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ORIGINAL ARTICLE

Temporal Variations in Diazotrophic Communities and *nif*H Transcripts Level Across the Agricultural and Fallow Land at Jaipur, Rajasthan, India

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Abstract Biological nitrogen fixation is one of the most important nutrient processes in the ecosystem. Many studies have estimated the variations in soil diazotrophic communities across the globe. To understand the dynamics of nitrogen fixing bacterial communities and their response to the environmental changes, it is important to study the temporal variability of these communities. Using DNA fingerprinting method, denaturing gradient gel electrophoresis and real time polymerase chain reaction of the *nif*H gene, we have found that the agricultural land harbored unique nitrogen fixing communities that revealed different temporal patterns and abundance. Variation in soil moisture, organic carbon content, ammonium and nitrate concentrations may be the main factors which influenced the diazotrophic community composition and *nif*H gene abundance. *Azospirillum* species were more dominant in the agricultural soil. Unique environmental factors and agricultural practices were responsible for the temporal shifts in bacterial community structures and *nif*H transcripts level. Our study expands understanding of the influence of environmental factors on diazotrophic population that contributes to the nitrogen pool.

Keywords Biological nitrogen fixation · Diazotrophic · Temporal · *nif*H · Denaturing gradient gel electrophoresis · Real time polymerase chain reaction

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Introduction

Biological nitrogen fixation (BNF) is the process of fixing nitrogen (N) in terrestrial ecosystem and a fundamental process of primary productivity [1]. Both symbiotic and free living bacteria convert N_2 into NH_4^+ and are contributors of N turnover in ecosystems [2]. These microbes are comprised of nitrogenase enzyme, whose one part is encoded by *nif*H gene. It is highly conserved gene and used as a marker to investigate the diversity and composition of nitrogen fixing bacterial communities [3, 4].

Nitrogen turnover is enormously damaged by global changes in soil, influencing nitrogen cycle processes [5]. Nitrogen fixation rate varied up to 50% across the seasons. There are factors that influence the nitrogen fixation such as soil pH, moisture, carbon content, nitrogen content, soil texture and soil aggregate size [6]. Previously it has been reported that in managed agricultural systems usually large variations were reported in bacterial communities unlike unmanaged systems [7]. Some reports have shown the down regulation of *nif*H gene expression under low phosphorus and high nitrogen conditions in a managed ecosystem [8]. Temporal analyses of soil microbial communities contribute in crucial understanding of the changes in bacterial communities due to change in the environmental factors [9] and this helps to understand the environmental niches which are not described previously [10].

In Rajasthan chemical fertilizers are used to fulfill the nitrogen requirements of crops [11]. A better knowledge of temporal pattern for the distribution and abundance of diazotrophs community may helps to use BNF for enhancing soil nitrogen content and lead to less usage of fertilizers. Here, we have investigated how the diversity of soil diazotrophs varied across the period of three years in agricultural and fallow land in Western part of the India.

Materials and Methods

Soil Sample Collection

The sample collection sites are agricultural and fallow land at the Agriculture Research Institute, Jaipur, India (Latitude: 24.5802 and Longitude: 76.15135). Texture of soil was sandy-loam. Wheat crop was followed by fallow period for rest of the year. Soil sampling was initiated on September, 2011 and ended in July, 2014 at an interval of 60 days throughout the year to include three seasons summer, winter and rainy. Simultaneously samples were collected from fallow land. From each plot, fifteen random replicates of soil cores were collected from the top 0–10 cm depth using a rectangular probe (5 × 5 × 10 cm). Samples were stored at −20 °C for molecular analysis and for chemical analysis at 4 °C.

Soil Physical and Chemical Properties

The soil pH was measured by J. Forster's method [12]. Soil moisture content was determined by comparison of fresh and dry weight of the samples (105 °C; 24 h). Walkley–Black method was used to determine total organic carbon [10]. Soil ammonium and nitrate content was determined by the Kandeler and Gerber, 1988 and Scharf and Wehrmann, 1976 respectively [13].

