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Daphy Meurial C
Ph. D. Scholar, Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India

K Kumar
Professor, Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India

Correspondence
Daphy Meurial C
Ph. D. Scholar, Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India

Identification of culturable anaerobic bacteria associated with paddy field soil and its influence on RSA modulations under *in vitro* condition

Daphy Meurial C and K Kumar

Abstract
Culturable anaerobic bacterial populations on paddy field soil were found on the order of 10^2 CFU^{-1} g of $soil^{-1}$, and the percentages of spores were usually less than 1% of the total anaerobes. Anaerobic bacteria were isolated from the soil samples by picking up colonies on the roll tube agar. The nitrogen fixing obligate and facultative anaerobes were isolated and characterised from paddy ecosystem. The phylogenetic analysis of 30 isolates based on 16S rRNA gene sequences revealed that the composition of dominant culturable anaerobic bacteria on rice soil was rather simple. Molecular analysis based on 16S rRNA gene sequencing revealed that the anaerobic N_2 fixing bacterial isolates NFTh2 showed maximum similarity with *Clostridium pasteurianum*. Higher nitrogenase activity was noticed for the screened obligate and facultative anaerobic bacteria and exhibited maximum activity on 132th day (19.81 n moles of C_2H_4 mg^{-1} protein h^{-1}. In the present investigation, the occurrences of anaerobic nitrogen fixer in the paddy ecosystem and their role in the RSA modulations as influenced by anaerobic nitrogen fixer under gnotobiotic condition have been examined.

Keywords: nitrogen fixing bacteria, diazotrophs, nitrogenase, *In vitro,* RSA

Introduction

Rice (*Oryza sativa* L.) is one of the world's oldest and most important crop species, having been domesticated about 8,000-9,000 years ago (Zeigler and Barclay, 2008) [29]. Different rice cultivars are adapted to a wide range of environments such as tropical and temperate climates, lowland and highland regions and a wide range of soil types. About 50% of rice is grown under intensively irrigated systems, which accounts for 75% of the global rice production. India has the largest absolute farm area under rice production, though it has the lowest productivity per hectare of the top three producers (Penili *et al*., 2009) [19]. The increase in productivity could be achieved by intensification of paddy cultivation rather than increasing the area under cultivation. About 80% of rice crop is grown in flooded condition. This flooded condition changes the chemistry, microbiological properties and nutrient supplying capacity of soil which leads to the differentiation of micro and macro environments differing by their redox potential, physical properties, light status and nutrient sources for the soil microorganisms (Kabir and Uphoff, 2007) [15].

Nitrogen is an essential plant nutrient, determinant for plant growth and crop yield (Hooper and Johnson, 1999) [9], as it is an essential component of proteins and nucleotides that is most commonly deficient in soils, contributing to reduced agricultural yields throughout the world. Because of the adverse impact of excess nitrogen on the global system, Socolow, (1999) [23] argues that management of food and nitrogen connection should be given as much importance as carbon and energy connection undertaken at the global level due to climate change. In rice cultivation the yield-limiting factor is the availability of nitrogen due to large losses in flooded soils through denitrification or leaching (Cassman *et al*., 1998) [3]. Generally, nitrogen fertilizer efficiency in flooded rice fields is poor. Flooded rice crops typically use only 20-40%, whereas upland crops frequently use 40-60% of the applied nitrogen. Since atmospheric nitrogen is a renewable energy source, Biological Nitrogen Fixation (BNF) is a sustainable source of N in cropping system. Hence BNF is becoming more important for not only reducing energy cost but also in seeking more sustainable agricultural production. The energy derived for BNF is virtually free and is derived from photosynthesis. For these reasons, BNF is the most environmentally friendly approach to supplying N to agro-ecosystem.

