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Isolation and Identification of Endophytes from Different Cultivars of Rice (*Oryza sativa* L.) under Wetland and Upland Conditions in South Assam

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Diversity of endophytic microbes both fungi and bacteria was evaluated from a wetland rice field in Karimganj district and upland rice field of Cachar district of South Assam. Three most popular varieties of Boro Rice of South Assam were selected for the study. *Cladosporium* sp, *Fusarium* sp, *Aspergillus* sp, *Penicillium* sp, and *Helminthosporium* sp., were identified and *Azospirillum* sp, *Herbaspirillum* sp, *Acetobacter* sp, *Enterobacter* sp and *Pantoea* sp were the bacterial endophytes identified in all the varieties in both the sites. Acetylene reduction assay showed that all the bacterial isolates are diazotrophic in nature. Comparison of the colonization rate revealed that wetland rice field and Mahsuri variety of rice harbored the highest diversity of endophytes. Maximum diversity of the endophytes was observed in root region followed by stem and leaves.

Key words: Diversity, Endophytes, Wetland and Upland rice field, Mahsuri variety, Acetylene reduction assay.

Rice is one of the primary cereal crop and is the main resources for more than two fifths of world population providing food security to the growing human population (Naik *et al*, 2009). One of the most important nutrients for increase in rice production is the availability of nitrogen which is supplied in the form of chemical fertilizers by the farmers. The yield of rice is also threatened with blast disease, sheath blight, bacterial leaf blight and rice bakanae disease. To get rid of such diseases fungicides are used by the farmers which in turn affect the ecological balance due to toxic residues of these chemicals also harmful to the health of human beings and other animals. There is a need to minimize the harmful effects of pesticides and fungicides and search for the ecofriendly alternative method. Endophytes are the organisms which survive inside the host tissues without producing any sympotoms. They are distributed in the root, stem and leaves and play a major role in physiological activities of host plants enhancing stress, insects, nematodes and disease resistance, and improvement of productivity (Shimizu *et al*., 2001). These can be an efficient biological control agent, good source of improvement of plant growth and nitrogen fixation in host plants. (Verma *et al*. 2001; Rahman & Saiga, 2005). Endophytic fungi produce aromatic compounds such as 2 Acetyl-1-pyrolene(2-AP) the major compound contributing jasmine like odour in cooked rice. Cho *et al*, (2007) have reported that

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the virulence of *Burkholderia glumae*, a casual pathogen of seedling rot and grain rot of rice is controlled by a different species of the same genus named *Burkholderia* sp. KJ006 possessing an N-acyl-homoserine lactonase (aiiA) gene. Some rhizobial strains also showed resistence towards a rice pathogen *Rhizoctonia solani* causing the sheath blight disease in rice. (Mishra *et al*., 2006).

Boro rice cultivation in Assam is practiced under waterlogged low-lying or medium lands with irrigation during November to May. It takes advantage of residual moisture in lands after harvest of *Kharif* paddy, longer moisture retention capacity of the soils, and surface water stored in nearby low-lying ditches, areas adjoining canals, roads, *chaur* lands and *Tal* lands. It is a short duration variety of rice and is commonly cultivated in south assam. The present study is an attempt of comparative analysis of the diversity of endophytic population growing in wetland and upland areas of south assam.

MATERIALS AND METHODS

Study site

Rice plants are collected from two sites of barak valley, one from upland rice field and the other from wetland rice field during the period of Decembar 2010 to April 2011. Site 1 was in Karimganj district, (between longitudes 92°15' and 92°35' east and latitudes 24°15' and 25°55' North) and site 2 was in Cachar district, (longitude 92.9167 and latitude 25.0833). Three varieties Boro68, Mahsuri and Krishna of Boro season mostly cultivated in Assam were selected for the study, Ten plants from each variety/site/season were collected, brought to the laboratory and processed within 12 hours of collection. From each plant 10 root and 10 stem fragments were analysed.