DNA Isolation and nifH Amplification

Bacterial DNA was isolated from soil using MOBIO kit (MoBio, USA). For first PCR reaction, PolF (5′-TGC GA(CT) CC(GC) AA(AG) GC (GCT) GAC TC-3′) and PolR (5′-AT(GC) GCC ATC AT(CT) TC(AG) CCG GA-3′) primers were used to amplify a 370 bp fragment [14]. In second PCR reaction, first PCR product was used as a template to amplify 320 bp product (including the GC clamp sequence) utilizing primers PolF and AQER (5'-ATT ACC GCG GCT GCT GG -3') with a GC clamp (5'- CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC CTA CGG GAG GCA GCA G -3́). Each 25 µl of PCR mixture contained 25 ng DNA, 1X Taq buffer, dNTP 200 µM each, primer 1 µM each, and Taq polymerase 1.5 U. PCR conditions as follows: 1 cycle at 94 °C (5 min), 30 cycles at 94 °C (1 min), 55 °C (1 min), 72 °C (2 min), 1 cycle at 72 °C (7 min), and finally hold at 4 °C in C1000TM Thermal Cycler (Bio-Rad). DGGE was done using D-Code system (Bio-Rad, Germany) at 60 V for 16 h at 60 °C [15]. Bands were detected and relative intensities were calculated using Image lab software (Bio-Rad). Unique bands were excised from the gel and reamplified using PolF and AQER without GC clamp and sequenced.

RNA Extraction and Real Time PCR

RNA was extracted from 2 g of soil with the MOBIO kit (MoBio, USA). 2 µg RNA was reverse transcribed with cDNA synthesis kit (Thermo scientific). First RNA was treated with DNase I (Thermo scientific) before cDNA synthesis. Reaction mixtures were heated to 95 °C for 15 min before completing 40 cycles of denaturation (95 °C, 15 s), annealing (60 °C, 30 s) and extension (72 °C, 15 s) with the Applied biosystems Viia7 (USA). PolF and PolR primers were used for *nifH* qPCR [16]. For normalization, 16S rRNA gene was chosen and 357F/518R primers were consumed. Pool of all samples was used as calibrator. The relative fold change in *nif*H gene expression was calculated using the $2^{(-\Delta\Delta Ct)}$ method [17]. A melt-curve was done at the end of 40 cycles to detect the occurrence of a unique PCR product.

Calculations and Statistical Analysis

Calculation of Shannon–Weaver diversity index (H') was done using formula $H' = -\Sigma\, pi \ln(pi)$, where pi is the ratio of relative intensity of band i and the relative intensity of the lane. Data were subjected to ANOVA analysis and Pearson's correlation coefficient calculation using the program Sigmaplot version 12.5. Canonical correspondence analysis (CCA) was utilized to estimate the effect of environmental factors on the diazotrophs community using MVSP (Multi-variate Statistical Program) software version 3. Heatmap was used to calculated relative abundance of diazotrophs with help of XLSTAT software program. Bray–Curtis similarity coefficient was utilized for diversity similarity matrices.

Results and Discussion

Analyses of temporal variation in microbial communities provide a complete understanding of their composition which influences the biogeochemical processes that leads to changing environmental conditions. We investigated the temporal variability of the diazotrophic communities in sandy-loam soil and their relationship with environmental factors like soil physico-chemical, seasonal changes and agricultural practices.

Meteorological data was recorded during the three years of analysis. Temperature ranged between 21.4 and 42.1 °C during the analysis. Maximum rainfall 196.3 mm was found in July month during 2012–2013. Soil moisture, pH, organic carbon, ammonium and nitrate were estimated for all soil samples. There was significant difference in the soil

physical properties and environmental factors across the sampling period. Moisture content was significantly higher ($p < 0.05$) in soil samples of the agricultural land in comparison to fallow land samples. This revealed that irrigation may be the main factor responsible for high percentage of moisture in soil. Moisture content of soil influences other factors like exchange of gases, movement and concentration of nutrients in soil. Across the samples of both land-use types H^+ ion concentration, ranged from neutral to slightly alkaline (7–7.8) but there was no significant difference. Soil samples of both land types showed variation in concentration of organic carbon content across the sampling. Highest carbon content was present in the fallow land samples during the month of September (3.7 ± 0.05, 4.7 ± 0.14 and 5 ± 0.07 respectively) and low values were observed in the fallow land samples during the month of January (1 ± 0.04, 0.5 ± 0.11 and 0.8 ± 0.05 respectively). Changes in soil organic carbon content in soil are controlled by environmental parameters such as soil texture, temperature, rainfall, farming practices like cropping system, application of fertilizers, tillage etc. [18]. Plant available forms of nitrogen, ammonium and nitrate concentration were significantly changed throughout the period of three years for both lands. Their concentration varied with time. Amount of mineral nitrogen forms like ammonium and nitrate in soil depends on the amount of humus, C:N ratio, fertilizers and climatic conditions. So these forms of nitrogen content varied greatly between the years and within the year [19]. Application of fertilizers in summer increases the soil nitrate and ammonium content [20]. Amount of mineral nitrogen also depends on the presence of plant species. Continuous cropping system also accumulates much amount of nitrate and ammonium in soil. In winter, wheat accumulates more nitrogen in the soil as compared to fallow land samples [21].

