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Assessment of the migration of the operational taxonomic units (OTUs) from rice rhizosphere to endosphere

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Abstract

An experiment was conducted to understand the migration of the OTUs from rice rhizosphere to endosphere at vegetative and flowering stages of the crop. The soil and plant samples were studied through culture independent molecular PCR-DGGE technique involving denaturing gradient gel electrophoresis (DGGE) of PCR-amplified V3 region of 16S rRNA gene. The DGGE analysis revealed higher species richness in soil, followed by root, stem and leaf. The analyses for range-weighted richness and Shannon diversity index indicated vegetative stage carries more diverse microflora in soil and root samples than stem and leaf samples. The Sorenson's similarity index indicates the migration of bacterial population from rhizosphere to endosphere. The soil samples shared about 28 to 35 % similarity with different plant compartments *viz*., root, stem and leaf at both crop stages. The fingerprinting of rhizosphere and endosphere samples would be a useful resource for plant microbe breeding through understanding of the associated microbial community.

Keywords: Rice, rhizosphere, DGGE, OTU, 16S rRNA, migration

Introduction

Microorganisms influence agriculture through their presence in rhizosphere and plant endosphere. It is crucial to understand the factors influencing the microbial communities, considering the enormous importance of plant microbe interactions in agricultural systems. Studies have revealed beneficial effects of endophytes in rice crop through N_2 fixation, phosphate solubilisation and anti-fungal or anti-bacterial properties (Kumar *et al.,* 2009; Naik *et al.* 2009; Ramesh and Mathivanan, 2009). Studies on the species diversity of bacterial endophytes have been mainly approached by cultivation-based methods. The study on movement of *gfp*-tagged rhizobia indicated surface colonization of the rhizoplane, followed by endophytic colonization within roots, and then ascending endophytic migration into the stem base, leaf sheath and leaves (Chi *et al.* 2005). However, a range of bacteria is not accessible to cultivation methods, because of their unknown growth requirements or their entrance into a viable but not culturable state. Diverse endophytic bacteria are present in various parts of rice plants as revealed by culture dependent and non-culture-based methods (Mano and Morisaki, 2008).

The 16S rRNA gene (rDNA) has become a frequently employed phylogenetic marker to describe microbial diversity in natural environments without the need for cultivation. Amplified ribosomal DNA restriction analysis (ARDRA) could reveal diverse taxa of endophytic bacteria in the SSU rDNA library of rice roots (Sun *et al.* 2007). Denaturing gradient gel electrophoresis (DGGE) analysis revealed bacterial and fungal communities in the intercellular fluid of rice leaf blades and sheaths were distinct from that in the surface washing fluids (Takahashi *et al*. 2011). Terminal restriction fragment length polymorphism (T-RFLP) and 16S rDNA cloning in the leaves of three rice varieties revealed that 74 % of communities were similar in all three rice varieties (Ferrando *et al.* 2012).

In an analysis of the actinomicrobiome present in rice, of the 393 actinobacterial OTUs discovered in root samples, 29 OTUs shared commonness in the grains and stem samples (Wang *et al*. 2016). Investigation on temporal changes in the root-associated microbial communities throughout the plant life cycle in field-grown rice indicated that root microbe composition varies with the developmental stage of the plants (Edwards *et al*. 2018).

However, our understanding of microbial structure in the rhizosphere and endosphere is still very poor. Although root-associated microbes are known to have the potential to be utilized to promote crop productivity, their exploitation has been hindered by a lack of understanding of the compositional dynamics of these communities. The rice root endophyte community suggested high potential for plant-growth promotion, improvement of plant stress resistance, biocontrol against pathogens and bioremediation, regardless of their culturability (Sessitsch *et al.* 2012). The objective of the present study was to understand the migration of the bacterial microflora from rice rhizosphere to endosphere at vegetative and flowering crop stages. Polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE) analysis is preferable over other methods because of its sensitivity, ability to monitor community shifts and cluster analysis. The results highlight relationship between plant growth stage and associated microbes that can be considered in strategies for the successful manipulation of microbial communities to enhance crop performance.

Materials and Methods

Seed and soil material

The seeds of rice genotype BPT5204 (Samba Mahsuri) were collected from the Agricultural Research Station (ARS), Mugad. Plants were raised in garden soil for conducting the pot experiments in greenhouse conditions.