Diverse N_2 fixing microorganisms (aerobes, facultative anaerobes, heterotrophs, phototrophs) grow in wetland rice fields and contribute to soil N pools. The major BNF systems known, include cyanobacteria and photosynthetic bacteria that inhabit flood waters and the soil surface and heterotrophic bacteria in the root zone (rhizosphere), or in the bulk soil (Ladha *et al*.,

1997) [16]. *Rhizobium, Azospirillum, Azotobacter, Gluconacetobacter, Azolla* and cyanobacteria are the popular diazotrophic microorganisms used as biofertilizers as source of BNF for various agro-ecosystems. These biofertilizer organisms are applied either through seed or soil or both, for endophytic or epiphytic colonization in the crop roots or as free-living in the rhizosphere region for active BNF. The present biofertilizer technology can contribute about 15 to 25 kg of nitrogen through BNF to various crops, with an yield increase of about 10 to 25 per cent. Apart from the fertilizer saving and yield increase, the biofertilizer application enhances the CO_2 fixation rate per mole of nitrogen assimilated; increases soil N uptake; reduces the CO_2 and N_2O emission; reduces the ammonia volatilization and N leaching. Increased soil fertility and soil N supply power are the long term effect of BNF in soil. It is well established that nitrogen fixation by bacterial genera including *Azotobacter, Clostridium, Azospirillum, Herbaspirillum* and *Burkholderia* can substitute for urea- N, whereas *Rhizobium* can promote the growth physiology or improve the root morphology of the rice plant (Choudhury and Kennedy, 2004; Jha *et al*., 2009) [4, 14].

Biological N_2 fixation is gaining importance in rice ecosystem because of current concern on the environmental and soil health that are caused by the continuous use of nitrogenous fertilizers and the need for improved sustainable rice productivity. Thus, biological fixation of atmospheric N, especially non- symbiotic N_2-fixation in the soil, has been subject of continuing interest in recent decades especially for low input agriculture. Therefore, the objectives of this paper are to assess the contribution of anaerobic nitrogen fixation in flooded rice soil especially *Clostridia.*

Materials and Methods

Soil sample collection

Soil samples for the anaerobic bacterial isolation were collected from rhizosphere of flooded rice field during active tillering stage. Random samples were collected from the field at the lower horizon (10 cm depth) under anaerobic conditions (Ramasamy *et al*., 1992) [20].

Anaerobic bacteria isolation by roll tube technique

Isolation of anaerobic bacteria was carried out with the anaerobic roll tube method using oxygen-free N_2 gas as headspace (Hungate, 1966) [12]. Soil sample (1g) was diluted by tenfold using sterile dilution buffer (100mL) containing sodium carbonate and sodium bi carbonate. After a thorough mixing, diluted sample (1mL) was transferred to another sterile dilution buffer (90mL), which conformed to 10^2 dilutions.

Each diluted sample was inoculated in triplicate into Hungates medium. The medium was flushed continuously with N_2 using the gassing manifold assembly, simultaneously the sterile test tubes were kept under N_2 atmosphere. The samples were also maintained under N_2 atmosphere. 1ml of the trace element solution and vitamin solution were added to one litre of the media before transferring to the sample. 1ml of the sample was transferred from the desired dilution to the sterile test tube of 25 ml capacity which again was kept under N_2 atmosphere. By using a sterile pipette with silicon tube at one end, 5 ml of the medium was transferred to the test tube with sample which was maintained under N_2 atmosphere. The test tube was immediately stoppered with sterile rubber corks by simultaneously drawing out the gassing jet. The test tube was rolled over the foam, soaked in cold water till the medium uniformly solidified on the sides of the test tube. The test tubes were incubated in an anaerobic jar (Hungate, 1957) [11]. The number of colonies that appeared on the agar medium during 12 days of incubation at 30 ^oC was counted to determine the number of culturable anaerobic microbes in the samples. The isolates were purified by repeating the colony isolation by the anaerobic roll tube method, and the strains finally purified were used in this study. The purity of the isolates was confirmed by uniform colony morphology on the roll tube agar and cellular morphology with Gram staining and motility of the cells, as well as observations by phase-contrast microscopy.

