MOLECULAR CHARACTERIZATION OF *NIF* GENES OF HETEROTROPHIC AND ENDOPHYTIC DIAZOTROPHS IN RICE

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Abstract

Diverse N_2 fixing microorganisms (aerobes, facultative anaerobes, heterotrophs, phototrophs) grow in wetland rice fields and contribute to soil N pools. The recent isolation and study of endophytic nitrogen fixing bacteria from several grasses represent an exciting period in the field of biological nitrogen fixation. 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. Targetd PCR finger printing of heterotrophic and endophytic diazotrophs from rice, using nif H primer, generated specific replicon with a molecular weight of approximately 750 bp. However, multiple replicons with molecular weight ranging from 500-1500 bp were observed in some isolates. The existence of considerable genetic and molecular diversity in the diazotrophic bacteria of rice and the scope for its better exploitation to achieve sustainable rice production.

INTRODUCTION

Symbiotic N_2 fixation by legumes is generally expected to be the dominant source of biological 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 nitrogen from root associated N_2 fixing bacteria in the plant rhizosphere (Mark and Crasswell, 1992). In non-legume agrosystems, 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 is naturally associated with field-grown rice (Balandreau, 1986). The free-living heterotrophic N_2 fixers are potentially important sources 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 of 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 within the root apoplast, i.e., the intercellular spaces and or the xylem vessels and may enter the plants via 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), which 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 in pure cultures (Frank et al., 1998), in soil (Widmer *et al*., 1999) and plants. Rapid and unambiguous identification of diazotrophs has greatly benefited from recent advances in DNA fingerprinting based on the Polymerase Chain Reaction (PCR), Random Amplified Polymorphic DNA PCR (RAPD-PCR) and the interspersed repetitive sequences PCR (rep-PCR) (Berg *et al*., 1994). This paper aims to briefly review the biodiversity of heterotrophic and endophytic diazotrophs in rice and the molecular characterization of their *nif* genes using *nif* H primer.

BIODIVERSITY OF HETEROTROPHIC DIAZOTROPHS IN RICE ECOSYSTEM

Diverse N_2 fixing microorganisms (aerobes, facultative anaerobes, heterotrophs, phototrophs) grow in wetland rice fields and contribute to soil N pools (Fig 1). The major BNF systems known, include cyanobacteria and photosynthetic bacteria, that inhabit flood waters and the soil surface and heterotrophic bacteria in the root zone (rhizosphere), or in the bulk soil (Ladha *et al*., 1997). Moist conditions are required in

paddy fields during most rice growth stages to produce a high yield, and aerobic soil conditions are prevalent during the rice growth period, except for a few days of flooding. As a result, aerobic N_2 fixers constitute a large portion of the total bacterial population (Trolldenier, 1977). Numerous studies have suggested that free – living heterotrophic N_2 fixes are a potentially important source of N_2 fixation in rice fields (Boddey *et al*., 1995, 1998; Mahadevappa and Shenoy, 2000).

