# Isolation and Characterization of Putative Nitrogen-Fixing Endophytes from Three Distinct Wild Rice Species in Sri Lanka

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**Abstract**

There are 22 recorded wild rice species on earth, of which 5 are found in Sri Lanka. Knowledge on the endophytic diazotrophs of wild rice species, which can be adopted for paddy cultivation, is still at a fledgling stage of discovery. The identification of stably maintained endophytic diazotrophic bacteria in the genus *Oryza* requires further attention, especially due to skyrocketing global prices of chemical fertilizers. The main objective of our study was the isolation and characterization of endophytic and root-surface colonizing nitrogen fixers from three distinct wild rice species found in Sri Lanka, namely *Oryza eichingeri*, *Oryza granulata* and *Oryza nivara*. Endophytic nitrogen fixers were isolated from surface–sterilized stems, leaves, and roots, while root surface colonizing bacteria were isolated from washed roots. There were eleven endophytic bacteria and four root surface colonizing bacteria isolated by using nitrogen-free yeast mannitol/malate agar plates; six isolates from *O. granulata*, five isolates from *O. eichingeri* and four isolates from *O. nivara*. Out of the 15 isolates, 13 were Gram negative and 2 stained Gram positive. Six of the eleven isolates secreted three enzymes tested, primarily pectinases, proteases, and cellulases, suggesting their prospective involvement in endophytic lifestyles. Most isolates showed either bulls eyes, dendritic or featureless patterns of swarming chemotaxis for the chemoattractant proline, a common promoter of bacterial motility. We also tested the genomes of the isolates for the presence of the *nifH* gene. DNA from seven isolates gave a PCR amplicon of the expected size (360 bp) using universal *nifH* primers, which proves that the genetic foundation for the production of nitrogenase reductase subunit was found in these seven isolates. We also tested each of the bacterial isolates against a devastating fungal pathogen of rice (*Rhizoctonia solani*). We found 4 potential candidates that show partial inhibitory activity against the growth of this rice sheath blight causative agent.

## 1. Introduction

The centrality and cruciality of nitrogen to all living systems is a widely accepted notion and remains as the single key ingredient in plant nutrition. Nitrogen is one of the most significant factors in plant development. However, plants cannot directly use atmospheric nitrogen, which makes up about 79% of the atmosphere. Basically, plants fulfil their nitrogen requirement via the absorption of available nitrogen from the soil through their roots. Nitrogen is absorbed by plants in the form of ammonium and nitrates (Santi *et al.*, 2013).

Synthetic nitrogen fertilizers are produced on large scale worldwide (Drechsel *et al.*, 2015). The Haber-Bosch process is the method which is used for the production of most synthetic nitrogen fertilizers using high temperature and pressure conditions. In this context, improving the use efficiency of the nitrogen fertilizer is essential for the establishment of sustainable agriculture and is one of the most

important challenges in the 21st century (Carvalho *et al.*, 2014). The exploitation of biological nitrogen fixation (BNF) in agriculture is a way forward, however, it is burdened with barriers such as host specificity, slow release of fixed nitrogen to plants, mutualistic cooperativity, and the mandatory requirement for a specific anatomical compartment. The capacity for the biological conversion of atmospheric di-nitrogen into plant available ammonium is only found in eubacteria and archaeobacteria and such microbes are called diazotrophs (Santi *et al.*, 2013). The supplementation of nitrogen to crops via diazotrophs is a suitable and promising alternative to synthesized nitrogen fertilizers.

Previous studies have proved the occurrence of endophytic diazotrophic bacteria in rice plants. In the Philippines, Japan and Thailand, researchers have been able to isolate endophytic diazotrophs from wild, traditional, and cultivated rice varieties. Most of the isolated diazotrophs were closely related to the genera, *Herbaspirullum, Azospirillum, Ideonolla, Azotobacter* and *Acetobacter* (Barraquio *et al*., 1997; Engelhard *et al*., 2000; Elbeltagy *et al*., 2001; Koomnok *et al*.,2007). Wild rice species are likely to harbour unique populations of diazotrophic bacteria that differ from those in extensively bred modern varieties of cultivated rice (Engelhard *et al*., 2000).

In recent times, the bacterial endophytes of the rhizome of the wild and perennial rice species, *Oryza longistaminata*, were identified by 16s rDNA sequencing as belonging to the genera, *Streptococcus, Bacillus,* and *Methylobacterium* (Peng *et al*., 2021). Especially the availability of members of *Methylobacteriaceae* in the rhizome, was significantly higher compared to the leaf and root (Peng *et al*., 2021). Another nitrogen fixer from *Oryza latifolia*, *Kosakonia oryzae* type strain Ola $51^T$, of which the genome was sequenced, was identified as a plant growth promoting bacterium due to the availability of auxin producing genes, while also containing both the *nif* and *anf* gene clusters for nitrogen fixation (Li *et al*., 2017).