Sampling details

Soil, root, stem and leaf samples were collected at vegetative and flowering stages of rice plants for studying the migration of soil microflora from rhizosphere to the plants. The soil samples were collected near the root zone at a depth of 10-15 cm and stored at -20 °C till further processing.

Protocol for surface sterilization of plant samples

Surface sterilization of plant samples for efficient removal of the rhizoplane bacteria was done according to the optimized method (Suryawanshi, 2018). Potted rice plants sown in garden soil were harvested and washed thoroughly under tap water. Roots were placed in sterile 50 ml Falcon tubes containing 25 ml phosphate buffer. Further, the Falcon tubes were vortexed at maximum speed for 15 s to remove any adhered rhizosphere soil from the roots. The roots were placed in fresh tubes containing phosphate buffer and sonicated at low frequency for 3 min (three 30 s bursts followed by three 30 s rests in between). The roots were chemically sterilized using 1.5 % sodium hypochlorite for 2 min by intermittent manual shaking and then rinsed twice with sterile distilled water. The roots were transferred to fresh bottles containing glass beads in 0.9 % NaCl solution and the rhizoplane bacteria were removed by vortexing at maximum speed for 10 min.

Extraction of DNA from plant samples

The genomic DNA was extracted from paddy leaves by using cetyltrimethylammonium bromide (CTAB/ NaCl) method of DNA isolation (Murray and Thompson, 1980).

Protocol for DNA isolation from soil sample

The extraction of DNA from soil samples was done using the lab protocol developed (Pasha *et al*, 2020) and standardized (Suryawanshi, 2018) earlier. Calcium chloride was added as a chemical flocculant suspended in DNA isolation buffer to remove humic substances from the soil. For cell lysis, 200 mg soil sample was taken in each 2 ml micro-centrifuge tube and 1 ml of soil DNA isolation buffer containing 100 mM Tris (pH 9.0), 100 mM Na_2EDTA (pH 9.0), 1.5 M NaCl, 80 mM $CaCl_2$, 1.25 % PVPP and 20 % SDS was added. The samples were incubated in the ThermoMixer (Eppendorf, Germany) at 1400 rpm and

70 °C for 50 min. After incubation, soil samples were centrifuged at 13,000 rpm for 10 min. The supernatant was transferred to fresh 2.0 ml micro-centrifuge tube, mixed with equal volume of chloroform: isoamyl alcohol (24: 1) and centrifuged at 13,000 rpm. The upper aqueous layer obtained was transferred to 1.5 ml micro-centrifuge tube, mixed with equal volume of chilled isopropanol, and incubated overnight at -20 °C. The DNA was pelleted by centrifugation at 13,000 rpm at 4 °C for 10 min. The DNA pellet was washed with 70 % ethanol, air dried and dissolved in $T_{10}E_1$ buffer for further use.

PCR amplification

For the PCR amplification, the hypervariable region (V3) of 16S rDNA was targeted and amplified using PRBA338F (5´ACTCCTACGGGAGGCAGCAG3´) and PRUN518R (5´ATTACCGC GGCTGCTGG3´) primer pair (Nakatsu *et al.,* 2000) with 40 bp GC clamp added to forward primer. Each PCR reaction contained 1X PCR buffer, 250 µM of each dNTP (GeNei, Bangalore), 5 µM of each primer (Sigma-Aldrich, USA), 1-unit *Taq* DNA polymerase (NEB, USA) and 100 ng of template DNA. The template DNA was denatured at 95 °C for 5 min followed by 30 cycles of denaturation at 94 °C for 50 s, annealing at 55 °C for 30 s, extension at 72 °C for 50 s and final extension of 5 min.

Denaturing Gradient Gel Electrophoresis (DGGE)

DGGE was carried out using 12 % polyacrylamide gel prepared with 30 % to 70 % chemical denaturant (40 % formamide and 7 M urea corresponding to 100 % denaturant) (Muyzer *et al*, 1993) as resolving gel, which was topped with 5 % polyacrylamide without any denaturant. The PCR product was loaded in each well carefully without contaminating adjacent wells. The gel was run in 1X TAE buffer for 17 hours at 100 Volts in DGGE system (Cleaver Scientific, UK) as per manufacturer's instructions. After the run, the gel was carefully removed and placed between two sheets of transparent plastic for proper handling during staining. The gel was stained by the silver staining protocol given by Gustavo and Gresshoff (1994).