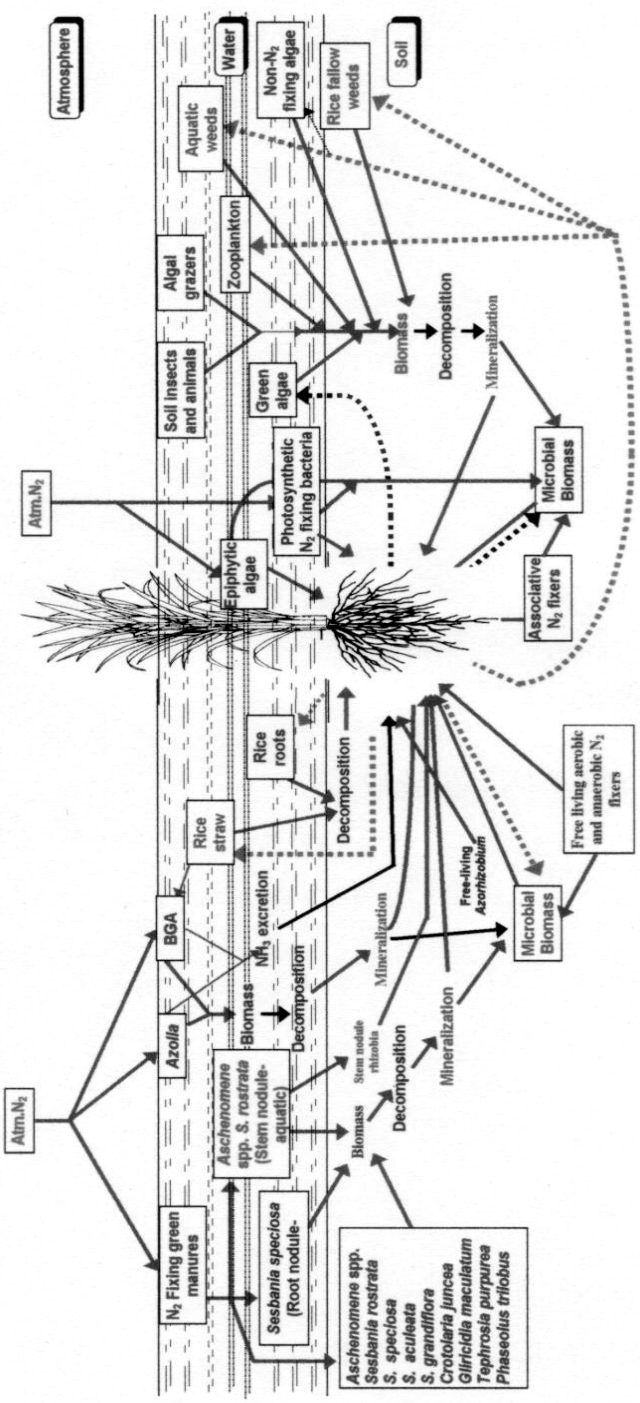

Fig. 1 Diazotrophic biodiversity in rice ecosystem

Several groups of symbiotic N_2 fixing bacteria have been identified in soils and flooded systems, such as *Azotobacter, Azomonas, Beijerinckia, Derxia* (aerobic), *Azospirillum, Aquaspirillum, Thiobacillus, Pseudomonas, Xanthobacter, Rhizobium, Methylosinus, Mycobacterium* (Microaerobic), *Klebsiella, Erwinia, Enterobacter, Citrobacter, Escherichia, Bacillus* (Facultative anaerobic) and *Desulfovibrio, Desulfotomaculum, Clostridium* anaerobic (Stewart, 1980; Havelka *et al*., 1982; Staley, 1989). The heterotrophic diazotrophs depend on carbon, eg., from straw, for energy and the most common isolates from soil are *Azotobacter, Azomonas, Beijerinckia, Derxia, Clostridium* and *Bacillus, Klebsiella* and *Enterobacter, Azospirillum, Desulfovibrio* and *Desulfotomaculum* (Roper and Halsall, 1986). The autotrophic bacteria such as *Rhodospirillum, Rhodopseudomonas, Rbodomicrobium, Chromatium, Thiocystis* and *Chlorobium*, derive their energy from photosynthesis (Havelka *et al*., 1982).

The contributions of cyanobacterial BNF are estimated to be 10-80 kg N ha^{-1} $crop^{-1}$, averaging about 30 kg N ha^{-1} $crop^{-1}$ and calculated that cyanobacterial inoculation increased rice yields only by an average of 337 kg grain ha^{-1} $crop^{-1}$. Heterotrophic bacterial BNF averages 7 kg N ha^{-1} (App *et al*., 1986), ranging from 11-16 kg N ha^{-1} and contributing to 16-21% of total rice N need (Shrestha and Ladha, 1996).

Malik *et al*. (1997) isolated various diazotrophs from rice roots and its rhizosphere, which were found to belong to the genera of *Azospirillum, Azotobacter, Flavobacterium, Pseudomonas, Xanthomonas* and *Zooglea*.

Velazco *et al*. (1999) showed that diazotrophs predominate among the complete bacterial microbiota of rice rhizosphere and they isolated the diazotrophs belonging to the genera of *Bacillus, Azospirillum, Pseudomonas* and *Flavobacterium*. In general *Azospirillum* was the dominant diazotroph in every ecosystem studied.

Khayyati and Anvari (2001) confirmed that all diazotrophic isolates of the rice rhizosphere soil were associative nitrogen fixers and belonged to the Enterobacteriaceae, particularly to the *Pseudomonas* and *Bacillus* genera. The isolates of the genus *Pseudomonas* were dominant, while those from *Serratia* and *Enterobacter* were present in smaller numbers. Mehnaz *et al*. (2001) estimated the population size of diazotrophic bacteria by ARA – based MPN in submerged roots and shoots and the results indicated about 10^5 - 10^6 counts/g dry weight at panicle initiation and grain filling stages.