DGGE gel analysis showed difference in banding pattern profile of *nifH* gene of the study (Figs. 1, 2, 3). Total 215 phylotypes (on the basis of relative front of each band) were detected in gel during 2011–2012. During the analysis of second year 2012–2013, 166 phylotypes were reported and during 2013–2014 analysis, only 144 phylotypes were observed in the gel. Banding profile was used for diversity index value calculation which changed with seasons in both lands across the sampling period. Highest value of diversity index, 2.7 was observed in July month agricultural soil sample during 2012–2013 analysis while lowest value was 0.004 found in March month agricultural soil sample during 2013–2014 analysis (Table 1).

Bacterial diversity in agricultural land was positively correlated with soil moisture and plant available forms of nitrogen i.e. ammonium and nitrate (Pearson $r = 0.4$, $r = 0.681$ and $r = 0.137$ respectively, $p < 0.05$) for first year. During second year of study, diversity showed positive correlation with soil moisture and temperature ($r = 0.12$ and $r = 0.40$ respectively, $p < 0.05$) while soil organic carbon ammonium and nitrate were poor predictors of diversity changes. During third year study, diversity showed positive correlation with organic carbon and ammonium ($r = 0.83$ and $r = 0.53$ respectively) and soil

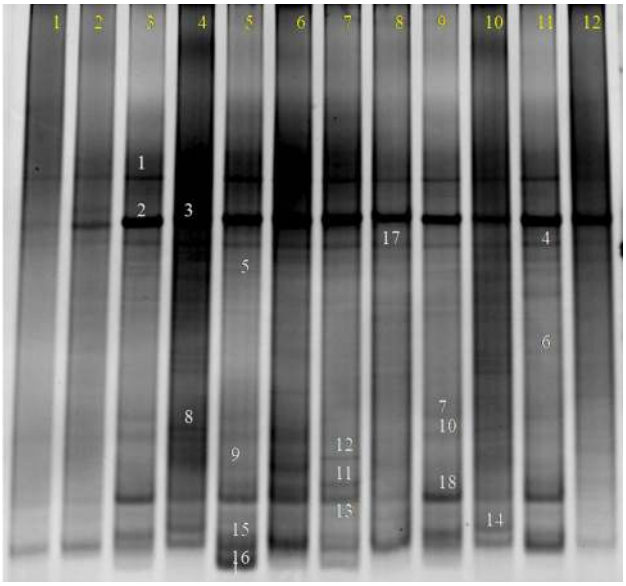

Fig. 1 8% Polyacrylamide gel showing DGGE profile of *nif*H gene for year 2011–2012. Upper numerical represent number of lanes while lower represents number of bands excised from DGGE gel. *Lane 1,3,5,7,9,11*: agricultural land and *Lane 2,4,6,8,10,12*: fallow land

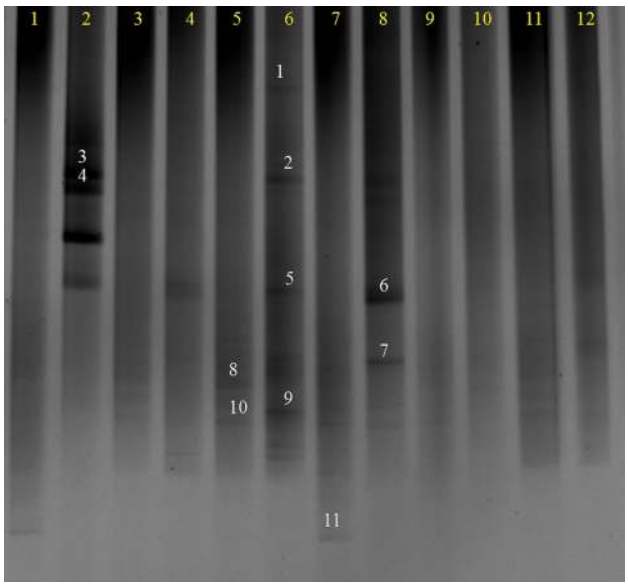

Fig. 2 8% Polyacrylamide gel showing DGGE profile of *nif*H gene for year 2012–2013. Upper numerical represent number of lanes while lower represents number of bands excised from DGGE gel. *Lane 1,3,5,7,9,11*: agricultural land and *Lane 2,4,6,8,10,12*: fallow land