Phenotypic characterization of isolates

Catalase activity of cells was tested by the O_2 generation in 3% H_2O_2 where bubble formation shows positive for catalase activity and no bubble formation shows negative for catalase activity. Utilization of different carbohydrates was tested with different carbon sources *viz.,* glucose, fructose, sucrose, cellulose and cellobiose and incubated anaerobically under N_2 atmosphere. Growth in each medium was monitored by measurement of the optical density at 660 nm with a spectrophotometer at periodical interval. Growth in the medium without supplements was used as control. Spore formation was examined by the observation of cell morphologies staining with malachite green and examined under oil immersion objective.

Analytical methods

Volatile fatty acids and CO_2 production were analysed with a gas chromatograph equipped with a flame ionization detector with N_2 as the carrier gas, by following the method of Holdemann *et al.,* (1977) [8]. The column, injector and detector temperatures were 135°, 150 ^{o} and 150 ^{o} C respectively. Gas samples were taken from the headspace of culture tubes with a pressure-lock syringe and analysed with a gas chromatograph equipped with a thermal conductivity detector. Denitrification activity was studied by adding 1% potassium nitrate and incubated under N_2 atmosphere and analysed with a gas chromatograph with Helium as the carrier gas. The column, injector and detector temperatures were 80 ^{o}, 125 ^{o} and 225 ^oC respectively. Nitrous oxide standard were prepared and the samples were identified and quantified with known standard.

16S rDNA sequencing and phylogenetic analysis

Cells cultivated in liquid medium were collected by centrifugation and used for DNA extraction. The sequential steps to identify the anaerobic bacterial isolate involve firstly extraction of genomic DNA hexadecyl-trimethyl ammonium bromide (CTAB) method with slight modifications (Melody, 1997) [17]. Then, the extracted DNA was used as a template for polymerase chain reaction (PCR) (Eppendorf Master cycler, Germany) for 16S rRNA gene amplification with universal primer pairs; MR forward (19 mer [5'-GAG TTT GAT CMT GGC TCA G-3']) and MR reverse (18 mer [5'-ACG GYT ACC TTG TTA CGA CTT-3']) (Weisberg *et al.,* 1991) [27]. Subsequently, the quality of amplified PCR product was examined by agarose gel electrophoresis based on the method given by Sambrook *et al.* (1989) [22]. Also, the PCR product was separated and purified using spin columns (Qiagen, Germany) according to the manufacturer's instructions. Finally, the purified PCR product was subjected to nucleotide sequencing using ABI prism terminator cycle sequencing ready reaction kit and electrophoresis of the products were

carried out on an Applied Biosystems (Model 3100) automated sequencer.
Multiple alignments of the sequences obtained and reference sequences in the GenBank database with the BLAST program were performed, and a phylogenetic tree with the neighbor-joining method of Saitou and Nei (1987) using MEGA 4.0 software (Tamura *et al*., 2007) [24] was constructed using the Clustal W program. All gaps and unidentified base positions in the alignment were excluded before calculations.

Preparation of sodium alginate slurry
The anaerobic bacterial isolates were inoculated in broth under nitrogen atmosphere and incubated anaerobically. They were grown until an optical density was reached to 0.6 at 600 nm. The cells were then harvested by centrifugation at 14000 rpm for 15 mins, washed with sterile 0.1% saline, again centrifuged and resuspended in 0.1% saline. The whole operation was performed by flushing nitrogen gas. Then the culture solution was added to sodium alginate slurry (Sodium alginate of 3.5 g and 100 ml of 0.1% calcium chloride) under nitrogen atmosphere and mixed thoroughly. Beads were prepared by passing the slurry through a tube and allowing the drop to fall into a 4% calcium chloride solution. Then the immobilized beads were transferred to a vial and stored under nitrogen atmosphere and used for further studies.