Twenty-two wild species of the genus *Oryza* are distributed worldwide, and they are rich repositories of valuable genes (Brar and Khush, 1997). Sri Lanka possesses five wild rice species: *Oryza eichingeri, Oryza granulata, Oryza nivara, Oryza rhizomatis* and *Oryza rufipogon*. Our quest is to find the whole compliment of nitrogen fixers in three such wild rice varieties (*Oryza eichingeri, Oryza granulata, Oryza nivara)* found in Sri Lanka, which are distinct in their taxonomical identities. *Oryza nivara* belongs to *Oryza sativa* complex and is closely related to *Oryza sativa* and *Oryza rufipogon* (Vaughan, 1994). The ploidy level of *O. nivara* is diploid (2n=24) and it belongs to the AA genome group. *Oryza eichingeri* is a member of the *Oryza officinalis* complex. The ploidy level is diploid (2n=24) and genome group is CC. *Oryza granulata* belongs to the *Oryza meyeriana* complex and it has the genome group GG. The ploidy level of *O. granulata* is diploid (2n=24).

In this study, we explore the complement of nitrogen fixers from four compartments of the rice plant in three wild rice species, namely, *Oryza eichingeri*, *Oryza granulata* and *Oryza nivara*. The importance of this study, is complimentary to the vast expansion of nitrogen fixing microbes in recent literature, burgeoned by the fact that the historical knowledge of nitrogen fixers as being of the genera, *Rhizobium* or *Nostoc*, is now renewed with a vast wealth of nitrogen fixers that are not always symbiotic, and thus not curtailed by host specificity, the requirement for nodular compartments, the need for



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mutualism or cooperation, and many other limiting characteristics that is making the challenge for a universal nitrogen fixer, less of an impossibility.

## 2. Materials and Methods

### *2.1. Optimized surface sterilization protocol*

The whole plant was washed with running tap water for about 30 minutes to remove adhering soil particles and surface residue. Then, the plant was separated into its parts; root, stem and leaf. All parts were cut into about 1-2 cm pieces and kept separately. Then, an optimized surface sterilization protocol was performed.

Small pieces of selected plant material (root or stem, or leaf) were dipped in 95% alcohol for 10 s. Then, they were transferred to 5% chlorox solution for 5 minutes (for each step, the solution was shaken well for proper sterilization, and sterilized beakers were used for dipping plant materials). Then, the plant materials were washed with sterilized distilled water five times (Somasegaran and Hoben, 1985). The solid nitrogen-free culture medium that was employed for the isolation of bacteria contained either mannitol or malate as the energy and carbon sources. 4 replicates were utilized for each wild rice species.

### *2.2. Chemotaxis assays*

A study was performed to check the ability to swarm by bacterial colonies as a result of chemotaxis movement, which was based on their perception of chemical stimuli such as the amino acid proline. Culture medium was prepared with 0.3% of agar, $10^{-4}$ M Proline and using mannitol medium. Sterilized plates were inoculated with 5 µL of 2 weeks old broth cultures and incubated at room temperature for 5-6 days. Due to the chemotaxis behavior; swarming patterns can be obtained on the plates (Benjamin *et al.,* 2014).

### *2.3. Optical microscopy*

To study cell morphology, single colonies from agar plates were used to prepare slides and the slides were stained with simple staining and Gram staining (Vincent, 1970) techniques and observed under 4×, 10×, 40×, 100× magnifications (Light Microscopy).

### *2.4. Biochemical assays for cellulose, pectin and protein utilization*

For cellulose, pectin and protein utilization, media plates were made with carboxy methyl cellulose, pectin and skim milk and the hydrolysis zones around the bacterial colonies were observed/measured to identify the respective hydrolysis-promoting bacteria (Teather and Wood, 1982, Vermelho *et al.* 1996, Soares *et al*. 1999).

*2.5. PCR amplification of nifH gene fragments*

Polymerase Chain Reactions were run with PolFor & PolR primers using the default cycling parameters (Poly *et al*., 2001). The primer sequences that were used are as follows;

| Gene | Primer sequence (5'-3') | Product size | Reference |
|---|---|---|---|
| ni*fH* | PolFor: TGCGACCCGAAGGCTGAC<br><br>PolR: ATGGCCATCATCTCACCGGA | 360 bp | Poly *et al*. (2001) |

*2.6. Phylogenetic reconstruction*

Phylogenetic analysis using Maximum Parsimony trees was constructed using protein sequences retrieved from NCBI GenBank (accession numbers are shown in the branch tips along with the species name) after ClustalW multiple sequence alignment using DAMBE (Xia, 2000). The tree was constructed using Mega (Molecular Evolutionary Genetics Analysis) version X (Kumar et al., 2018). The bootstrap value was adjusted as 500, but there was no outgroup attribution.