Xie *et al*. (2003) found that the number of cultivable N_2 – fixing bacteria isolated from the paddy field ranged between 1.41 x 10^6 cfu and 1.24 x 10^8 cfu/g dry weight of the soil. They identified that the isolates were belonging to the genus *Bacillus, Burkholderia, Agrobacterium, Pseudomonas, Derxia, Alcaligenes, Aeromonas, Citrobacter* and the corynebacter –form group.

Kumar and Kumari Sugitha (2004) reported that the proportion of total diazotrophs to total heterotrophs was in the range of 12.39 to 20.65% in the rhizosphere of different rice cultivars and the distribution pattern of these diazotrophs was in the order of *Pseudomonas* > *Azospirillum* > *Azotobacter* > *Beijerinckia* > *Derxia* > *Klebsiella* > *Enterobacter*.

BIODIVERSITY OF ENDOPHYTIC DIAZOTROPHS IN RICE

One of the major limitations of associative nitrogen fixation is that rhizospheric diazotrophs utilize the products of nitrogen fixation for their own growth, but release little while they are alive (Alazard, 1990). Identification of stably maintained diazotrophic endophytic bacteria in rice tissues is one of the best approaches to mitigate nitrogen loss and to improve the N use efficiency (de Bruijn *et al.*, 1995 and Ladha *et al.*, 1997). The term endophytic bacteria has been used in a number of different ways but the generally accepted definition now is the most straight forward that is the 'bacteria' found within tissues internal to the epidermis (Kloepper *et al.*, 1992). Endophytic diazotrophs usually live with in the root apoplast i.e., the intercellular spaces and or the xylem vessels and may enter the plants via the root or epidermal cracks at lateral root junctions.

Endophytic diazotrophs have been proposed to be responsible for the supply of biologically fixed N to their host plant (Boddey *et al.*, 1995). If endophytic diazotrophs are really responsible partly or wholly for BNF in sugarcane and kallar grass, it is possible that rice varieties that show significant heterotrophic BNF may also be obtaining their fixed N from bacteria living within their tissues (Shrestha and Ladha, 1996). 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. Indeed, numerous diazotrophs, were isolated from surface sterilized rice plants using N deficient media (Barraquio *et al.* 1997; Stoltzfus *et al.* 1997).

According to Dobereiner *et al.* (1995), endophytic diazotrophs, by inhabiting the interior of the plants, can avoid the competition with rhizospheric bacteria and derive nutrients directly from the host plants. In return, as the plant interior may provide an environment conducive to N_2 fixation by being low in O_2 and relatively high in carbon, the bacteria can fix N_2 more efficiently to the host (James and Olivares, 1998).

Baldani *et al.* (1997) proposed to divide the endophytic diazotrophs into two groups: facultative and obligate. Facultative endophytes are described as those bacteria that survive in the soil and or on plant surfaces as well as being able to colonize the interior of some plants. Most endophytic *Azospirillum* strains are regarded as being facultative endophytes. However, *Herbaspirillum* sp., *G. diazotrophicus, Burkholderia* sp. and other endophytes are obligate endophytes, as they survive poorly in the soil and appear to have a requirement for living within a host plant. On the other hand, Olivares *et al.* (1997) had reported that *H. rubrisubalbicans* would clearly live for some time on leaf surface. The scope for nitrogen fixation in cereals by means of endophytic nitrogen fixation has been increased, the endophytic association of *A. caulinodans* for nodulation and nitrogen fixation in cereal crops was reported (Kannaiyan and Kumar, 2003).

NITROGENASE ACTIVITY OF RHIZOSPHERIC AND ENDOPHYTIC DIAZOTROPHS

Nitrogenase, the enzyme highly essential for reducing nitrogen to ammonia, is composed of Fe (dinitrogenase) and Mo-Fe protein (dinitrogenase reductase) The isolation of *nif*$^-$ mutants of *A. brasilense* devoid of nitrogenase activity using ethyl methane sulfonate mutagenesis has been attempted. Partial diploids were constructed by introducing plasmids PAB 35 and PAB 36 into the n*if*$^-$ mutants. The two plasmids were derivation of the broad host ranged plasmid vector, PRK 290. The restoration of a nif$^-$ phenotype by PAB 35, but not by PAB 36, was observed in the case of mutant, which might be impaired in the structural gene for the nitrogenase complex.