Analysis of DGGE profiles

The DGGE profile was documented in Syngene G box gel documentation unit and processed by GeneTools software (Syngene). The faint band was scored as 1 (or the brightest band was scored as 10) and used as reference for the densitometric based scoring of other bands in profile. The number of bands was taken as measure of different operational taxonomic units (OUT's) and the respective intensity as their proportion in the population.

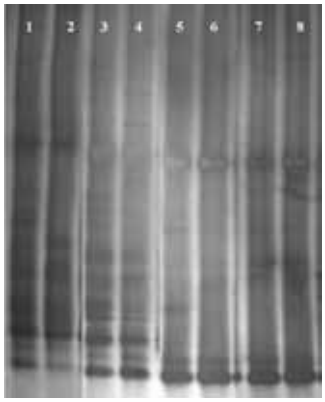
Species richness was calculated by range-weighted richness (Rr) = ($N^2 \times Dg$) (Marzorati *et al*, 2008), where N is the total number of bands in the pattern and Dg is the denaturing gradient in which the uppermost and lowermost bands were obtained. Shannon diversity index (H) was calculated using the equation: $H = -\Sigma Pi \times \log (Pi)$ (Shannon, 1948), where Pi is the proportional intensity of each band or OTU. The statistical analysis for Shannon diversity index was performed according to Hutcheson's modified t test (Hutcheson, 1970). Sorenson's similarity index (Cs) was calculated by formula $Cs = 2j/(a+b)$ (Sorensen, 1948), where j is the number of OTUs common for both samples, a and b are the number of OTUs present in first and second samples respectively.

Results

The soil and plant samples were studied through DGGE analysis to understand the migration of the OTUs from rhizosphere to leaf tissues at two crop growth stages *viz*., vegetative and flowering. The amplification of approximately 220 bp size product (including GC clamp) by PRBA338-PRUN518 primer pair for V3 region of 16S rDNA was obtained from all the samples. The DGGE profiles generated from amplified products for all the samples studied were distinct (**Figure 1**). Different bands, each representing an OTU were observed in all the samples. The number of OTUs was higher in soil followed by roots, stems and leaves. The vegetative stage had a relatively higher number of OTUs than the flowering stage in all the samples.

The range-weighted richness (*Rr*) for the analysed samples varied from 61.44 to 156.88 (**Figure 2A**). Soil showed higher *Rr,* followed by root, stem and leaf. For any sample, vegetative stage had higher *Rr* than flowering stage; implying broader carrying capacity at vegetative stage. The Shannon diversity index for the analysed samples ranged between 1.95 and 2.44 (**Table 1, Figure 2B**). The highest diversity index (2.44) was observed in soil at vegetative stage, while lowest diversity index (1.95) was observed in leaf at flowering stage. In soil and root samples, the Shannon diversity index at vegetative stage was significantly higher than flowering stage. No significant difference in the Shannon diversity index was observed in stem and leaf samples at vegetative stage and flowering stage.

The similarity index of soil with root, stem and leaf was compared within the stage, to determine the migration of bacterial population from soil to plant tissues. The similarity between samples was calculated using Sorenson's similarity index (**Table 2**). At vegetative stage, soil had 34.92 %, 32.97 % and 32.77 % similarity with root, stem and leaf respectively. At flowering stage, soil had 31.64 %, 29.10 % and 28.37 % similarity with root, stem and leaf respectively.



1: Soil (Vegetative stage)
2: Soil (Flowering stage)
3: Root (Vegetative stage)
4: Root (Flowering stage)
5: Stem (Vegetative stage)
6: Stem (Flowering stage)
7: Leaf (Vegetative stage)
8: Leaf (Flowering stage)

Figure 1: DGGE profile for assessment of migration of microflora from soil to plants

Table 1. Shannon diversity index of soil and plant samples

Stage vs Sample	Soil	Root	Stem	Leaf
Vegetative stage	2.44**	2.4*	2.13	1.98
Flowering stage	1.97	2.26†	2.07	1.95

*Significant at 1 % within sample, ** Significant at 5 % within sample, † Significant at 1 % within stage