*2.7 Inhibitory assays against Rhizoctonia solani*

The stock culture of causing agent of sheath blight disease, *Rhizoctonia solani* was provided by Rice Research and Development Institute (RRDI), Bathalagoda, Sri Lanka. The spores of the *Rhizoctonia solani* were placed at the center on three PDA plates while the isolates were inoculated in the periphery with a suitable negative control. The plates were incubated at room temperature for 2 to 3 days and the cultures with inhibition zones were observed and photographed.

## 3. Results and Discussion

Eleven presumptive diazotrophic endophytes were isolated (Figure 1 and Figure 2). Separation from surface sterilized plant parts is the *de facto* method in use for the isolation of bacteria, as also reported by Elbeltagy *et al.* (2000) and Koomnk *et al.* (2007). Out of the 15 isolates, 2 were Gram positive and the remainder were Gram negative (Table 3). Due to the limited finances associated with an undergraduate program, we were not able to characterize the bacteria using the gold standard of bacterial identification, i.e. sequencing of the 16s rDNA loci.

Diazotrophic bacteria commonly isolated from rice plants are *Herbaspirullum* sp. (Barraquio *et al.*, 1997; Elbeltagy *et al*., 2001; Koomnok *et al*., 2007), *Azospirullum* sp. (Elbeltagy *et al*., 2000; Elbeltagy *et al*., 2001; Koomnok *et al*., 2007), *Acetobacter diazotrophicus* (Barraquio *et al*., 1997), *Azoarcus* sp. (Barraquio *et al*., 1997), *Ideonella* sp. (Elbeltagy *et al*., 2001), and *Klebsiella oxytoca* (Elbeltagy *et al*.,



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2000). *Herbaspirullum* spp. are Gram negative, and has curved rods with polar flagella (Baldani *et al*., 1986; James and Olivares, 1997). *Azospirillum* sp. is also Gram-negative, and is a curved rod with single flagellum (Baldani et al., 1986). *Acetobacter diazotrophicus* is a small, Gram-negative rod shaped bacterium (James and Olivares, 1997). *Klebsiella oxytoca* is Gram-negative and rod-shaped while *Azoarcus* sp. is a rod-shaped bacterium with polar flagella. The majority of the nitrogen fixing bacteria isolated from *O.eichingeri, O.nivara* and *O.granulata* were Gram-negative and were rod-shaped bacteria.

Six out of the fifteen isolates of this study were able to secrete all three enzymes, pectinases, cellulases and proteinases (Table 3). It can be hypothesized that endophytes secrete tissue degrading enzymes such as cellulases, pectinases and proteases to enter the plant. The production of those enzymes facilitates endophytic behavior. For the root surface colonizing diazotrophs, the secretion of cell wall degrading enzymes is an ecological advantage over bacteria that can only colonize plants epiphytically. Because some associative bacteria invade internal tissue (Carvalho *et al.*, 2014), they are able to produce such enzymes to enter to internal plant tissue without any aid from natural openings and wounds. Colonization of the internal tissues of plants is thought to provide a uniform and protective environment for microorganisms than plant surfaces (Lodewyckx *et al*., 2002). Previous studies also support that idea and show cellulase and pectinase degrading abilities as proof of the endophytic behavior of isolated bacteria (Elbeltagy *et al*., 2000; Elbeltagy *et al*., 2001; Lodewyckx *et al*., 2002).

Motility is a significant feature of endophytic life since they need to migrate towards the host plant (Elbeltagy *et al*., 2000). Associate-living diazotrophs, especially *Azospirillum braziliense* have been studied in great detail on their chemotactic behavior (Alexandre and Zhulin, 2007). Most of the bacterial cultures used in our study showed three characteristic patterns of motility to the chemoattractant proline; bulls' eye, dendritic and featureless (Figure 1; Table 4). Motility is indicative of their natural ability to sense and move towards a chemoattractant like proline and was stronger with proline than in the absence of proline in the medium (Table 4). Chemotaxis is metabolism-dependent in the root surface colonizing diazotroph *A. brasilense*. Amino acids are chemo-attractants for *A. brasilense* that fix nitrogen under microaerophilic conditions (Alexander and Zhulin, 2007). Amino acids such as proline and histidine have been shown by hydroponics to be part of the secreted material on the surface of roots of rice plants (Bacilio-Jimenez *et al*., 2003). Chemoattraction to proline is mediated by the McpU chemoreceptor in proteobacteria such as *Sinorhizobium melliloti* which is strongly expressed in this motile bacterium (Webb *et al*., 2014). We used PSI-BLAST, which is more sensitive for identifying distant members of query proteins, to search for homologs of the McpU receptors, and built a phylogenetic tree with 19 distinct proteobacteria that we identified using our search. The search query was the McpU receptor sequence from *Sinorhizobium melliloti.* We were able to see two clear divisions; The *Ensifer* (*Sinorhizobium*) clades and the *Rhizobium/Agrobacterium* clade (Figure 3). All the bacteria were alpha proteobacteria except for a lone *Pseudomonas* species. Symbiotic bacteria that colonize legume roots enter through a root hair or a crack on the root surface, which, too is the preferred mode of infection of rice plants by endophytic rhizobia. However, Rhizobia can enter the non-legume genus *Paraponia* and the water-tolerant legume *Sesbania rostrata*. However, in contrast to intracellular symbiotic nodule tissue formation in such genera, in the rice plant, rhizobia can only inhabit the intercellular compartment. Methyl-accepting chemotaxis proteins are found in the cultivated rice endophyte *Azospirillum* sp. B510 (Kaneko et al., 2010). Therefore, the availability of such proteins in other endophytes of rice is a promising avenue for future research.