Isolation of endophytes

Stem and roots of different rice varieties were collected and cleaned under running tap water and cut into 1 cm segments. Surface sterilization was done with 70% ethanol for 20 min, sodium hypochlorite solution (2% available chlorine) for 5 min and 70% ethanol for 30s followed by two rinses in sterile distilled water. For isolation of fungal endophytes, the segment were placed on 9 cm petri plates containing Czapek's Dox agar medium for fungal growth amended wih streptomycin (250 mg/L) to slow down the bacterial growth. For isolation of bacterial endophytes the tissues were aseptically cut into small pieces and macerated in sterile water. Serial dilution were prepared and spread on plates containing NA medium (Koomnok *et. al.,* 2007). Petri plates were incubated in a light chamber at 28 ± 1°C for 1 – 12 days. The well separated colonies were then purified several times by repeated streaking to obtain pure culture and stored at 4°C.

Effectiveness of surface sterilization:

Effectiveness of the surface sterilisation was carried out using the method of Song *et al*., (1999) and Schulz *et al*. (1993).

Identification of endophytic isolates

Endophytic fungi were identified on the basis of cultural characteristics and morphology of fruiting bodies and spores. Isolation percentage was calculated following Hata and Futai (1996). Endophytic bacteria were characterized morphologically and biochemically and were finally identified following Bergey's manual of systematic bacteriology. (Kreyg *et al*., 1994)

Acetylene Reduction Assay

Nitrogenase activity of bacterial strains was determined in semisolid nitrogen free malate medium (by using acetylene reduction assay, HARDY *et al*. 1973). Pure bacterial colonies were inoculated on to NFM medium in Mc Cartine vials of 10 ml capacity and incubated at 28 ± 2 °C for 48 h. Acetylene (10% vol/vol) was injected to the vials. After incubation for 16 h at 28 ± 2 °C, gas samples (100 ìl) were analyzed on a gas chromatograph (Carlo Erba-Model GC 180-series 2150) using Porapak-Q column and H2 flame ionization detector.

RESULTS AND DISCUSSION

1 ml of flow out water after surface sterilization was inoculated in PDA and NA plates. The plates were then incubated at 25°C for 2 weeks. Results of viability have shown that 25 viable fungal and bacterial cultures were removed by surface sterilization,confirming that epiphytes were eradicated and the isolates were only endophytes.

Most of the endophytic taxa in this study have been reported as endophytes from previous studies on rice plants. (Tien *et al*.,2004; Naik *et al*.,

2009). The diversity of both the endophytic bacteria and fungi was found higher in the varieties growing in wetland rice field than upland rice. There were also significant differences in the endophytic counts in different rice varieties and plant age. Among all the varieties studied Mashuri variety showed the highest endophytic count during the vegetative stage followed by the Boro 68 and Krishna variety of rice. Previous study in this area reported that cultivars and plant growth stage were strong determinants of microbial community. (Naher *et.al*, 2009). Colonization rate of both the endophytic fungi and bacteria varied at a significant value in both the sites also. This fact is also reported by Barraquio *et al*, (1997). The process of nitrogen fixation is oxygen sensitive in most of diazotrophs. So the growth conditions might be most favorable in anoxic flooded soil with microaerobic niches resulting from air flow through the aerenchymatic root tissues. (Armstrong, 1978; Hurek, 2000). A total of 80 endophytic fungi were isolated from Site I and 65 from site II. The fungi belong to the genus *Cladosporium*, *Fusarium*, *Penicillium , Aspergillus and Helminthosporium*. Among all the isolates *Cladosporium* and *Fusarium* were found from both the sites. *Penicillium , Aspergillus* and *Helminthosporium* were found in higher number in site I. Microclimatic factors may affect the rate of infection of endophytes. (Carroll, 1995).

Table 1. Percentage of endophytic fungal population from rice plants.

Endophyic fungal isolates	Site I			Site II		
	Mahsuri	Boro 68	Krishna	Mahsuri	Boro 68	Krishna
Cladosporium sp.	43	32	45	50.6	61.2	56.6
Fusarium sp.	42.5	36.6	39.3	33.6	19.5	29.3
Penicillium sp.	0	10.3	16.5	15.5	0	0
Aspergillus sp.	25.5	15.9	0	0	12.3	10.9
Helminthosporium sp.	15.0	17.3	10.0	0	12.9	11.0

The endophytic fungi varied significantly in different varieties of rice ($p<0.05$).