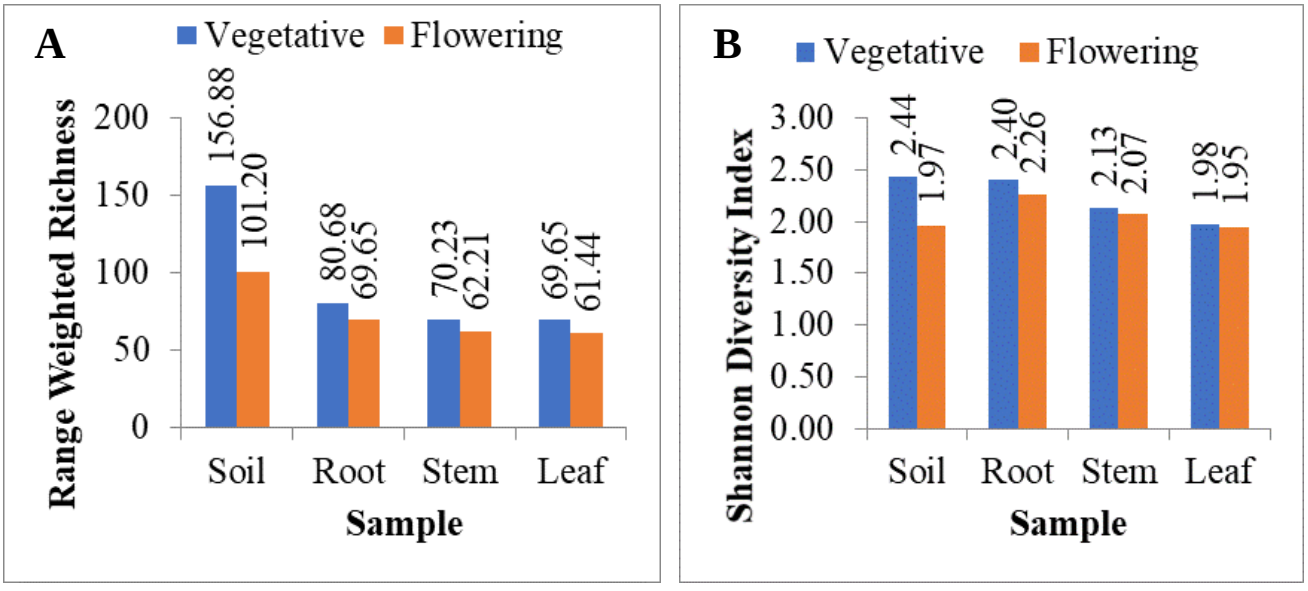


Figure 2: Assessment of migration of bacterial community from soil to plants

A. Range weighted richness, B. Shannon diversity index

Table 2. Sorenson's similarity index for migration of OTUs in soil and plant tissues

Sample	Stage	Soil		Root		Stem		Leaf	
		V	F	V	F	V	F	V	F
Soil	Vegetative	1							
	Flowering	38.06	1						
Root	Vegetative	**34.92**	31.59	1					
	Flowering	36.92	**31.64**	41.60	1				
Stem	Vegetative	**32.97**	29.41	39.44	30.42	1			
	Flowering	28.29	**29.10**	25.04	31.80	31.78	1		
Leaf	Vegetative	**32.77**	28.86	24.78	30.25	40.51	39.85	1	
	Flowering	27.36	**28.37**	24.47	31.51	28.13	44.67	46.38	1

V: Vegetative, F: Flowering

Discussion

The soil and plant samples were studied through culture independent DGGE analysis to understand the migration of the OTUs from rhizosphere to leaf tissues at two crop stages *viz.*, vegetative and flowering. Here in migration we assume, the soil as source of the bacteria, some of which would move into the root and further into the stem and leaf.

The number of OTUs was higher in soil samples followed by roots, stems and leaves. The vegetative stage samples had relatively higher number of OTUs than at flowering stage in all the soil and plant samples. The range-weighted richness (*Rr*) > 30 is typical of habitable environments with broad carrying capacity. Soil showed higher *Rr,* followed by root, stem and leaf. This implies decreased microbial diversity from soil to leaves; and leaves have relatively narrow carrying capacity than soil habitat. Similar results were observed by Ramond *et al.* (2013), Bodenhausen *et al.* (2013) and Wang *et al.* (2016) during their studies in sorghum, *Arabidopsis* and rice respectively. Soils are rich in carbon sources and other nutrients, which supports higher richness and diversity (Mendes *et al.*, 2013). On the other hand, plants represent relatively stable environment for microbial survival due to limited availability of carbon sources and other growth promoting factors (Moronta *et al*., 2018). Among all the samples herein, vegetative stage had higher *Rr* than flowering stage, implying broader carrying capacity at vegetative stage.

