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Full Length Research Paper

Identification of *nif* genes of heterotrophic and endophytic diazotrophs associated with rice (*Oryza sativa* L.,) by targeted DNA finger printing

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Heterotrophic and endophytic diazotrophs were isolated from rhizosphere soil, rhizoplane, roots and stems of different rice varieties. A total of thirteen isolates obtained were subjected to acetylene reduction assay (ARA) and eight isolates recorded significant amount of nitrogenase activity in a range of 31.65 to 91.95 nmoles of ethylene mg^{-1} cells h^{-1}. Targeted PCR fingerprinting using *nif* H primers generated specific DNA band of approximately 750 bp, confirming the presence of *nif* genes in these isolates. Two isolates of heterotrophic diazotrophs, HDM 7 and HDT 1, generated multiple bands ranging from 500 – 1000 bp.

Key words: Acetylene reduction assay, endophytes, heterotrophs, *nif* gene, targeted PCR fingerprinting.

INTRODUCTION

Symbiotic N_2 fixation by legumes is generally expected to be the dominant source of biological of N input in the earth. In recent years, N balance and ^{15}N techniques have provided convincing evidence that non-legumes such as wetland rice can, under certain conditions, derive a considerable amount of N from associated N_2 fixing bacteria in the plant rhizosphere (Chalk, 1991; Mark and Craswell, 1992). In non-legume agro systems, rice fields are considered to be ideal niches for biological nitrogen fixation because of their characteristic ecological conditions. It is well known that a remarkable diversity of N_2 fixing bacteria are naturally associated with field-grown rice (Balandreau, 1986). The free living heterotrophic N_2 fixers are a potentially important source of N_2 fixation in rice fields (Boddey et al., 1995, Mahadevappa and Shenoy, 2000).

The recent isolation and study of endophytic nitrogen fixing bacteria from several grasses (Baldani et al., 1997) represent an exciting phase in the field of biological nitrogen fixation. The search for natural association and endophytic interaction of diazotrophs with rice is considered very promising especially in primitive rice varieties not bred to efficiently respond to N fertilizer (Barraquio et al., 1997; Stoltzfus et al., 1997). Endophytic diazotrophs usually live with in the root apoplast that is the intercellular spaces and or the xylem vessels, and may enter the plants *via* the root or epidermal cracks at lateral root junctions and might interact more closely with the host with less competition for carbon sources and a more protected environment for N_2 fixation (Reinhold and Hurek, 1998).

Nitrogenase, the enzyme highly essential for reducing nitrogen to ammonia, is composed of Fe (dinitrogenase) and Mo-Fe protein (dinitrogenase reductase) is encoded by *nif* gene. A substantial molecular diversity of N_2 fixing bacteria has been detected in field grown rice based on retrieval of *nif* H or *nif* D gene fragments from root DNA (da Rocha et al., 1986). Since the *nif* H gene only occurs in nitrogen fixing microorganisms, it has been used to monitor the presence of these diazotrophs, for example, in pure cultures (Frank et al., 1998), in soil (Widmer et al., 1999) and plants. In view of the above, this study was aimed to identify the diazotrophic nature of bacteria isolated from rice rhizosphere soil of different locations and popular rice cultivars of Tamil Nadu, India, using targeted PCR finger printing with *nif* H primer.

MATERIALS AND METHODS

Isolation of heterotrophic bacteria from the rhizosphere

Heterotrophic diazotrophs were isolated from rhizosphere soil sam-

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Table 1. Nitrogenase activity of the heterotrophic and endophytic diazotrophic isolates.

S/N	Isolate	Nitrogenase activity (n moles of ethylene mg^{-1} of cells h^{-1})
1	HDC 4	0.00
2	HDPY 1	61.8
3	HDPY 2	0.00
4	HDMY 2	0.00
5	HDT 1	53.4
6	HDC 8	91.8
7	HDM 7	53.1
8	EDA 1	91.95
9	EDC 5	68.05
10	EDA 2	31.65
11	EDM 2	53.75
12	EDC 3	0.00
13	EDC 6	0.00

HD = Heterotrophic diazotrophs; ED = endophytic diazotrophs.
The isolates are subjected to acetylene reduction assay. Acetylene was converted to ethylene by the organisms due to the presence of nitrogenase enzyme and the amount of ethylene was quantified in a gas chromatography.

ples collected from different rice growing regions in Tamil Nadu State, India using the medium for total diazotrophs as standardized by Watanabe and Barrquio (1979) consisting of (per liter) glucose (0.5 g), malic acid (0.5 g), yeast (0.1 g), K_2HPO_4 (0.5 g), $FeSO_4$ (0.04 g), $MgSO_4$ (0.02 g), $NaMoO_4$ (trace), $CaCl_2$ (0.2 g), boric acid (0.15 g), $ZnSO_4$ (0.07 g), $CuSO_4$ (trace), $MnCl_2$ (trace), agar (20 g) and pH 7.0.