The primer pair PolFor/PolR gave a single PCR band of the expected size (360 bp) from the genomic DNA of seven isolated diazotrophic bacteria from wild rice species (Figure 2). Based on these

results, the genetic foundation for the production of the nitrogenase enzyme of seven isolates was confirmed. One endophytic isolate (OG/ISO C) of *O. granulata,* two endophytic isolates from *O.nivara* (ON/ISO J, ON/ISO U) and one endophytic and three root surface colonizing isolate (OE/ISO F, OE/ISO K1, OE/ISO K2 and OE/ISO K′ respectively) from *O. eichingeri*.

*NifH* gene encodes the nitrogenase reductase subunit of the nitrogenase heterotetramer and is universally available for all nitrogen fixers, including those of alternate vanadium and iron nitrogenases. Phylogenetically, *nifH* genes are divided into five clusters, of which the first is aerobic and facultatively anaerobic organisms that belong to bacterial *phyla Proteobacteria, Cyanobacteria, Firmicutes and Actinobacteria.*

Other clusters make up alternate nitrogenases (Cluster II); *Archaea*, *Treponema*, *Clostridium* and sulfate-reducing and sulfur-reducing species of *Deltaproteobacteria (Cluster III)* and paralogs that do not take part in fixing elemental nitrogen (Cluster IV and V) (Gaby and Buckley, 2012 and 2014). We suggest most of our isolates belong to cluster 1, since chemotaxis by swarming (as shown in this study) is only found in proteobacteria (alpha and gamma) and firmicutes; therefore, the availability of the *nifH* locus with their respective chemotaxis propensities, helps us to zoom into their prospective identities as belonging to cluster I.

A much sought-after discovery is a universal plant-growth promoting nitrogen fixer for cultivated rice varieties, including the elite cultivars that are able to positively influence plant growth promotion and grain yield. Quantifying nitrogen fixation would be the next step forward to this preliminary study and performing hydroponics studies to look at growth promotion would be invaluable.

*Rhizoctonia solani,* a soil-borne fungus, causes rice sheath blight, impacting grain yield. When we co-inoculated plates of our isolates with a culture of *Rhizoctonia solani* at the center, we observed a partial inhibitory effect by four of the isolates compared to the negative control. The culture plates are shown in Figure 4. The cultures OE/ISO K2, OE/ISO F, ON/ISO J and ON/ISO F were able to inhibit the growth of *Rhizoctonia solani* stronger than that of the negative control (on the same plate) [Figure 4] and will be contenders for downstream hydroponics/pot studies. Conventional breeding offers little potential to *Rhizoctonia solani* resistance and thus our findings have emergent potential for the development of a bio-fungicide, uniquely or as a concoction.

We name Strain F as the key microbe which has the most positive attributes, to be taken to the field for trials and for the development of a suitable biofertilizer for rice cultivation. Strain F is a likely nitrogen fixer (presence of ni*fH* locus) and a beneficial bacterium to fight *Rhizoctonia solani*, the pathogenic agent of rice sheath blight, while also showcasing a catalog of enzymes helpful for endophytic lifestyles, as well superior swarming motility in the presence of proline.