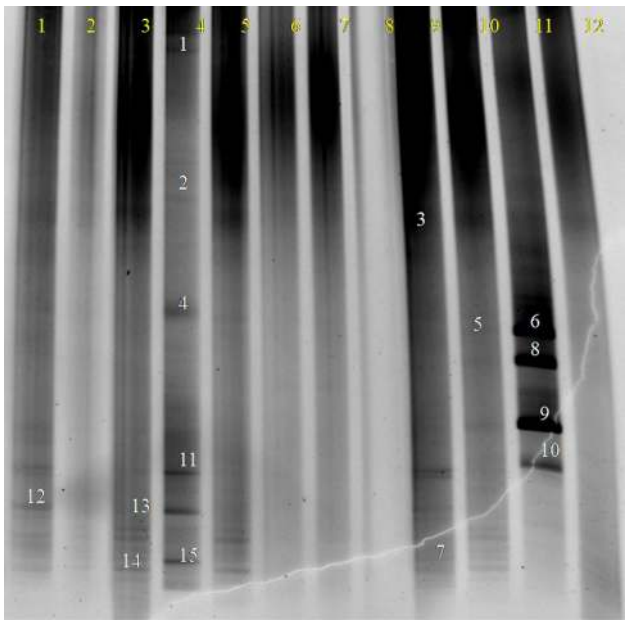

Fig. 3 8% Polyacrylamide gel showing DGGE profile of *nif*H gene for year 2013–2014. Upper numerical represent number of lanes while lower represents number of bands excised from DGGE gel. *Lane 1,3,5,7,9,11*: agricultural land and *Lane 2,4,6,8,10,12*: fallow land

moisture, nitrate and pH showed poor relation with diversity (r = 0.25, r = 0.179 and r = 0.008 respectively). Canonical correspondence analysis is best utilized for the complex ecosystem for exploratory purposes to identify significant environmental factors contributing to variation in species composition.CCA demonstrated that both environmental conditions including agricultural practices influenced the nitrogen fixing bacterial diversity. The results of the CCA for the first year showed that CC1 explains greater than 73.8% of the variance, whereas CC2 explains <13.74% (Fig. 4). For second year CC1 showed 50.2% of the variance and CC2 represented only 31.96% (Fig. 5) while for third year of analysis CC1and CC2 explains 48.5 and 30.6% of the variance respectively (Fig. 6). CCA data revealed that different factors influenced different phylotypes of the diazotrophic bacteria in sandy-loam soil. In an ordinate diagram form, this model describes the differential habitat preferences (niches) of bacterial population. This also explains the extent of variations in population caused by environmental factors.

Both Pearson's correlation and CCA results showed that no specific factor was responsible for changes in diazotrophic diversity during three years of study. In first 2 years of analysis strong correlation between moisture content and diazotrophic diversity could be as a result of moisture which is beneficial for the microorganisms. It is a nutrient source and provides hydrogen and oxygen for their growth and act as a carrier of other food nutrients. The

Table 1 Shannon–Weaver index values for two lands during sampling period

Soil samples	Agricultural land						Fallow land					
	Sep	Nov	Jan	Mar	May	July	Sep	Nov	Jan	Mar	May	July
Shannon–Weaver index												
2011–2012	1.7 ± 0.05^{a}	1.8 ± 0.02^{a}	2.1 ± 0.05^{bd}	1.9 ± 0.03^{abd}	2.2 ± 0.04^{d}	1.7 ± 0.2^{ad}	1.9 ± 0.08^{abd}	2.4 ± 0.06^{bd}	2 ± 0.03^{abd}	2.2 ± 0.06^{abd}	1.27 ± 0.07^{c}	1.98 ± 0.07^{abd}
2012–2013	0.8 ± 0.04^{a}	2.2 ± 0.04^{b}	0.5 ± 0.03^{c}	1.9 ± 0.02^{d}	0.7 ± 0.3^{ac}	2.7 ± 0.04^{e}	1.04 ± 0.1^{f}	1.94 ± 0.04^{d}	0.87 ± 0.2^{acf}	0.77 ± 0.03^{ac}	0.88 ± 0.2^{acf}	2 ± 0.05^{bd}
2013–2014	1.2 ± 0.08^{a}	0.8 ± 0.05^{b}	0.8 ± 0.05^{b}	0.004 ± 0.7^{c}	0.91 ± 0.3^{d}	0.95 ± 0.02^{d}	0.49 ± 0.3^{e}	1.14 ± 0.12^{a}	0.52 ± 0.34^{e}	0.48 ± 0.04^{e}	0.81 ± 0.09^{b}	0.38 ± 0.06^{e}