Isolation of heterotrophic diazotrophs from rhizoplane

To isolate the diazotrophs from rhizoplane rice seedlings were uprooted carefully from the field and the shoot portion was cut with sterile scissors. The excised root system along with the soil particles were washed carefully in a beaker containing water without disturbing the root system. One gram of roots washed free of soil was transferred to 10 ml of distilled water, serially diluted upto 10^{-6} dilutions and plated in Watanabe and Barraquio (1979) medium for isolation of total heterotrophic diazotrophs.

Isolation of endophytic bacteria

Plant samples of five different rice varieties (ADT 38, ADT 39, White ponni, ASD 16 and TPS 1) were collected and washed with tap water to remove all soil particles and the stems, roots and leaves were cut with sterile scissors. The roots and leaves were surface sterilized with 70% ethanol for 5 min and then treated with 0.1% $HgCl_2$ for 30 s. The stems were further cut into small bits, surface sterilized by dipping in 70% ethanol and then treated with 0.1% trypic soy agar (TSA plates). They were then homogenised, under sterile conditions with pestle and mortar in phosphate – buffered saline. The serially diluted homogenate was poured in tubes with N-free medium (Dobereiner and Day, 1976) consisting of (per liter) malic acid (5 g), K_2HPO_4 (0.5 g), $MgSo_4$, 7 H_2O (0.2 g), NaCl (0.1 g), $CaCl_2$ (0.2 g) and 0.5% bromothymol blue in 0.2 N KOH (2 ml), 1.64% Fe-EDTA solution (4 ml) and agar (2 g). The final pH was adjusted to 7.0 by 0.1 N KOH.

Purification and maintenance of cultures

Single, well separated and morphologically, different colonies growing on the plate were picked and purified by streak plate method. The purified colonies were transferred into agar slants and mass multiplied in the respective medium. Thirteen isolates were obtained and were used for further studies. The slant cultures were stored at 4°C. The isolates were designated according to the location from which it was isolated. The first two letters of the diazotrophic isolate names denote heterotrophic (HD) and endophytic (ED) and the remaining letters represent the location from which it was isolated with the numerals indicating the isolate number from each location.

Diazotrophy of the isolates

Diazotrophy of the isolates were determined on the basis of ARA and *nif* H gene amplification by PCR.

i) Assay of acetylene reduction activity (ARA)

The ARA was carried out for all the thirteen isolates grown in N free medium (Watanabe and Barraquio, 1979) for total diazotrophs by following the method of Hardy et al. (1968).

ii) Identification of the nif genes of diazotrophs using nif H primer

The total genomic DNA of the diazotroph was isolated using the method of Chen and Kuo (1993). The respective cultures were grown till log phase and bacterial cells were harvested by centrifugation for 5 min at 6000 rpm at 4°C. To the cell pellet, 200 µl of washing buffer (0.15 M NaCl 7.5 ml, 0.1 M EDTA 10 ml and the final volume made upto 50 ml) was added, thoroughly vortexed and again centrifuged at 6000 rpm at 4°C for 5 min. Ten per cent SDS was added to the pellet and again spun at 8000 rpm for 10 min at 4°C. The top aqueous layer was transferred to new tubes, added with 2.5 volume of cold ethanol and 1/10 volume of 3 M sodium acetate and then the contents were precipitated at-20°C overnight. After centrifugation at 12,000 rpm at 4°C for 5 min, the pellet was washed with 100 µl of 70% ethanol, again spinned at 12000 rpm at 4°C for 5 min and the pellet was dissolved in 50 µl TE buffer (Tris HCL 10 mM 2 ml, EDTA 1 mM 0.4 ml and volume made upto 100 ml) and stored at 4°C. The quality of DNA obtained was checked by loading 5 µl DNA on 0.8% agarose gel, and viewing the lands in UV transilluminator.

3 µl of this DNA was used as the template for amplification of *nif* H by PCR with primers (5' GTTTTACGGCAAGGGCGGTATCGGCA-3' and 5'-TCCTCCAGCTCTCCATGGTGATCsG-3'). PCR wa carried out in a PTC-100™ programmable thermocycler with an initial denature-tion for 5 min at 94°C, then 35 cycles of 1 min at 94°C, 1 min of 50°C, 2 min at 72°C and a final extension for 10 min at 72 and 4°C for storage.