Root architecture study by inoculation of *Clostridium pasteurianum* NFTh2
In vitro experiment was performed in rice (cultivar Co51) to assess the RSA modulations as influenced by anaerobic nitrogen fixer under gnotobiotic condition. The modified Hoagland's nutrient solution was prepared and added to all the tubes. The pre-germinated rice seed was placed over it and the tube was stoppered with rubber cork and flushed with nitrogen gas and incubated in plant growth chamber with 12 h light (200 mole/m2/s) at 28 °C and root architecture was observed.

Root imaging
The root architecture of inoculated and uninoculated seedlings was photographed on 30th day of bacterial inoculation. The tubes were placed inside a cuboid glass container covered on 3 sides with black chart and filled with water in order to avoid reflection while capturing the images. The images were captured in high resolution camera (Sony cyber-shot, 12.5 mega pixels) and area covered by the root was cropped with Picasa photo editor software. The images of uninoculated and anaerobic nitrogen fixer inoculated rice roots were compared.

Use of Gia Roots software for RSA parameters
The root images were processed and fed into software [Gia Roots obtained from http://giaroots.biology.gatech.edu/ (Galkovskyi *et al*., 2012) [5]] which was exclusively designed for analyzing the various root architecture traits of rice. The RSA traits include network perimeter, solidity, convex area, network area and bushiness index (Iyer-Pascuzzi *et al*., 2010) [13].

Results and Discussion
The lowland rice field soils favour the growth of anaerobic microflora due to the less concentration of oxygen and have been studied in greater details, for the distribution of various kinds of nitrogen fixing microorganisms. In India, 90 per cent of the rice fields are grown under submerged conditions, as they experience three conditions *viz.,* aerobic, microaerophilic and anaerobic conditions which favour the activities of the respective group of microorganisms. Important activities of the flooded soil are nitrogen fixation, denitrification, reduction of iron, manganese, sulphur and methane emission. However, the roles of anaerobic bacteria in all these processes in the lowland rice soil ecosystem are not fully understood. Hence, the present investigation was carried out to isolate the anaerobic nitrogen fixer and to assess the role of anaerobic microflora in RSA modulations as influenced by anaerobic nitrogen fixer under gnotobiotic condition. The experiments were carried out for isolation of anaerobic nitrogen fixer from flooded rice field having the soil texture of sandy clay loam with a bulk density of 1.27 mg/m3. The porosity and water holding capacity of the soil was 51.32 and 48 per cent and available NPK are 246, 14.3 and 431.8 kg ha-1 respectively. A total of 54 strains were selected as representatives of the bacterial groups classified by anaerobic growth ability and cellular morphology. Phylogenetic analysis and some phenotypic characterizations, including catalase activity, nitrogenase activity, fermentation products from glucose, were carried out on all 54 strains selected.

Anaerobic nitrogen fixer
The importance of nitrogen fixing activity of *Clostridium pasteurianum* and *Klebsiella pneumoniae* in the flooded soil is known (Hoshi *et al*., 1989; Hill, 1976 and 1988 and Paul and Newton, 1960) [10, 6, 7, 18]. The cultures isolated in the present study from the flooded soil samples were characterized. The isolates were straight terminal spore forming gram positive rods, which produced acetate and propionate, utilized sucrose more efficiently than the other carbon sources. During growth it released CO_2 and reduced acetylene and showed nitrogen fixing activity of about 19.81 n moles of C_2H_4 mg^{-1} protein h^{-1}. Burns (1982) [2] described a *Clostridium pasteuranium* from flooded rice ecosystem and *Clostridium* is considered to be more widely distributed than Azotobacter. Yamagata, (1924) [28] found that *Clostridium* occurred in 95 per cent of paddy fields and their population was high.

