule volume (P 0.01), ARA ($P<0.001$) and abundance of *nifH* ($P < 0.001$) (Table 3). However, CH_4 concentration had positive effect on the abundance of pmoA gene copies ($P < 0.0001$). CH_4 had similar effect for both soybean and pigeon pea.

Table 3. Pearson's product-moment correlation between the factor (rhizospheric CH_4 concentration) and plant parameters to exhibit the relative effect of rhizospheric CH_4 concentration on plant growth.

Crop	Correlation	Shoot		Root		Nodule			ARA	*pmoA*	*nifH*
		Length	Dry wt	Length	Dry wt	Number	Dry wt	Volume			
Soybean	*R*	-0.8193	-0.861	-0.9409	-0.9188	-0.8828	-0.8661	-0.8139	-0.9284	0.9867	-0.8245
	P	0.0011	0.0003	<0.0001	<0.0001	0.0001	0.0002	0.0012	<0.0001	<0.0001	0.0009
Pigeon pea	*R*	-0.9468	-0.7876	-0.936	-0.9299	-0.9247	-0.9617	-0.9175	-0.8539	0.975	-0.9335
	P	<0.0001	0.0023	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0004	<0.0001	<0.0001

The parameters were shoot length, shoot dry weight (wt), root length, root dry wt, nodule numbers, nodule dry weight, nodule volume, acetylene reduction activity (ARA), and abundance of *pmoA* and *nifH* genes. The crops (soybean and pigeon pea) were grown in soil enriched with different CH_4 concentration (0 ppm, 50 ppm, 200 ppm). Correlation is presented in terms of r with p values as level of significance.

CHAPTER- V

DISCUSSION

CH_4 in soil gas phase exhibited inhibitory effect on the growth of both soybean and pigeon pea. The negative effect of CH_4 was in proportion to its concentration, which means that the effect of CH_4 at 200 ppm was more than 50 ppm. The extent of inhibition was higher in soybean than pigeon pea. Shoot length, shoot biomass, root length and root biomass decreased due to CH_4 and the extent of inhibition was more at higher CH_4 concentration. The extent of negative effect was higher in root (54% inhibition) than the above ground biomass (35% inhibition) in case of soybean. Similarly, in case of pigeon pea, there was about 47% decline in shoot biomass and 51% decline in root biomass due to CH_4 in rhizosphere. The overall negative effect of CH_4 on plant growth was related to the deleterious effect on root biomass growth. Nodule numbers also declined (54% in soybean and 48% in pigeon pea) significantly due to CH_4 enrichment. This could be due to the interference of CH_4 in plan-microbial interaction affecting the rhizobial association with the legumes. Acetylene reduction assay declined significantly at 200 ppm of CH_4 which indicated that CH_4 potentially affected rhizobium activity and its association with plant.

CH_4 consumption rate increased in soil during CH_4 exposure, which denoted that rise in CH_4 concentration and exposure time, resulted higher CH_4 uptake potential. This was due to the enrichment of methanotrophs, where CH_4 was used as substrate. CH_4 consumption was completed within 12 days in soil samples collected at 0 DAS, whereas, the headspace CH_4 was consumed faster in soil samples collected at 15, 30, and 45 DAS. CH_4 consumption rate varied from 3.5 ng CH_4 consumed g^{-1} soil d^{-1} to 7.0 ng CH_4 consumed g^{-1} soil d^{-1}. Similar values has been observed in other studies (Mohanty *et al.,* 2015, 2017).

Microbial abundance highlights that the abundance of methanotrophs and nitrogen fixing organisms varied inversely, as the former group peaked with CH_4 and the later declined with CH_4. Methanotrophs use CH_4 as carbon and energy source