A Shannon diversity (H) index is a mathematical measure of species diversity in a community based on the species richness (the number of species present) and species abundance (the number of individuals per species) (Shannon, 1948). All the samples in current study showed moderate Shannon diversity index. In soil and root samples, the Shannon diversity index at vegetative stage was significantly higher than flowering stage samples; indicating more diverse bacterial population is present in soil and root during vegetative stage. This indicates that the microbial diversity (species richness and abundance) at flowering stage is lower than vegetative stage. Andreote *et al.* (2010) and Hussain *et al*. (2012) also reported reduced microbial diversity in potato and rice roots at reproductive stage as compared to vegetative stage. Edwards *et al*. (2018) investigated changes in the root-associated microbial communities throughout the plant life cycle in field-grown rice. Results indicated that root microbe composition varies with the developmental stage of the plants, suggestive of distinct root microbe associations for the juvenile and adult plant phases. The reduced microbial diversity at reproductive stage may be due to lesser rhizodeposition (Mougel *et al.*, 2006).

The Sorenson's similarity index indicates the percent of bacterial population that might have been shared between samples. In other words, it helped to determine the migration of bacterial population from rhizosphere to endosphere. The soil samples shared 28 to 35 % similarity with different plant compartments at both crop stages in current study. Wang *et al*. (2016) analysed the actinomicrobiome present in rice indicating more diverse OTUs were present in roots than in stems. The grains and stem samples shared 7 % commonness with root samples.

Conclusion

The carrying capacity of soil is higher than root, followed by stem and leaf. The vegetative stage carries more diverse microflora in terms of species richness and abundance in soil and root samples. There is huge similarity in bacterial population of rice rhizosphere and endosphere. Further studies on the identification of soil and plant bacterial communities would be a useful resource for improvement of soil and plant health.

References

Andreote FD, Ulisses NR, Welington LA, Joao LA and Overbeek LS. 2010. Effect of bacterial inoculation, plant genotype and developmental stage on root-associated and endophytic bacterial communities in potato (*Solanum tuberosum*). *Antonie van Leeuwenhoek*, 97: 389-399.

Bodenhausen N, Horton MW and Bergelson J. 2013. Bacterial communities associated with the leaves and the roots of *Arabidopsis thaliana*. *PloS one*, 8(2): e56329.

Chi F, Shen SH, Cheng HP, Jing YX, Yanni YG and Dazzo FB. 2005. Ascending migration of endophytic rhizobia, from roots to leaves, inside rice plants and assessment of benefits to rice growth physiology. *Applied and Environmental Microbiology,* 71(11): 7271-7278.

Edwards JA, Santos-Medelloan CM, Liechty ZS, Nguyen B, Lurie E, Eason S. 2018. Compositional shifts in root-associated bacterial and archaeal microbiota track the plant life cycle in field-grown rice. *PLoS Biology,* 16(2): e2003862.

Ferrando L, Manay JF and Scavino AF. 2012. Molecular and culture-dependent analyses revealed similarities in the endophytic bacterial community composition of leaves from three rice (*Oryza sativa*) varieties. *FEMS Microbiology Ecology*, 80(3): 696-708.

Gustavo CA and Gresshoff PM. 1994. Staining of nucleic acid with silver: an alternative to radioisotopic and fluorescent labelling. *Promega Notes*, 45:13-20.

Hussain Q, Pan GX, Liu YZ, Zhang A, Li LQ, Zhang XH and Jin ZJ. 2012. Microbial community dynamics and function associated with rhizosphere over periods of rice growth. *Plant Soil and Environment*, 58(2): 55-61.

Hutcheson K. 1970. A test for comparing diversities based on the Shannon formula. *Journal of Theoretical Biology*, 29: 151-154.

Kumar VKK, Reddy MS, Kloepper JW, Lawrence KS, Groth DE. and Miller ME. 2009. Sheath blight disease of rice (*Oryza sativa* L.) - An overview. *Biosciences Biotechnology Research Asia,* 6(2): 465-480.