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Table 1(A) Diazotrophic bacteria isolated from stems and roots of *O.granulata*

| Label of the bacteria strain | Isolated plant part |
|---|---|
| OG/ ISO A1 | Root |
| OG/ ISO A2 | Root |
| OG/ ISO B | Root |
| OG/ ISO D1 | Root |
| OG/ ISO D2 | Root |
| OG/ ISO C | Stem |

*Table 1(B)* Diazotrophic bacteria isolated from root and root surface of *O.eichingeri*

| Label of the bacteria strain | Isolated plant part |
|---|---|
| OE/ ISO K1 | Root surface |
| OE/ ISO K2 | Root surface |
| OE/ ISO K′ | Root surface |
| OE/ ISO E | Root surface |
| OE/ ISO F | Root extract |

Table 1(C) Diazotrophic bacteria isolated from stems and roots of *O.nivara*

| Label of the bacteria strain | Isolated plant part |
|---|---|
| ON/ ISO G | Root |
| ON/ ISO H | Root |
| ON/ ISO J | Stem |

| | |
|---|---|
| ON/ ISO U | Stem |

Table 2 Identities of bacterial strains isolated from different sources of plant parts and their extracts of wild rice species using two culture media.

| Wild rice species | Source | Bacteria strain | Medium |
|---|---|---|---|
| 1.*Oryza granulata* | Root extract | A1 | malate |
| | | A2 | malate |
| | | B | malate |
| | | D1 | mannitol |
| | | D2 | mannitol |
| | Stem extract | C | mannitol |
| 2. *Oryza eichingeri* | Root surface | K1 | mannitol |
| | | K2 | mannitol |
| | | K′ | mannitol |
| | | E | malate |
| | Root extract | F | mannitol |
| 3. *Oryza nivara* | Root extract | G | mannitol |
| | | H | mannitol |
| | Stem extract | J | mannitol |
| | | U | mannitol |

Table 3 The enzymes that were found in each isolate as part of its secretome. The candidates tested were cellulases, proteinases and pectinases. The positive chemotaxis of the bacterial culture towards the chemoattractant proline too is mentioned in the penultimate column. The pictures of the chemotaxis plates are provided as figure 1.



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| Wild rice species | Plant material | Bacteria strain | Gram strain | Cellulase test | Pectinase test | Proline test | Protease test |
|---|---|---|---|---|---|---|---|
| 1.*Oryza granulata* | Root extract | A1 | - | + | + | + | - |
| | | A2 | - | + | + | + | + |
| | | B | - | + | + | + | - |
| | | D1 | - | + | + | + | + |
| | | D2 | - | - | + | + | + |
| | Stem extract | C | - | + | + | + | - |
| 2. *Oryza eichingeri* | Root surface | K1 | - | + | - | + | + |
| | | K2 | + | + | - | + | + |
| | | K′ | - | + | - | - | - |
| | | E | - | + | + | + | + |
| | Root extract | F | + | + | + | + | + |
| 3. *Oryza nivara* | Root extract | G | - | + | + | + | + |
| | | H | - | + | + | + | + |
| | Stem extract | J | - | + | + | + | + |
| | | U | - | + | - | + | + |

Table 4 Results of swimming motility/chemotaxis behavior of diazotrophic bacteria isolated from *O.granulata, O.eichingeri* and *O.nivara* in the presence and absence of proline.

| Bacterial isolates | Diameter of swarm ring in absence of Proline | Diameter of swarm ring in presence of Proline |
|---|---|---|
| OG/ ISO A1 | ++ | + |
| OG/ ISO A2 | + | +++ |
| OG/ ISO B | + | ++ |
| OG/ ISO D1 | + | +++ |
| OG/ ISO D2 | ++ | +++ |
| OG/ ISO C | ++ | + |
| OE/ ISO K1 | ++ | +++ |

| | | |
|---|---|---|
| OE/ ISO K2 | +++ | +++ |
| OE/ ISO K′ | - | - |
| OE/ ISO E | ++ | + |
| OE/ ISO F | ++ | +++ |
| ON/ ISO G | ++ | +++ |
| ON/ ISO H | + | +++ |
| ON/ ISO J | ++ | +++ |
| ON/ ISO U | ++ | +++ |