Diazotrophs can turn off nitrogenase activity in the presence of NH_4^+ and turn it on again when the NH_4^+ is exhausted. This is accomplished by dinitrogenase reductase ADP – ribosyl transferase (DRAT) and reactivated when NH_4^+ exhausted by dinitrogenase reductase activating glycohydrolase (DRAG), which removes the inactivating group. Some species of the genus *Azospirillum* possess the DRAT and DRAG system (*A. lipoferum* and *A. brasilense*), whereas *A. amazonense* in the same genus lacking DRAT and DRAG.*A. amazonense* responds to NH_4^+, which is influenced by dissolved oxygen concentration (DOC), but does not exhibit modification of dinitrogenase reductase (Burris, 1981).

According to Vande Broek *et al.* (1993), the fact that BNF is apparently not involved in plant growth promotion cannot be simply attributed to the absence of nitrogenase expression. Using a translational *nif* H- *gus* A fusion, it was observed that *Azospirillum nif* genes are expressed during the association with wheat roots.

Incorporation of atmospheric nitrogen into the host plant by *Azospirillum* was evaluated mainly by acetylene reduction assay and Watanabe and Lin (1984) observed an increase in the nitrogenase activity of *Azospirillum* inoculated in wetland rice at early flowering stage.

Line and Loutit (1971) reported ARA activity in *B. macerans* and *B. circulans*. The results obtained were equivalent to those obtained for some *P. azotofixans* strains (approximately 100 to 200 n moles of ethylene ml^{-1} h^{-1}).

Fujii *et al*. (1987) investigated that not all soil isolates belonging to Enterobacteriaceae were capable of reducing acetylene. Although all soil isolates of *Klebsiella penumoniae* reduced acetylene, only 80% of the *Enterobacter cloacae* and 30% of the *Enterobacter herbicola* isolates reduced acetylene.

Lima *et al*. (1987) reported that a novel nitrogen fixing *Acetobacter* species, isolated from semi–solid sugarcane juice inoculated with dilutions (10^{-7}and10^{-8}) of

sugarcane roots and stems, showed higher nitrogenase activity (above 50 nmoles C_2H_4 h^{-1}). They suggested that when *Acetobacter* colonies were grown on N–free semisolid sucrose medium, they consistently yielded nitrogenase activity at the rate of >200 n moles C_2H_4 h^{-1}.

Cavalcante and Dobereiner (1988) opined that the *Herbaspirillum* isolates exhibited increased nitrogenase activity with increasing concentrations of yeast extract as 'N' source, and also reported that the complete inhibition or repression of nitrogenase activity occurs when complemented with NH_4Cl even at a concentration of 10 mM.

You *et al*. (1991) suggested that the maximum nitrogen fixing activity of *Alcaligenes faecalis* was 40 mg of N assimilated g^{-1} malic acid consumed. Malik *et al*. (1997) found maximum ARA activity in *A. lipoferum* N-4 (686 n moles C2H4/mg protein/hr) followed by *Zogloea* KY-1 (544 n moles) and *Azoarcus* K-1 (290 n moles). Kumari Sugitha (2003) isolated heterotrophic and endophytic diazotrophs from rhizosphere soil, rhizoplane and macerated samples of root, stem and leaf tissues of different rice varieties. When thirteen selected isolates were subjected to ARA, their nitrogenase activity was in the range of 15.15 - 98.25 n moles of ethylene mg^{-1} cells h^{-1} (Table 1).

Table 1. Nitrogenase activity of heterotrophic and endophytic diazotrophs isolated from different rice varieties

S. No.	*Isolate Name*	Location	Tentative Identification	Nitrogenase activity n moles of C_2H_4 mg^{-1} of cells h^{-1}
1	HDC 4	Coimbatore	*Pseudomonas* sp.	0.00
2	HDC 8	Coimbatore	*Azospirillum* sp.	91.80
3	HDM 7	Marthandam	*Azospirillum* sp.	53.10
4	HDMY 2	Mayilandipatnam	*Rhizobium* sp.	0.00
5	HDPY 1	Poondy	*Beijerinckia* sp.	61.80
6	HDPY 2	Poondy	*Rhizobium* sp.	0.00
7	HDT 1	Thondamuthur	*Azotobacter* sp.	53.40
8	EDA 1	***Ambasamudram***	*Azospirillum* sp.	91.95
9	EDA 2	Ambasamudram	*Serratia* sp.	31.65
10	EDC 3	Coimbatore	NI	0.00
11	EDC 5	Coimbatore	*Azospirillum* sp.	68.05