Mano H and Morisaki H. 2008. Endophytic bacteria in the rice plant. *Microbes and Environment*, 23(2): 109-117.

Marzorati M, Wittebolle L, Boon N, Daffonchio D and Verstraete W. 2008. How to get more out of molecular fingerprints: practical tools for microbial ecology. *Environmental Microbiology*, 10: 1571-1581.

Mendes R, Garbeva P and Raaijmakers JM. 2013. The rhizosphere microbiome: significance of plant beneficial, plant pathogenic, and human pathogenic microorganisms. *FEMS Microbiology Reviews*, 37(5): 634-663.

Moronta BF, Gionechetti F, Pallavicini A, Marys E and Venturi V. 2018. Bacterial microbiota of rice roots: 16S-based taxonomic profiling of endophytic and rhizospheric diversity, endophytes isolation and simplified endophytic community. *Microorganisms*, 6(1): 14-33.

Mougel C, Offre P, Ranjard L, Corberand T, Gamalero E, Robin C and Lemanceau P. 2006. Dynamic of the genetic structure of bacterial and fungal communities at different developmental stages of *Medicago truncatula* Gaertn. cv. Jemalong line J5. *New Phytologist*, 170(1):165-175.

Murray MG and Thompson WF. 1980. Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Research*, 108(19): 4321- 4325.

Muyzer G, De Waal EC and Uitterlinden AG. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology*, 59: 695-700.

Naik BS, Shashikala J and Krishnamurthy YLA. 2009. Study on the diversity of endophytic communities from rice (*Oryza sativa* L.) and their antagonistic activities *in vitro*. *Microbiology Research*, 164: 290-296.

Nakatsu CH, Torsvik V and Ovreas L. 2000. Soil community analysis using DGGE of 16S rDNA polymerase chain reaction products. *Soil Science Society of America Journal*, 64: 1382-1388.

Pasha MA, More SA and Krishnaraj PU. 2020. Optimization of soil DNA extraction protocol using Na_2EDTA, SDS, heating, vortexing and $CaCl_2$ and its validation for metagenomic studies. *International Journal of Environment and Climate Change*, 10(6): 1-13.

Ramesh S and Mathivanan N. 2009. Screening of marine actinomycetes isolated from the Bay of Bengal, India for antimicrobial activity and industrial enzymes. *World Journal of Microbiology and Biotechnology*, 25: 2103-2111.

Ramond JB, Tshabuse F, Bopda CW, Cowan DA and Tuffin MI. 2013. Evidence of variability in the structure and recruitment of rhizospheric and endophytic bacterial communities associated with arable sweet sorghum (*Sorghum bicolor* (L) Moench). *Plant and Soil*, 372(1-2): 265-278.

Sessitsch A, Hardoim P, Doring J, Weilharter A, Krause A, Woyke T and Mitter B. 2012. Functional characteristics of an endophyte community colonizing rice roots as revealed by metagenomic analysis. *Molecular Plant Microbe Interactions*, 25(1): 28-36.

Shannon CE. 1948. A mathematical theory of communication: The Bell System. *Technical Journal*, 27: 379-423.

Sorensen T. 1948. A method of establishing groups of equal amplitude in plant sociology based on similarity of species and its application to analyses of the vegetation on Danish commons. *Kongelige Danske Videnskabernes Selskab*, 5(4): 1-34.

Sun L, Qiu F, Zhang X, Dai X, Dong X and Song W. 2007. Endophytic bacterial diversity in rice (*Oryza sativa* L.) roots estimated by 16S rDNA sequence analysis. *Microbial Ecology*, 55: 415-424.

Suryawanshi PP. 2018. Molecular analysis of rice - *Rhizoctonia solani* - actinobacteria interactions *Ph.D. Thesis*, University of Agricultural Sciences, Dharwad (India).

Takahashi H, Sekiguchi H, Ito T, Sasahara M, Hatanaka N, Ohba A, Hase S, Ando S, Hasegawa H and Takenaka S. 2011. Microbial community profiles in intercellular fluid of rice. *Journal of General Plant Pathology*, 77(2): 121-131.

Wang W, Zhai Y, Cao L, Tan H and Zhang R. 2016. Illumina-based analysis of core actinobacteriome in roots, stems and grains of rice. *Microbiology Research*, 190: 12-18.