Facultative nitrogen fixing isolate
The isolates with glistening and raised colonies in the roll tube were found to be straight rods, gram negative. During growth, they released volatile intermediates like acetate or formate along with CO_2 and H_2. Their VFA profile also exhibited acetate and formate. The nitrogenase activity (15.78 n moles of C_2H_4 mg^{-1} protein h^{-1}) of the cultures was found to be positive. The isolates utilized different carbon sources *viz.,* cellulose, glucose, sucrose, cellobiose and fructose were utilized for the growth by the isolate and found that sucrose to be preferred carbon source. Based on the morphological and physiological properties the isolate was identified as *Klebsiella sp.* The present study was conducted under flooded conditions which encouraged the anaerobic and facultative anaerobic populations. The abundance of *Clostridium* and *Klebsiella* under flooded conditions observed in the present study was the result of conducive environment of the rice rhizosphere like low redox potential (-200 mV), availability of organic matter from rice root, rice stubbles, weeds, phytoplankton and hydrolysable carbohydrates.

Authenticity and phylogenetic analysis of anaerobic bacterial isolates
Fig 2 shows the 16S rDNA-based phylogenetic placement of all 30 strains derived from the rice field soil samples. The

screened anaerobic and a facultative anaerobic N_2 fixing bacterial isolates had a PCR product of expected size (1500 bp) (Fig 1). After purification, the amplified PCR products were sequenced (Merck, Bangalore). The BLASTn search of these sequences with the most similar 16S rRNA gene sequences of the GenBank database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) revealed the closest sequence identities. Molecular analysis based on 16S rRNA gene sequencing revealed that the anaerobic N_2 fixing bacterial isolates NFTh2 showed maximum similarity with *Clostridium pasteurianum* to about 99 per cent, facultative anaerobic N_2 fixing bacterial isolate NFT3 showed maximum similarity with *Klebsiella pneumonia* to about 94 per cent and the isolate NFP2 showed maximum similarity with *Enterobacter cloacae* to about 99 per cent respectively. Weber *et al.,* 2001 [26] used the molecular techniques to show the dominance of members of different clostridial clusters in the bacterial community degrading rice straw under anoxic conditions. In the study, the bacterial counts obtained from the heat-treated samples of plant residue were less than 1% of the total anaerobes during the flooded period, and most of the strains belonging to the clostridial group were isolated from the heat-treated samples, although the percentages of spores of soil samples were much higher than those of plant residue. Akasaka *et al*., 2002 [1] enumerated culturable anaerobic bacterial populations on rice plant residue (straw and stubble with roots) in paddy field soil on the roll tube agar and were found on the order of 10^9 CFU (colony-forming units) g^{-1} dry weight of plant residue, and the percentages of spores were usually less than 1% of the total anaerobes. The phylogenetic analysis of 47 isolates based on 16S rRNA gene sequences revealed that the most dominant group was closely related to the *Cellulomonas* species in the Actinobacteria phylum and accounted for more than 60% of the isolates for most of the samples. Most of the strains affiliated to the clostridial group were isolated from the heat-treated samples. Thus, hydrolytic and fermentative anaerobic bacteria decomposing these polymers should have been enriched in soil of the field.

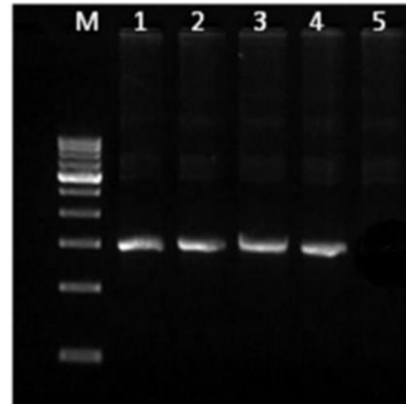

Fig 1: PCR amplification of 16S rRNA gene of the bacterial isolates M- 500bp DNA ladder; 1- NFTh2; 2- NFT3; 3- NFP2; 4- Positive control; 5- Negative control