RESULTS AND DISCUSSION

Among the 13 isolates subjected to ARA, nitrogenase activity was not detected in 5 isolates. All the other 8 isolates recorded significant amount of nitrogenase activity in a range of 31.65 to 91.95 moles of ethylene as depicted in the Table 1. A maximum of 91.95 n moles of ethylene mg^{-1} cells h^{-1} was exhibited by the isolate EDA 1 followed by HDC 8 (91.8 n moles of ethylene mg^{-1} cells

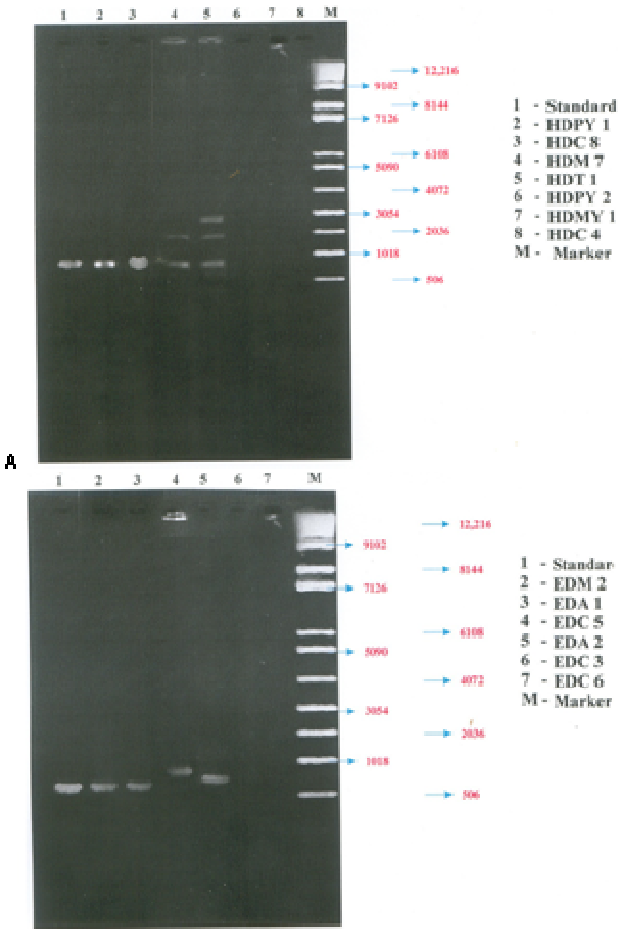

Figure 1. Characterization of *nif* genes of heterotrophic and endophytic diazotrophs using nif H primers.

h^{-1}), HDPY 1, HDT 1, HDM 7, EDC 5, EDA 2 and EDM 2 recorded a maximum of 61.8, 53.4, 53.1, 68.05, 31.65 and 53.75 n moles of ethylene mg^{-1} of cells h^{-1} respectively.

Investigation on the nitrogen fixing capabilities based on acetylene reduction activity of the heterotrophic and endophytic isolates of rice revealed that 57.14 per cent and 66.66 per cent were nitrogen fixers respectively. Some strains isolated in this study showed relatively low ARA. This could have been due to the reason that the O_2 level was not controlled during incubation and diazotrophs have a varying ability to tolerate O_2 (Postgate, 1998).

Identification of diazotrophs using *nif* gene

The DNA extracted from different cultures was resolved in 0.8% agarose gel. The amplified DNA was found to be in the range of 15 to 20 kbp. The *nif* H primers produced informative and reproducible genetic markers in standard *Rhizobium* culture (BMBS 1) and eight bacterial isolates, confirming their diazotrophic nature. The molecular weight of the replicon was approximately 750 bp for the standard culture, HDPY 1, HDC 8, EDM 2, EDA 2, EDC 5 and EDA 1 (Figure 1). Two isolates of heterotrophic diazotrophs, HDM 7, and HDT 1 generated multiple DNA bands ranging from 500-1500 bp. The remaining 5 isolates did not generate any replicon.

Rapid and unambiguous identification of diazotrophs has greatly benefited from recent advances in DNA fingerprinting based on polymerase chain reaction (PCR). Though short-term kinetic measurements such as Acetylene Reduction Assay (ARA) and reduction of $^{15}N_2$ or $^{13}N_2$ are available, they are not reliable and also more time consuming (Rennie and Rennie, 1983). In the present investigation, targeted PCR fingerprinting (TPF) using *nif* primers was used to as a molecular tool to identify the *nif* H gene of the selected isolates.

Identification of specific replicon with a molecular weight of approximately 750 bp in HDPY 1, HDC 8, EDM 2, EDA 1, EDC 5 and EDA 2 confirmed the presence of *nif* genes in the isolates. Perrett and Broughton (1998) employed TPF using primers specific for the *nif* H and *rec* A genes to discriminate between *Rhizobium* species NGR 234 and *R. fredii* USDA 257, the closely related bacteria in which the symbiotic loci are 98% homologous. Incase of HDT 7 and HDT 1, multiple replicons were formed, this might be due to minute variations in the concentration and purity of template DNA in these culture as observed by Berg et al. (1994).

The diazotrophs has been intensively studied in the last few decades because of their widespread ecological and knowledge of the physiological characteristics of *nif* H will be highly useful to study the diversity and role that these diazotrophs play in the environment.

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