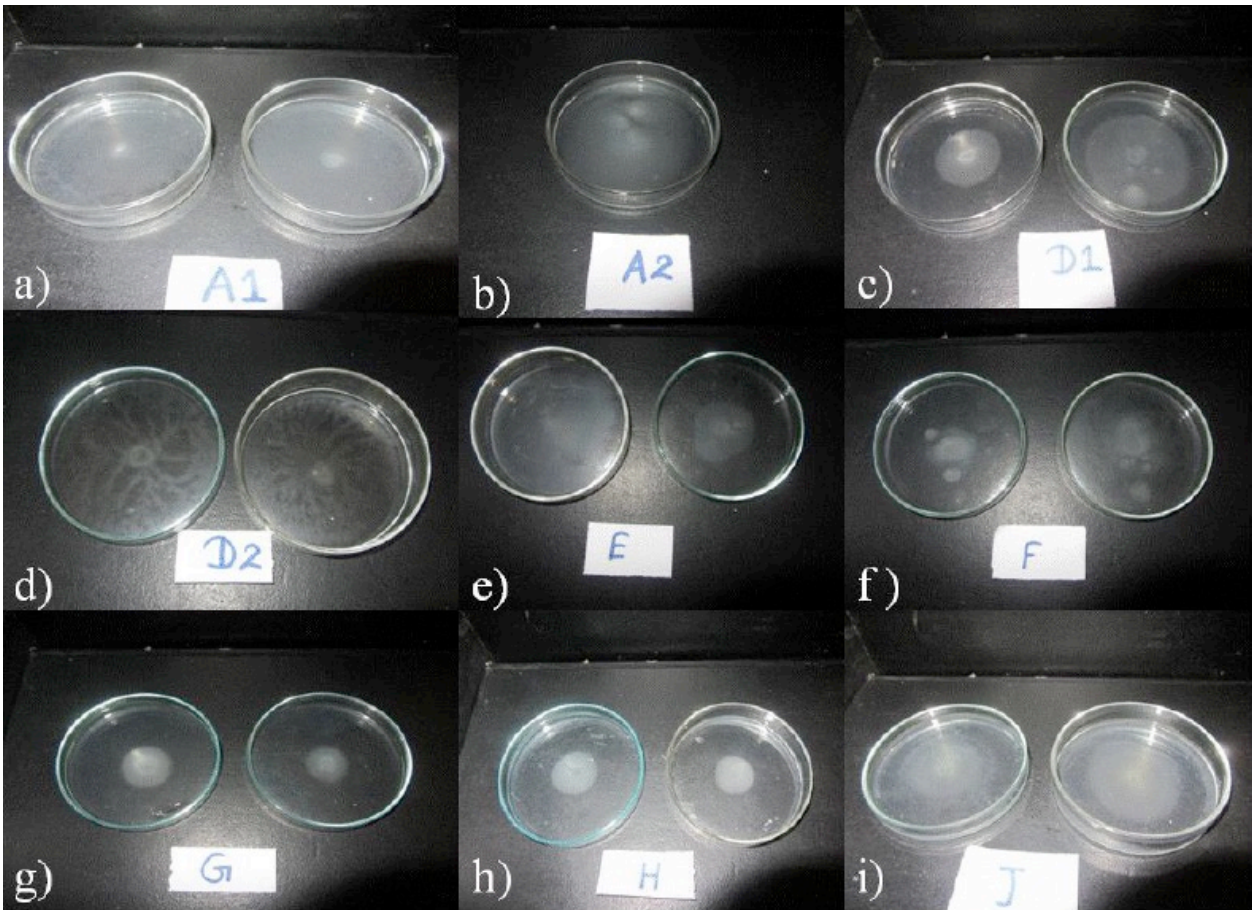




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Figure 1 Assessment of chemotaxis behavior of diazotrophic bacteria isolated from O.*granulata*, [ a).OG/ISO A1, b)OG/ISO A2, c).OG/ISO D1 and d).OG/ISO D2],from O.*eichingeri*, [ e).OE/ISO E and f).OE/ISO F ] and from O.*nivara*, [ g). ON/ISO G, h). ON/ISO H and i). ON/ISO J ]. All plates except K' showed swarming patterns on 0.3% agar plates containing proline. The commonest swarming pattens were bulls-eye (e.g – D1), dendritic (e.g - D2) and featureless (e.g. - A1)