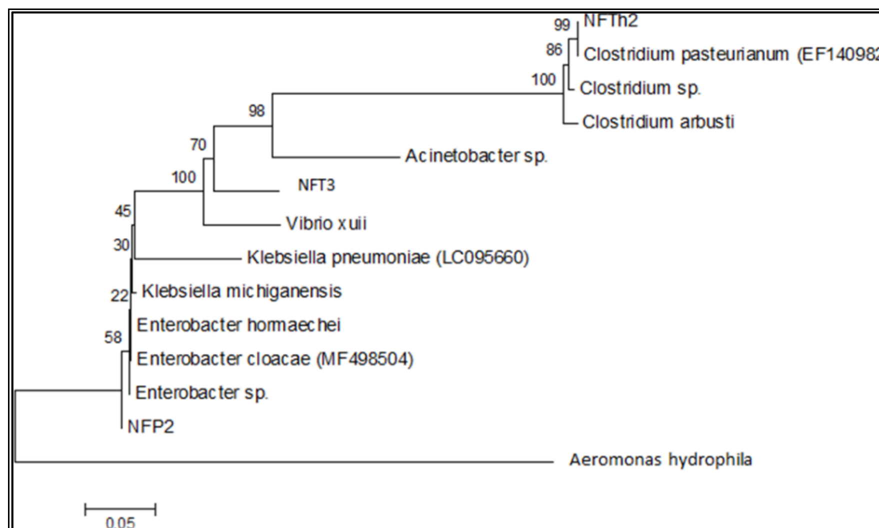

Fig 2: Neighbour-joining phylogenetic tree based on partial 16S rRNA gene of anaerobic bacterial isolates. The related species, strain name and accession number were collected from NCBI, GenBank database. The boot strap value of more than 50 % (out of 1000) were denoted in each node of the tree. The scale bar indicates per cent changes in nucleotide position (5 out of 100 bp)

Root system architecture (RSA) of rice seedlings

The rice seedlings, grown as single plant in modified Hoagland medium in culture tubes, were observed for RSA variables using root imaging system (Fig 3). The RSA variables at 0th, 5th, 10th and 15th day after inoculation were compared between uninoculated (Uninoculated) and anaerobic nitrogen fixer *Clostridium pasteurianum* NFTh2-inoculated (Inoculated) rice roots. Maximum number of roots (MXNR), average root width (AVRW), median number of roots (MDNR), number of connected compounds (NOCC) and specific root length (SPRL) were treated as primary variables of RSA. The mean values of those primary variables recorded on 0, 5, 10 and 15 days of incubation are presented in Table 1. After *Clostridium pasteurianum* NFTh2 inoculation, MXNR, AVRW, MDNR and NOCC significantly increased (p=0.00) in inoculated rice root than those of uninoculated control in all the observed days (5, 10 and 15 days). Among the four positively responded RSA primary variables, MXNR and MDNR had maximum response for treated than control (57%, 47%), while AVRW also had maximum response due to *Clostridium pasteurianum* NFTh2 inoculation.

Ellipse represents the over-all shape and size of the root system, which was measured by three parameters *viz*., major ellipse axes (MAEA) and minor ellipse axes (MIEA) represent the lengths of the major and minor axes of an ellipse best fit to the overall shape and size of the root system. Ellipse axes aspect ratio (ELAX) represents the ratio of the length of the minor ellipse axis to the length of the major ellipse axis. All these ellipse variables for inoculated and

uninoculated samples for 0, 5, 10 and 15 days after *Clostridium pasteurianum* NFTh2 inoculation are presented in Table 1. MAEA, MIEA and ELAX did not differ significantly due to *Clostridium pasteurianum* NFTh2 inoculation ($p>0.05$). However, MAEA was found to be higher for inoculated than that of uninoculated control.

Network represents the over-all coverage of the image by the root and it is differentiated from the background. It includes the bushiness (NWBS), solidity (NWSD), depth (NWDP), length (NWLN), distribution (NWLD), width (NWWI) and perimeter (NWPM). All these above network related RSA-variables are recorded for inoculated and uninoculated control samples on 0, 5, 10 and 15 days and mean values were presented in Table 1. Among the network variables, the depth (NWDP) increased due to *Clostridium pasteurianum* NFTh2 inoculation (3351) from 5th day to 15th day, as compared to uninoculated control (2418). The remaining variables *viz*., NWSD, NWWI and NWPM showed slight increase due to *Clostridium pasteurianum* NFTh2 inoculation.