There was a significant difference between the occurrence of the endophytic fungal isolates in different tissues of the plant from in both the sites ($p<00.1$). In both the sites *Fusarium* sp. was found dominate in the roots (60.0%), *Cladosporium* sp. was found dominant as shoot endophyte (46.6%). *Helminthosporium* sp. was found only in the stem and leaves but not in the roots. Occurrence of *Fusarium, Aspergillus* and *Penicillium* in the roots was also reported by Tien *et al*., (2004). Previous reports have indicated that colonization of endophytes was higher in root than in shoot. (Fisher *et al*., 1994; Rodrigues *et al*, 1994; Photita *et al*, 2001; Stierle *et al*, 1993). The difference in endophytic occurrence in different tissue types might be a capacity of the endophyes to utilize the substrates (Rodrignes, 1994) in addition to the physicochemical factors of the tissue.

Table 2. Average percentage of fungal endophytes isolated from different tissues of rice plant

Communities of fungal endophytes	Leaves	Roots	Stem
Cladosporium sp.	35.5	35.6	60.0
Fusarium sp.	29.9	46.6	38.6
Penicillium sp.	14.2	22.2	19.0
Aspergillus sp.	18.2	35.2	28.2
Helminthosprium sp.	6.0	0	8.0

Significant difference in endophytic fungal count in different tissues ($P<0.01$)

The number of bacteria varied between 5.2×10^5 to 1.1×10^5 cfu per gram fresh weight of plant parts inoculated. The highest count was observed in the roots ranging from 10.3×10^5 to 1×10^5 cfu per gram fresh weight in Mahsuri rice

Table 3. Average number of colony (cfu/ml) X 10^5 bacterial isolates in NA plates (means of three replicates, ±standard error) from rice varieties.

Sites	Plant Tissue	Seedling	Vegetative	Reproductive
Site I	Root	2.3±0.09	10.3±0.09	4.3±0.09
	Stem	2.03±0.09	7.3±0.09	2.3±0.09
	Leaf	1.3±0.09	4.3±0.09	1.1±0.09
Site Ii	Root	1.3±0.09	6.3±0.09	3.3±0.09
	Stem	1.03±0.09	4.1±0.09	2.0±10.09
	Leaf	0.3±0.09	3.3±0.09	1.3±0.09

Number of endophytes varied at a singnificant rate in different growth stages of rice ($p<0.01$).

Table 4. Biochemical characteristics of diazotrophic bacteria isolated from various tissues of the rice plant

Isolate name	Characteristics	ARA activity (nmole/mg protein/hour)	Genus
EI1,EI4,EI5,EI2,EI3,EI7, EI9,EI12,EI16,EI15,EII5, EII3,EII12,EII13,EII17, EII29,EII20,EII41	Gram negative rod, motile, oxidase and catalase positive, large white colony and slimy in nitrogen free medium.	457± 0.9	*Aspergillus* sp.
EI32,EI33,EI34,EI35,EI39, EI41,EI40,EII7,EII6,EII8, EII9,EII10,EII14,EII18	Gram negative, short curved rods, oxidase and catalase positive, small a white colony, slimy on nitrogen free media.	190± 0.8	*Herbaspirillum* sp.
EI22,EI21,EI24,EI25, EI44,EI33,EII1,EII2	Gram negative, rods, slightly curved, motile, oxidase negative, catalase positive, capable of gelatin liquification. Small white colony in nitrogen free media.	220.26± 0.2	*Acetobacter* sp.
EI6,EI8,EI9,EII49,EII40.	Gram negative, rod shaped, facultatively anaerobic, motile, oxidase negative and catalase positive, capable of producing acid from glucose, sucrose, lactose. Production of white small flat colony in nitrogen free media.	24.8 ± 0.2	*Enterobacter* sp.
EII30,EII38,EII31, EI35,EI27	Gram-negative rods. Anaerobic, Catalase -positive and oxidase-negative, Small colonies greyish to yellow in colour, in nitrogen free media.	5.601 ± 0.5	*Pantoea* sp.

