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Long-term aromatic rice cultivation effect on frequency and diversity of diazotrophs in its rhizosphere

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ABSTRACT

Diazotrophs are one of the most important microbial communities which play a key role in rhizosphere to enhance plant growth-promotion by supplying fixed nitrogen to the plants. The aim of present study was to analyze the frequency and diversity of diazotrophs in the rhizospheres of five genotypes of aromatic rice (*Oryza sativa* cultivars Nua Kalajeera, Ketekijoha, Nua Desheri, Nua Chandan and Kala Namak) grown in ten years old long-term aromatic rice-rice (LARR) cropping system under sub-humid tropical condition. In this study, it was observed that very less frequency of active diazotrophs (0–14.28%) in the rhizosphere of aromatic rice genotypes based on polymerase chain reaction (PCR)-based *nifH* gene amplification, dot blot hybridization and acetylene reduction assay (ARA). The following five isolates (B10, B12, B29, BK16, NKR16) from rhizosphere of aromatic rice and the reference strain *Azospirillum brasilence* showed *nifH* amplification and positive signal for dot blot. None of these isolates showed positive in ARA test except *A. brasilence*. Basic local alignment search tool (BLAST) homology of *nifH* sequences of B10, B12, B29, BK16 and NKR16 matched with *Gluconoacetobacter diazotrophicus*, *Pseudomonas stutzeri*, *Klebsiella* sp., *Klebsiella pneumoniae* and *Sinorhizobium meliloti*, respectively. Whereas, isolates B10, B12 and BK16 were identified as *Bacillus cereus, Enterobacter* sp., and *Lysinibacillus* sp., respectively based on 16S-rDNA sequencing. As per *nifH* sequences, these diazotrophs (B10, B12, B29, BK16, NKR16) fall under α and γ-proteobacter, whereas 16S-rDNA sequences revealed that they belonged to firmicutes and γ-proteobacter. Multiple sequence alignment of *nifH* gene sequences showed wider variation among diazotrophs, however 3D structure of NifH protein revealed the less diversity among isolates. Moreover, restriction digestion of 16S-rDNA using tetracutters (*MspI, MboI and HaeIII*) deciphered a unique pattern among small population of diazotrophs.The results of this finding provided an interesting data and novel information of diazotroph diversity in LARR cropping system. Finally, the present study indicated that the continuous application of high dose of inorganic nitrogenous fertilizers (nitrogen at the rate of 60 & 80 kg N ha^{-1} $year^{-1}$ in wet and dry seasons, respectively) limit the frequency and diversity of rhizospheric diazotrophs in the LARR cropping system.

1. Introduction

Aromatic rice is one of the most popular cultivar among rice (*Oryza sativa* L.) in Indian sub-continent. It may be due to many special characters like long grain, delicious taste and presence of characteristic subtle aroma (2-Acetyl-1-pyrroline) (Kumar et al., 2012, 2013b). India is the largest producer and exporter of aromatic rice in the world which accounts 70% (3.7 million metric tones) of basmati rice production in 7.76 million hectares and contributes 194 million rupees (2.85 million US dollar) in rice export (NBHC, 2015). Overuse of nitrogenous fertilizers in aromatic rice leads to robust plant foliage, tall and weak stature of plant which causes lodging, poor response of the fertilizers, reduces the spikelets per panicle and susceptible towards pest and diseases (Bashyal et al., 2016; Kumar et al., 2013a,b; Singh et al., 2016). One of the possible alternatives to mitigate the above problems is exploring the possibility of using beneficial microbes particularly diazotrophs (Govindasamy et al., 2008, 2010; Kumar and Dangar, 2013; Kumar et al., 2013a, 2014, 2016a,b; Kumar and Mishra, 2014).