The area (NWAR, convex area (NWCA), surface area (NWSA), volume (NWVL) and width depth ratio (NWWD) of root network observed for inoculated and uninoculated control at time course of incubation are presented in Table 1. The network area (NWAR) for inoculated was recorded to be higher (783353) when compared with uninoculated control. The other variables showed slight increase due to *Clostridium pasteurianum* NFTh2 inoculation to that of uninoculated control.

Principal component analyses were done for the root traits revealed a clear separation in the variation of the root-traits between different treatments. In the present investigation first principal component, this explained 97.4 % of the variation in the data (Inoculated) and second principal component which explained 5.5 % variation in the data (uninoculated) (Fig 4).

In the present study, both immobilized facultative and obligate anaerobes augmented the N uptake. This finding suggested that the nitrogen fixed by nitrogen fixing bacteria inoculated in the rice rhizosphere can be utilized by the rice plant rapidly and favour the N supply from the atmosphere. In general, the prolonged submerged rice system is favourable for reducing the soil nitrogen into ammoniacal form. However, the existence of facultative and obligate anaerobes could also favour the fixation of atmospheric nitrogen into ammoniacal form which could be utilized by the growing rice plants. This fixation is clearly evidenced with the increased soil N contribution. Though the N_2 fixation by the anaerobes seems to be of meager quantity if the mechanism of fixation is known explicitly, this phenomenon could be improved.

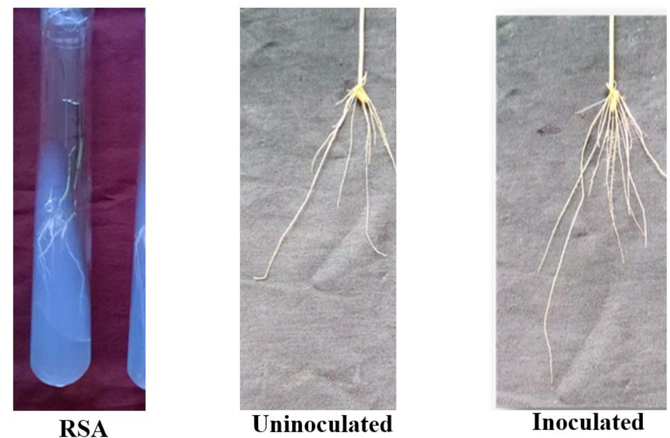

Fig 3: Effect of inoculation of *Clostridium pasteurianum* NFTh2 on the root traits of rice grown in seedling agar tubes

Table 1: Effect of inoculation of *Clostridium pasteurianum* NFTh2 on the root traits of rice grown in seedling agar tubes

Root traits	T_1-Uninoculated	T_2-Inoculated
Average Root Width (Diameter) (cm)	3.142812	3.724523
Network Bushiness (n/n)	1.83721	1.77778
Number of Connected Components	43	19
Cropping rect	0 0 0 0	0 0 0 0
Network Depth (cm)	2418	3351
Ellipse Axes Ratio (cm/cm)	0.347838	0.581759
Network Length Distribution	0.436213	0.649483
Major Ellipse Axis (cm)	3178.025	4406.053
Maximum Number of Roots (n)	32	57
Network Width (cm)	1482	1826
Median Number of Roots (n)	24	47
Minor Ellipse Axis (cm)	1631.471	1942.702
Network Area (cm^2)	453458	783353
Network Convex Area (cm^2)	4282367	5808171
Network Perimeter (cm)	395727	498617
Network Solidity (cm^2/cm^2)	0.108463	0.148328
Specific Root Length (cm/cm^3)	0.097483	0.109554
Network Surface Area (cm^2)	2464768	2826779
Network Length(cm)	129481	244866
Network Volume (cm^3)	2464215	3625543
Network Width to Depth Ratio (cm/cm)	0.516853	0.701030