variety. In stem tissue sample the number of bacteria ranged between 7.2 X 10^3 to 1.0 X 10^5. Lowest number of bacterial colony count was observed in the leaf tissue of Krishna variety ranging between 2.3 X 10^3 to 3.5 X 10^5 per gram fresh weight. 50 isolates are purified by repeated streaking. From morphological and biochemical characterization of the isolates five genera of bacteria were identified. 18 isolates were belonging to the *Azospirillum* genus, 14 isolates belong to the *Herbaspirillum* genus, 8 isolates belonging to the *Acetobacter* sp, 5 isolates belong to *Pantoea*

sp and 5 isolates belong to *Enterobacteria* sp. The bacterial isolates are able to produce cellulase, a enzymatic activity. Since pectinolytic activity has been proposed to be responsible for plant invasion by endophytes, so the production of cellulase and pectinase by bacterial isolate proves its endophytic nature. All the bacterial isolates were capable of reducing acetylene indicating their nitrogen fixing ability as plant growth promoting rhizobacteria (PGPR).

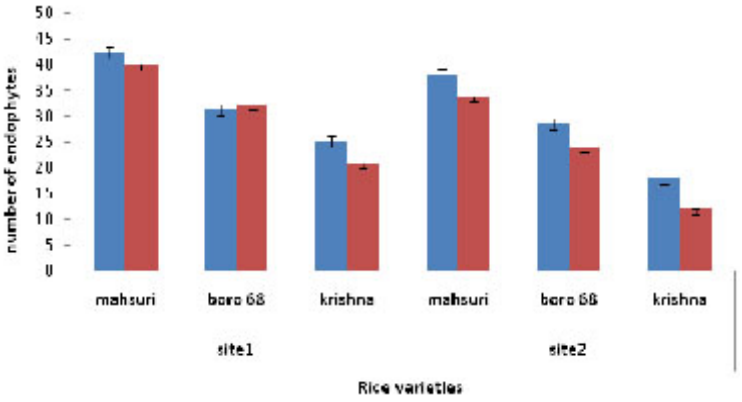

Fig 1. Colonization rate of endophytic fungi and percentage of diazotrophic bacterial isolates in all the three variety in both the sites varied significantly ($p<0.05$).

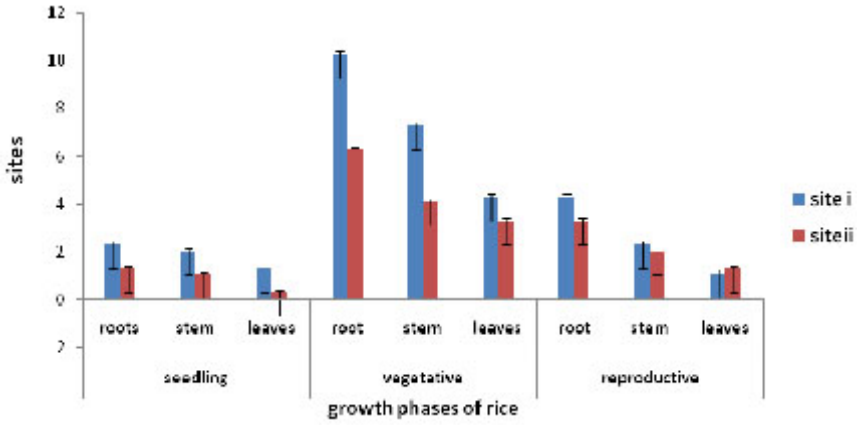

Fig. 2. Population of endophytic bacteria from different rice tissue of both the sites at different growth stages. The endophytic bacterial count significantly affected ($p<0.05$) by the type of tissue ($F=9.207704$) and the type of the soil ($F=13.32432$)

CONCLUSION

The surface sterilization viability test proved the efficacy for the isolation of endophytic microbes. Wetland rice fields harbored the higher number of endophytes than the upland ones. Root tissues contained the higher diversity of endophytes than other tissues. All the endophytic bacterial isolates were nitrogen fixing diazotrophs in nature as PGPR. The fungal isolates were reported to show antagonistic activity, (Naik *et al*, 2009).

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