which stimulated their abundance (Conrad, 1995). CH_4 stimulated methanotrophs *pmoA* gene abundance by a factor of 1.6 (50 ppm CH_4) to 3.2 (200 ppm CH_4) in soybean and 1.75 (50 ppm CH_4) to 3.05 (200 ppm CH_4) times in pigeon pea. Abundance of nitrogen fixing organisms (*nifH* gene copies) declined by CH_4 enrichment by 2.81-fold at 50 ppm CH_4 and 5.73 fold at 200 ppm CH_4. The inverse relation between the *pmoA* gene and *nifH* gene could be due to nutrient limitation or selective inhibition of N fixers. High CH_4 favored methanotrophs and these microbial groups are competitive in nutrient acquisition compared to N fixers. Methanotrophs exhibited higher CH_4 consumption rate and their abundance increased significantly in relation to CH_4 concentration. Other mechanism that regulated *pmoA* and *nifH* variation was through selective inhibition of N fixers by CH_4. Abundance of *nifH* declined drastically under CH_4 atmosphere. Many methanotrophs are capable of fixing N2 and dominance of this organism possibly created an air phase with limited N2 restricting metabolism of N fixers (Khadem *et al.,* 2010).

Further research required to unravel how CH_4 influenced plant growth, rhizobia metabolism and plant-rhizobial interaction. This will provide crucial information on the role of CH_4 in soil gas phase to regulate plant-microbial interaction.

CHAPTER- VI

SUMMARY, CONCLUSION AND SUGGESTION FOR FURTHER WORK

The global emissions and terrestrial sinks of methane (CH_4), an important anthropogenic greenhouse gas, are regulated by microorganisms in the environment. With increasing anthropogenic activities and industrialization resulting in climate alterations, there is an alarming increasing in the CH_4 level. This can have direct effects on the biotic factors like micro- and macro-fauna, plants, etc. It has been well established that plant-associated beneficial microorganism boosts plant growth and triggers resistance to biotic and abiotic stresses. Increased CH_4 concentrations in the environment will also have significant impact on the dynamics of plant-microbe interactions as well as crop growth and quality.

The present investigation was aimed to establish the linkage between rhizospheric CH_4 and growth of legumes to get the crucial information on the effect of CH_4 in the rhizosphere of legumes (soybean and pigeon pea). The research program included setup of microcosm for evaluating the effect of different levels of CH_4 on soybean and pigeon pea. After incubation period, soil was analysed for CH_4 consumption potential and microbial abundance, and plant parameters like shoot length, shoot weight, root length, root weight, number of nodules, nodules weight, and acetylene reduction assay were estimated.

SUMMARY

- The rhizosphere was enriched with two concentrations of CH_4 (50 and 200 ppm), and 0 ppm as control. The amount of CH_4 consumption (ng CH_4 consumed g^{-1} soil d^{-1}) of soil ranged from 3.50 at 0 DAS and highest (7.27) in soybean at 45 DAS.
- Irrespective of treatments, the CH_4 consumption rate was higher in soybean than pigeon pea. Inhibition of plant growth, in terms of biomass, was observed as expressed with increasing CH_4 concentration.

- At 200 ppm shoot biomass, root biomass, and nodule number decreased significantly by 35%, 54% and 65% respectively.
- The nitrogen fixing potential, as measured by Acetylene reduction activity (ARA), was also reduced by 59% and 90% in soybean and pigeon pea, respectively, at 200 ppm CH_4.
- Microbial abundance in the soils was determined to exhibit how the microbial groups representing methanotrophs and nitrogen fixers vary in the rhizosphere of legumes under CH_4 enrichment. Abundance of *pmoA* gene copies increased by 2.37 folds while *nifH* gene copies decreased by 0.53 folds by 200 ppm CH_4, compared to control.
- Pearson's product moment correlation indicated that CH_4 exerted significant negative effect (P 0.05) on plant growth attributes. Methanotrophs exhibited higher CH_4 consumption rate and their abundance increased significantly in relation to CH_4 concentration.

Study highlight that CH_4 in soil gas phase can affect growth and productivity of soybean and pigeon pea, which can be managed by enhancing methanotrophic activities.

CONCLUSION AND SUGGESTION FOR FURTHER WORK

Methane (CH_4) is mainly produced by methanogens in the anaerobic compartments of soil. The generated CH_4 is emitted to atmosphere directly by diffusion or through plants. However, majority of the CH_4 is retained in soil creating an enriched CH_4 environment in soil ecosystem. It has been found that CH_4 concentration in soil may reach up to 20000 ppm. Interestingly, major fraction (~80%) of the biogenic CH_4 gets oxidized in soil itself by methanotrophs. However, soil's gas phase often contains high CH_4 concentration. Keeping this as background, this study was undertaken to explore how CH_4 in soil regulates plant growth, and plant-microbial interaction. Two legumes including soybean and pigeon were taken as test crop under 3 levels of CH_4 (0 ppm, 50ppm, and 200 ppm). CH_4 was continuously injected into the rhizosphere of plants which were grown under controlled environment.