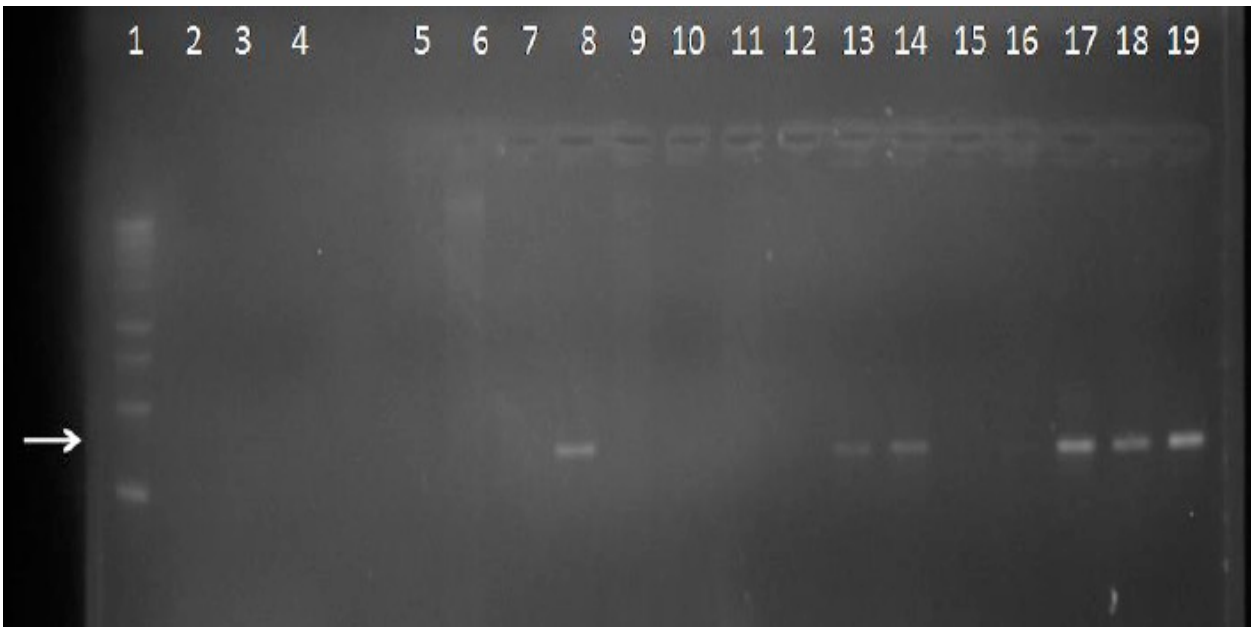


Figure 2 PCR amplification of nifH fragment of 360bp (indicated by the arrow) using the universal primers PolFor and PolR. O. *granulata*, O. *nivara* and O. *eichigeri* respectively. Ladder and PCR product have been loaded in following order.Lane 1,1kb ladder (Promega); Lane 2, *Anabaena azollae* (positive control); Lane 3, *Escherichia coli* (negative control );Lane 4, control; Lane 5,OG/ISO A1; Lane 6, OG/ISO A2; Lane 7, OG/ISO B; Lane 8, OG/ISO C; Lane 9, OG/ISO D1; Lane 10, OG/ISO D2; Lane 11, ON/ISO G; Lane 12, ON/ISO H; Lane 13, ON/ISO J; Lane 14, ON/ISO U; Lane 15, OE/ISO E, Lane 16, OE/ISO F; Lane 17, OE/ISO K1; Lane 18, OE/ISO K2; Lane 19, OE/ISO K′.