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Fixed nitrogen is often the limiting factor of productivity of aromatic rice and therefore, affects plant communities and ecosystems (Tan et al., 2003). In order to boost the yield of aromatic rice, the inorganic nitrogeneous fertilizers are generally used. However, indiscriminate use of these nitrogenous fertilizer in wet land rice can lead to nitrate pollution of surface or ground water (Tang et al., 2012). Therefore, the special attention to be given on biological nitrogen fixation to identify the major taxonomic groups of bacteria which may considerably reduce the use of inorganic fertilizers input in aromatic rice cultivation.

Biological nitrogen fixation (BNF) is carried out by prokaryotic organisms containing a nitrogenase enzyme complex that is encoded by *nifHDK* genes (de Brujin, 2015). The *nifH* gene encodes the iron (Fe)-protein subunit of nitrogenase and is highly conserved among all nitrogen fixing microorganisms (Liu et al., 2012). It is a useful marker in culture-dependent and independent studies, to offer evidence for potential nitrogen fixation in diverse terrestrial environments (Coelho et al., 2008). An analysis of the indigenous diazotrophs community in diverse paddy soil, the *nifH* gene, is a commonly used marker for studying the assemblage, diversity and activity of diazotrophs (Zehr et al., 2003). Polymerase chain reaction (PCR) amplification of the *nifH* gene of community DNA and sequencing gives information on the diazotrophs composition in an environment (Demba Diallo et al., 2008; Poly et al., 2001) to genus and group specific (Huang et al., 1999). Few reports also revealed a strong correlation between the *nifH* and 16S rRNA genes for the purposes of bacterial genus identification (de Brujin, 2015).

Several N_2 fixing bacteria have been isolated from rice fields (Vaishampayan et al., 2001; Xie et al., 2003; Park et al., 2005; Singh et al., 2006; Mårtensson et al., 2009) and strains of *Azospirillum* sp ., *Azotobacter* sp., *Herbaspirillum* sp. *and Burkholderia* sp. have been tested and found suitable for use as bio-fertilizers (Chowdhury et al., 2007). Other studies have demonstrated the presence of nitrogen-fixing organisms in other crops like sugarcane (*Saccharum officinarum*) (James et al., 2001), maize (*Zea mays*) (da Silva et al., 2003) and winter wheat (*Triticum aestivum*) (Pedersen et al., 1978).

Based on the extensive survey of scientific literatures, there is no information on the effects of long-term nitrogen fertilizer use on the diazotrophic frequency and diversity in aromatic rice-rice cropping system in sandy clay loam soil under sub-humid tropical condition. Therefore, in the present study, the *nifH*-PCR, dot blot hybridization and acetylene reduction assay (ARA) techniques were adopted to identify the frequency of active diazotrophs from the rhizosphere of five genotypes of aromatic long-term rice-rice cropping system at ICAR-National Rice Research Institute (NRRI) research farm, Cuttack, India and further used different molecular tools to assess the genetic diversity of selected diazotrophs.

2. Materials and methods

2.1. Study site

The experimental site is situated at ICAR-National Rice Research Institute (NRRI), Cuttack, India (latitude 20° 45′ 08″ N, longitude 85° 93′ 29″ E and mean sea level 24 m). This experiment site is exclusively allotted for cultivating aromatic rice in both wet and dry seasons since 2001. The experiment was laid out in a randomized complete block design comprising five aromatic rice cultivars (*Oryza sativa* var. Nua Kalajeera, Ketekijoha, Nua Desheri, Nua Chandan and Kala Namak) with three replications. Fertilizers were applied as per the treatments requirement at the rate of 60–40–40 and 80–40–40 kg ha^{-1} N-P_2O_5-K_2O in the form of urea, single super phosphate and murate of potash for wet and dry seasons, respectively. Mean annual temperatures fall in the range of 22.5–39.2 °C.