Plant growth parameters comprising shoot biomass, root biomass, nodule number, nodule biomass, acetylene reduction assay was evaluated to determine how CH_4 shaped the plant growth. Abundance of methanotrophs and rhizobia were estimated in terms of gene copies of pmoA and nifH. Results highlight that CH_4 in the soil gas phase inhibited plant growth and secondly, the inhibitory effect was through the decline of rhizobial abundance. Study provided insightful information to manage soil for having low CH_4 concentration in gas phase to minimize negative effect of CH_4 on crop. However, further research is essential to understand the mechanistic process regulating CH_4 driven plant-microbial interaction in soil ecosystem.

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ABSTRACT

Methane (CH_4) is a potent greenhouse gas and its concentration in atmosphere is rising alarmingly. CH_4 is mainly produced by soil microorganisms and constitutes a major fraction in the gas phase of soil. However, it is not clearly understood how CH_4 in the soil influences plant's growth. An experiment was conducted under controlled environment to evaluate the effect of CH_4on soybean and pigeon pea. The treatments included enrichment of rhizosphere of plants with CH_4at 0 ppm (control), 50 ppm and 200 ppm. CH_4 consumption potential (ng CH_4 consumed g^{-1} soil d^{-1}) of soil increased from 3.50 at 0 DAS and highest (7.27) in soybean at 45 DAS. CH_4 consumption rate was higher in soybean than pigeon pea irrespective of treatments. Growth of plants in terms of biomass was inhibited by CH_4. The trend of negative effect of CH_4 on plant increased with CH_4 concentration. Shoot biomass, root biomass, and nodule number decreased by 35%, 54% and 65% respectively at 200 ppm of CH_4. Acetylene reduction activity (ARA) was inhibited by 59% in soybean and 90% in pigeon pea at 200 ppm CH_4. Abundance of *pmoA* gene copies increased by 2.37 folds while *nifH* gene copies decreased by 0.53 folds by 200 ppm CH_4 than control. Pearson's product moment correlation indicated that CH_4 exerted significant negative effect (P 0.05) on plant growth attributes. Study highlight that CH_4 in soil gas phase can affect growth and productivity of soybean and pigeon pea, which can be managed by enhancing methanotrophic activities.

Appendix

ANOVA:										
Soybean	**Shoot**		**Root**							
SUMMARY	**Length (cm)**	**Dry wt (g)**	**Length (cm)**	**Dry wt (g)**	**Nodule no/plant**	**Nodules dry wt/plant**	**Nodule vol (ml)**	**ARA (umol C_2H_2 reduced/g nodule)**	**Length (cm)**	**Total**
CH4 0 ppm										
Count	4	4	4	4	4	4	4	4	4.00	36.00
Sum	176	1.94	109	0.77	44	1.665	2.42	17.7398	116.60	470.13
Average	44	0.485	27.25	0.1925	11	0.41625	0.605	4.43495	29.15	13.0593
Variance	15.33333	0.000967	4.036667	0.000158	2	0.000206	0.005967	0.100646	16.03667	247.199
CH4-50 ppm										
Count	4	4	4	4	4	4	4	4	4	36
Sum	137	1.48	80.8	0.57	31	1.11	1.28	12.3773	97.7	363.3173
Average	34.25	0.37	20.2	0.1425	7.75	0.2775	0.32	3.094325	24.425	10.09215
Variance	0.916667	0.0002	0.613333	0.000292	0.916667	0.000625	0.005933	0.061916	0.109167	152.3386
CH4 200 ppm										
Count	4	4	4	4	4	4	4	4	4	36
Sum	119	1.25	50.1	0.35	20	0.86	0.84	7.2007	57	256.6007
Average	29.75	0.3125	12.525	0.0875	5	0.215	0.21	1.800175	14.25	7.127797
Variance	6.25	0.000292	3.555833	0.000358	0.666667	3.33E-05	0.0016	0.135727	0.776667	94.25461
Total										
Count	12	12	12	12	12	12	12	12	12	
Sum	432	4.67	239.9	1.69	95	3.635	4.54	37.3178	271.3	
Average	36	0.389167	19.99167	0.140833	7.916667	0.302917	0.378333	3.109817	22.60833	
Variance	44.72727	0.006008	41.68447	0.002227	7.537879	0.007952	0.033906	1.343671	46.78083	
ANOVA										
Source of Variation	*SS*	*Df*	*MS*	*F*	*P-value*	*F crit*				
Sample	633.2891	2	316.6446	165.9222	2.26E-29	3.109311				
Columns	16352.66	8	2044.082	1071.102	8.7E-79	2.054882				
Interaction	775.4975	16	48.4686	25.39761	5.38E-25	1.769953				
Within	154.5798	81	1.908392							
Total	17916.03	107								