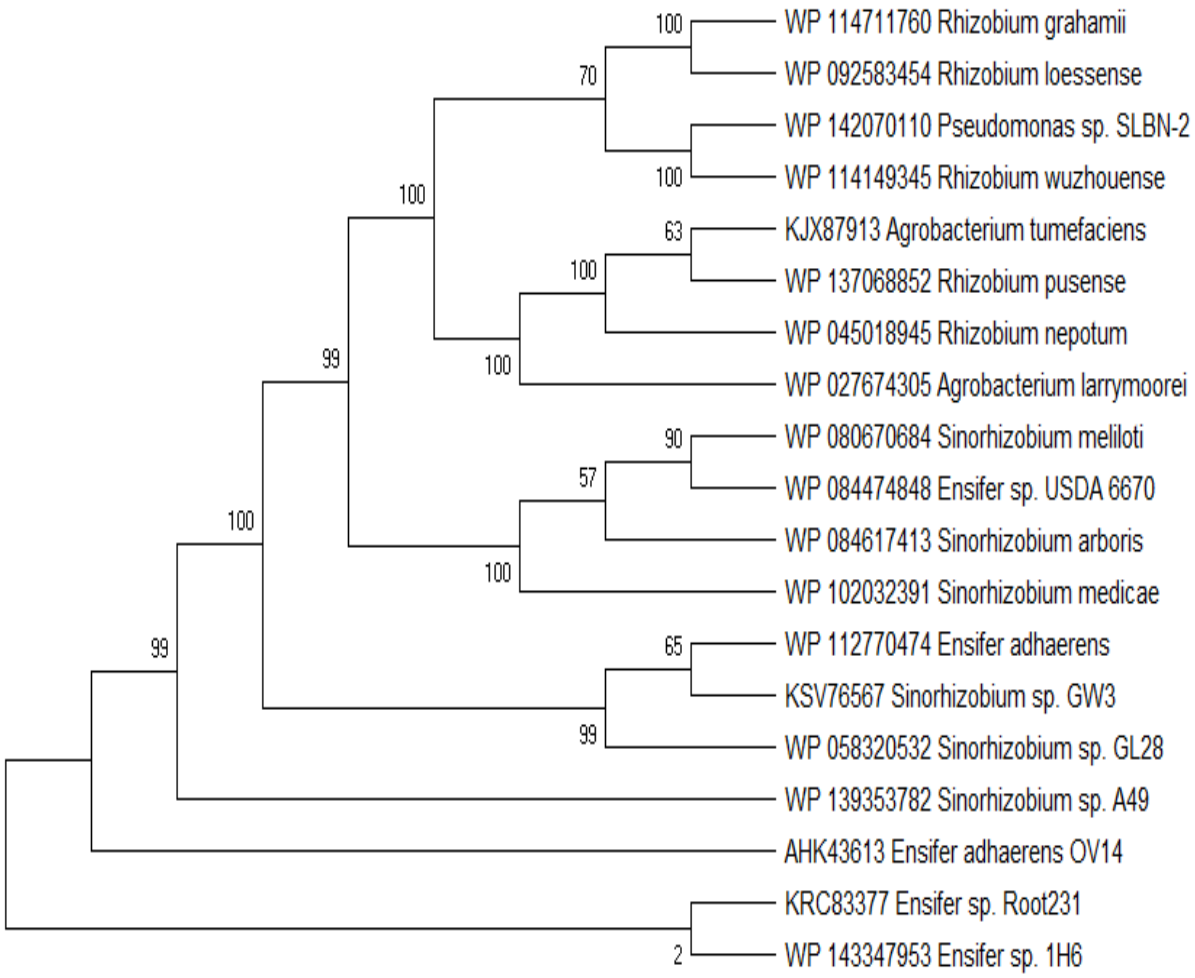


Figure 3 Phylogenetic inferences after ClustalW multiple sequence alignment of 19 McpU protein sequences using DAMBE (Xia, 2000). The tree was constructed using Mega (Molecular Evolutionary Genetics Analysis) version X (Kumar *et al*., 2018). The bootstrap value was adjusted as 500 and was not anchored by any out-group.



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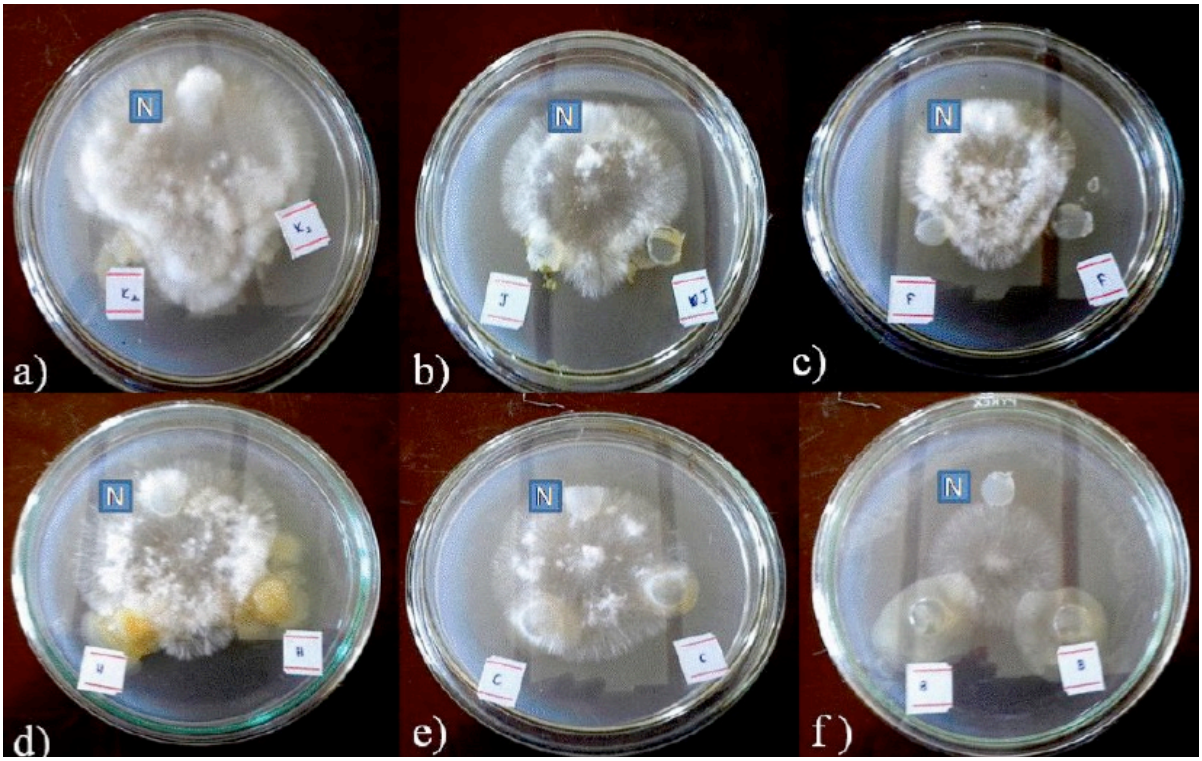


Figure 4 Dual culture plate assay of *Rhizoctonia solani*, pathogen of sheath blight disease in rice and dazotrophic bacteria isolated from wild rice species, *O. granulata, O. eichingeri* and *O. nivara;*after three days of incubation, a).OE/ISO K2,b).ON/ISO J, c).OE/ISO F, d). ON/ISO H, e).OG/ISO C, f).OG/ISO B and N- negative control. *Rhizoctonia solani* was inoculated at the center of the plate and the negative contro l and the duplicated isolates are in the periphery.