S. No.	*Isolate Name*	Location	Tentative Identification	Nitrogenase activity n moles of C_2H_4 mg^{-1} of cells h^{-1}
12	EDC 6	Coimbatore	*Pseudomonas* sp.	0.00
13	EDM 2	Marthandam	*Gluconacetobacter* sp	53.75

MOLECULAR CHARACTERIZATION OF *NIF* GENES OF DIAZOTROPHS USING PCR AMPLIFICATION, SEQUENCING OF 16S rRNA AND *NIF H PRIMERS*

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 (Ueda *et al*., 1995). Palus *et al.* (1996) used PCR amplification for the identification of a diazotrophic bacterial endophyte, *Klebsiella*, isolated from the stems of *Zea mays* L. by amplifying portions of *nif* H and 16 S rRNA genes from this organism. The *nif* H gene, which codes for dinitrogenase reductase, from this organism is closely related to *nif* H from *K. pneumoniae*. Stoltzfus *et al.* (1997) developed highly conserved DNA primers for PCR mediated detection of *nif* D genes and used to screen the collection for the presence of *nif* genes. Perret and Broughton (1998) employed Targeted PCR Fingerprinting (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.

Cabellero –Mellado and Martinoz-Romero (1994) used multilocus enzymes profiles, plasmid and *nif* HDK restriction enzyme patterns of isolates in Mexico and Brazil to determine that populations of *A. diazotrophicus* are clonal. Based on genomic fingerprinting using BOX, ERIC, and REP PCR provide more evidence for the existence of greater genetic diversity in *A. diazotrophicus* isolates (Sevilla and Kennedy, 2000).

Nitrogen fixation by diazotrophic bacteria is a significant source of new nitrogen in salt marsh ecosystems. Five hundred and twenty-one isolates cultivated from the rhizoplanes of salt marsh grasses like *Juncus roemarianus, Spartina patens* and *Spartina alterniflora* were screened for the presence of plasmids. Analysis of the RAPD-PCR patterns using *nifH* primer indicated as many as 71 different plasmid genotypes occurring in diazotroph bacterial assemblages within and between the four different salt marsh grass rhizoplane habitats investigated (Beeson *et al*., 2002).

Kumari Sugitha (2003) identified *nif* H genes of heterotrophic and endophytic diazotrophs isolated from rice by TPF using *nif* primers. The *nif* H primers produced informative and reproducible genetic markers in standard *Rhizobium* culture (BMBS 1)

and eight (HDPY 1, HDC 8, HDM 7, HDT 1, EDM 2, EDA 1, EDC 5 and EDA 2) of the thirteen diazotrophic isolates tested, confirming the presence of *nif* H in these isolates. The molecular weight of the replicon was approximately 750 bp. Two isolates of heterotrophic diazotrophs (HDM 7 and HDT 1) generated multiple replicons with molecular weight ranging between 500 and 1500 bp. Three heterotrophic isolates (HDPY 2, HDMY 1 and HDC 4) and two endophytic isolates (EDC 3 and EDC 6) did not generate any replicon (Plates 1&2).

Kabir *et al.* (1995) designed species-specific oligonucleotide probes for *Azospirillum* strains such as Al, Ai and Aba based on partial sequences of the 16S rRNA molecules. They also reported that few non target organisms also hybridized with the different *Azospirillum* probes and hence its usage in bulk soil hybridization is not permitted. However, their use together with specific isolation techniques is validated.

Chen *et al.* (2000) using CRIC-REP-PCR profiles and 16 S rDNA of sequences identified and characterized *Rhizobium isolates* and detected high level of genetic diversity among the strains.

Weid *et al.* (2002) isolated a novel nitrogen fixing species from the maize rhizosphere and, based on amplified rDNA–restriction analysis (ARDRA) and 16 S rRNA gene sequencing, identified the isolate as *Paenibacillus brasilensis.*

Whole genome comparisons by genomic interspecies microarray hybridization of *K. pnuemoniae,* a maize endophyte, were shown to rapidly identify thousands of genes in a previously, uncharacterized bacterial genome provided that the genome of a close relative has been fully sequenced (Dong *et al.,* 2001).