Pigeon pea	Shoot		Root						
SUMMARY	**Length (cm)**	**Dry wt (g)**	**Length (cm)**	**Dry wt (g)**	**Nodules no**	**Nodules dry wt**	**Nodule vol (ml)**	**ARA (umol C2H2 reduced/g nodule)**	**Total**
CH_4 0 ppm									
Count	4	4	4	4	4	4	4	4	32
Sum	116.6	1.86	113.4	0.99	128	0.064	1.45	2.236	364.6
Average	29.15	0.465	28.35	0.2475	32	0.016	0.3625	0.559	11.39375
Variance	16.03667	0.005233	1.77	0.000225	4.666667	0.000002	0.000158	0.013832	213.7414
CH_4-50 ppm									
Count	4	4	4	4	4	4	4	4	32
Sum	97.7	1.359	90.3	0.76	100	0.05	1.04	0.8788	292.0878
Average	24.425	0.33975	22.575	0.19	25	0.0125	0.26	0.2197	9.127744
Variance	0.109167	6.02E-05	3.549167	0.000467	0.666667	0.000003	0.0006	0.001514	137.8321
CH_4 200 ppm									
Count	4	4	4	4	4	4	4	4	32
Sum	57	1.12	59.4	0.51	71	0.021	0.69	0.2015	189.9425
Average	14.25	0.28	14.85	0.1275	17.75	0.00525	0.1725	0.050375	5.935703
Variance	0.776667	0.001	6.563333	9.17E-05	6.25	9.17E-07	0.000825	0.000433	60.27202
Total									
Count	12	12	12	12	12	12	12	12	
Sum	271.3	4.339	263.1	2.26	299	0.135	3.18	3.3163	
Average	22.60833	0.361583	21.925	0.188333	24.91667	0.01125	0.265	0.276358	
Variance	46.78083	0.008199	36.6075	0.002833	40.08333	2.35E-05	0.007009	0.053091	
ANOVA									
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*			
Sample	481.218	2	240.609	142.8908	8.58E-26	3.123907			
Columns	11889.46	7	1698.494	1008.687	3.6E-69	2.139656			
Interaction	756.5148	14	54.03677	32.0909	2.47E-25	1.831607			
Within	121.2383	72	1.683866						
Total	13248.43	95							

VITA

The author of this thesis Ms. Seema Choudhary D/O Shri Shivram Choudhary was born on 11 November 1996 at VPO- Doongri kalan, Tehsil- Kishangarh Renwal, Dist- Jaipur (Raj.), I completed my education from S. J. P. S. Secondary School (10 & 12th) Doongri kalan (Jaipur) with 78.17 & 74.00 per cent marks respectively.

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I further joined Department of Soil Science & Agricultural Chemistry, College of Agriculture, RVSKVV, Gwalior, (M.P.) for the post-graduation degree programme. I am submitting my Thesis for M.Sc. (Ag) Soil Science degree in partial fulfillment of the requirements for the degree of **Master of Science**, was allotted the research problem entitled **"Plant Microbial Interaction Under the Influence of Methane Consumption in the Rhizosphere of Legumes".** which is duly completed by me and is presented in the form of Thesis and successfully passed my all academic subjects of post graduate degree (Ag.) Soil Science in 2020 with 76.30 percent marks.

Place: Gwalior

Date: / / **(Seema Choudhary)**