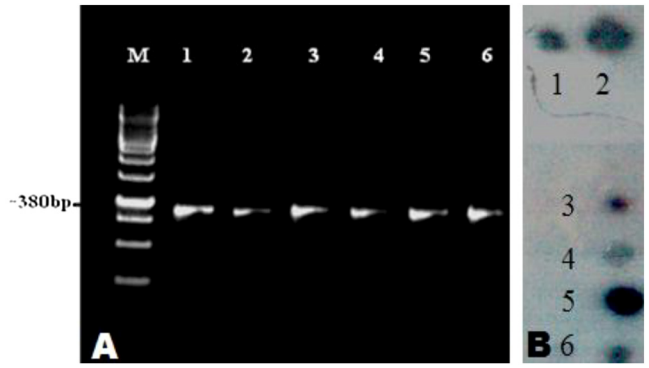

Fig 1. (A) PCR amplification of *nifH* gene isolates of different diazotrophs from aromatic rice rhizosphere (B) Dot blot hybridization using *nifH* gene probe (M: 100 bp ladder; 1: *Azospirillum brasilense*; 2: B10; 3:NKR16; 4:B29; 5:BK16 & 6: B12).

Annual precipitation is 1500 mm of which 75–80% is received during June to September. The texture of soil at the experimental site falls under Aeric Endoaquept soil type (31% clay, 17% silt and 52% sand). Physico-chemical properties of the experimental field was as follows *viz.,* bulk density (BD) 1.40 Mg m^{-3}, cation-exchange capacity 15.2 cmol (p+) kg^{-1}, pH 6.2, organic carbon 0.66%, available-N 151 kg ha^{-1}, available-K 105 kg ha^{-1} and available-P 31 kg ha^{-1}.

2.2. Isolation of diazotrophs in aromatic rice rhizosphere

Rhizosphere (soil that adheres to roots) soils were collected at the depth of 0–20 cm from treatments comprising five different varieties of aromatic rice at panicle initiation stage. In each treatment, five soil samples were collected and mixed to form a composite sample. Immediately after sample collection, a part of the soil sample was air-dried and ground, then passed through a 2 mm sieve. Organic carbon, available-N, P, K and pH of soil were analyzed by using standard protocols (Bray and Kurtz, 1945; Piper, 1966; Subbiah and Asija, 1956). Remaining soil samples were stored in refrigerator at 4 °C for enumeration of diazotrophs. The diazotrophs were isolated by using N-free diazotrophic (NFD) medium (Stella and Suhaimi, 2010). Briefly, rhizospheric soils (10 g in 90 ml, 0.85% sterile saline distilled water) suspensions were diluted to 10^{-2}–10^{-6} level, 100 μl suspension was spread plated on NFD medium and maintained three replications for each dilutions. The plates were incubated at 30 ± 0. 1 °C for 3–7 days in a bacteriological incubator and the numbers of colonies were counted. Distinct colonies were isolated, purified and sub-cultures for more than six times in NFD medium.

2.3. Polymerase chain reaction (PCR) amplification and dot-blot hybridization of nifH gene

Rhizospheric isolates were grown in 5 ml Luria Bertani (LB) broth and incubated at 30 ± 2 °C for 24 h. Genomic DNA was extracted based on phenol-chloroform method (Masterson et al., 1985) and resuspended it in 50 μl 1× TE (Tris-EDTA) buffer. Further, the quality of genomic DNA was checked on 0.8% agarose gel (containing 0.5 μl ml^{-1} ethidium bromide (Etbr)) and visualized under UV light (Alfa Infotech corp.). Polymerase chain reactions (PCR) were carried out to detect the presence of *nifH* gene. Reference strain *Azospirillum brasilence* (JQ952670) received from Indian Agricultural Research Institute (Division of Microbiology), New Delhi, India, was used as positive control for *nifH* gene amplification at amplicon size ~ 380 bp. PCR amplification of *nifH* gene was done by using a thermal cycler (Eppendorf, Master cycler gradient). 25 μl PCR reaction mixture was prepared by adding 4 ng genomic DNA per μl of reaction, 10 x