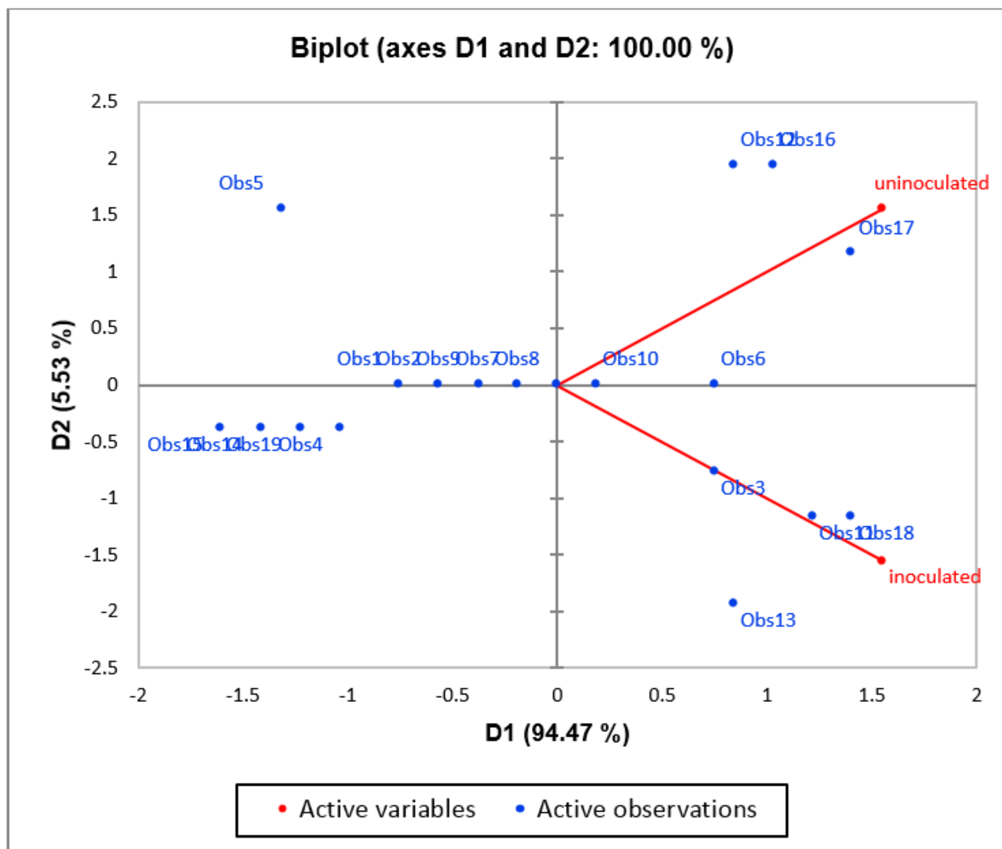

Fig 4: Effect of of inoculation of *Clostridium pasteurianum* NFTh2 on the Root system architecture (RSA) of rice grown in seedling agar tubes

Conclusion

BNF is an important aspect of sustainable and environment friendly food production at long term crop productivity. Hence, identifying suitable N_2 fixers for each cropping system and agro ecological condition, and optimizing their efficiency through integrated approach would be the future thrust to enhance the BNF for sustainable and eco-friendly agriculture. In conclusion, the nitrogen-fixing bacterial species within the genus *Clostridium* and *Klebsiella* are traditionally considered representatives of anaerobic, facultative anaerobic, free-living nitrogen-fixers. These anaerobic nitrogen fixers, because of their free-living lifestyle, were not considered active contributors of fixed nitrogen for supporting plant growth and productivity. The isolated nitrogen-fixing clostridia from paddy ecosystem are phylogenetically close to known nitrogen-fixing species, *Clostridium pasteurianum.* The paddy ecosystem was considered as the niche for nitrogen-fixing clostridia and also suggests a more significant role for the obligate anaerobes in supporting plant growth via nitrogen fixation.

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