Thakuria *et al*. (2004) isolated three groups of rhizobacteria from rice rhizosphere, *viz*., phosphate solubilizing, fluorescent bacteria and Azospirilla group. RAPD analysis of the *Azospirillum* isolates indicated that they belonged to four distinct genotypes. For all RAPD amplifications, the single primer used was 1253 (5´-GTTTCCGCCC-3´).

The diazotrophic endophyte of rice, *Serratia* sp. was marked with *egfp*-Km marker gene by biparental mating and used for colonization studies in rice. The conjugants established themselves endophytically in rice root, stem and leaves, of which the stem was preferentially colonized. The flavonoids, quercetin and diadzein, significantly increased the endophytic colonization ability of *Serratia* sp. than growth hormones. The induced colonization of *Serratia* sp. due to quercetin proportionally increased the *in planta* nitrogenase activity which reflected in the increased plant height, protein and chlorophyll contents of rice seedlings (Sandhya *etal*., 2005).

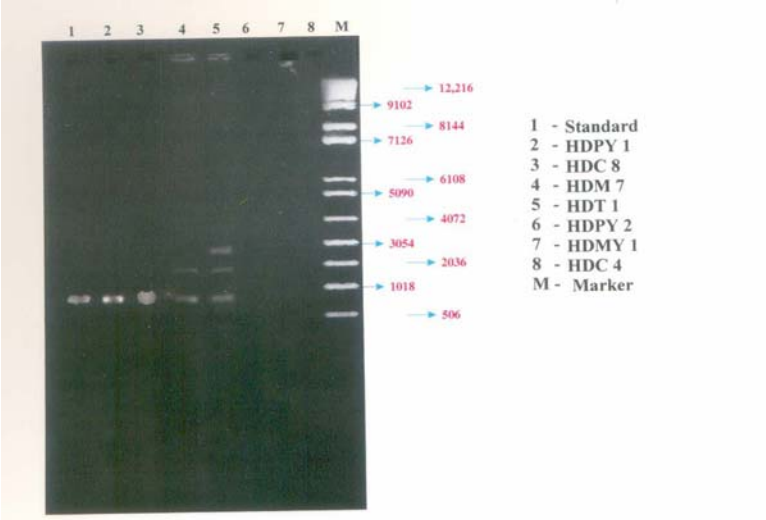

Plate 1. Identification of *nif* genes of heterotrophic diaztrophs using *nif* H primer

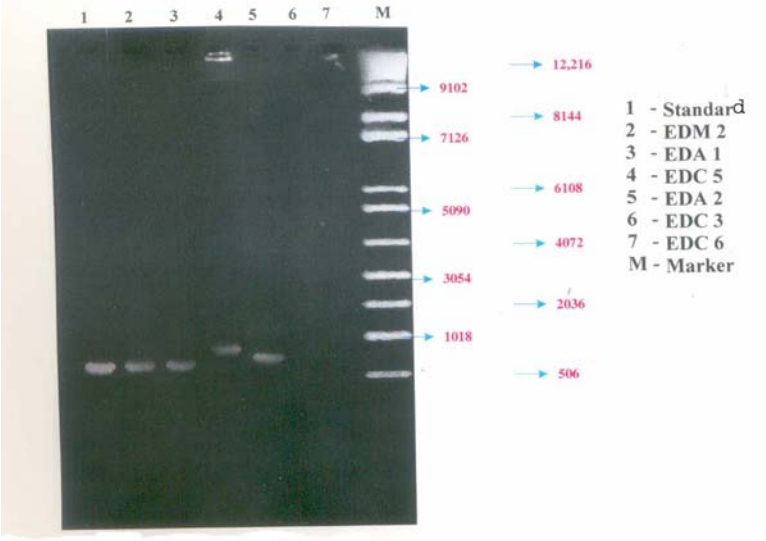

Plate 2. Identification of *nif* genes of endophytic diaztrophs using *nif* H primer

CONCLUSION

Several new endophytic diazotrophs have been reported in rice in the recent past and the exploitation of their potential will be the future strategy for sustained rice production. It has become the need of the hour to extend the molecular tools in conjunction with microbiological methods for analysing the genetic diversity of diazotrophic endophytes and establishing a stable endophytic association with rice. More intensive research is essential to identify the right diazotrophic endophyte of rice and study the host genes that are specifically expressed during the development of association with endophytes for achieving a significant breakthrough in this approach.

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