Each value represents average of the replicates (mean ± SD). Different letters represent significant difference and same letter are not significantly different ($p < 0.05$)

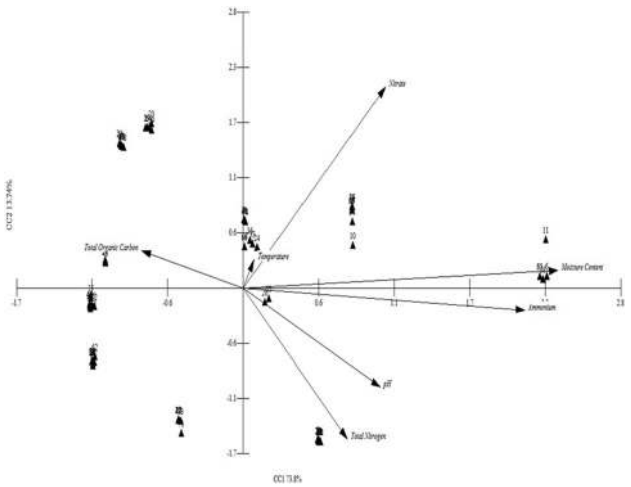

Fig. 4 Changes in structure of the diazotrophic communities from DGGE fingerprints across different time intervals for year 2011–2012 using canonical correspondence analysis (CCA)

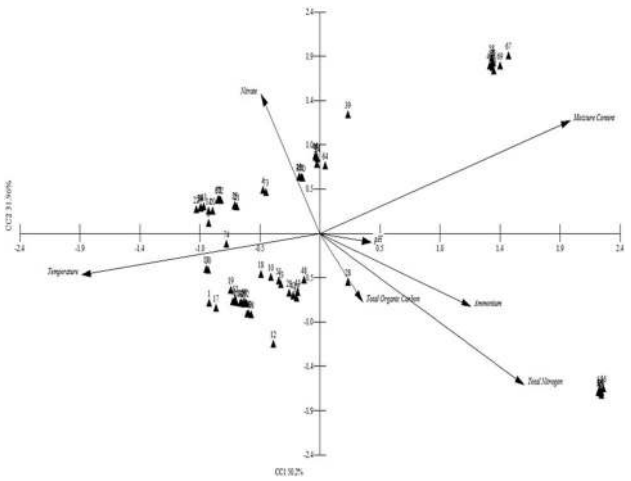

Fig. 5 Changes in structure of the diazotrophic communities from DGGE fingerprints across different time intervals for year 2012–2013 using canonical correspondence analysis (CCA)

relationship between diversity and soil moisture content varied between two lands. Previous studies also reported positive relationship between bacterial diversity and moisture content in agricultural soil [22]. But for third year samples, soil carbon content showed strong correlation with diversity index of diazotrophic population. Soil carbon is an important source of energy for most of the microorganisms. It affects the population and activity of microorganisms directly or indirectly. It influences the structure and texture of soil and thereby activity of the microorganisms. After soil moisture and carbon content, plant available forms of nitrogen including ammonium and nitrate were another factor which strongly influenced the diazotrophic population. Application of fertilizers increases the level of ammonium and nitrate which leads to shift of

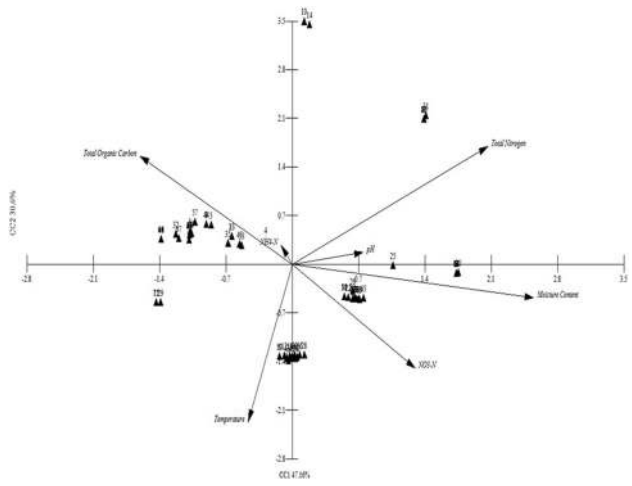

Fig. 6 Changes in structure of the diazotrophic communities from DGGE fingerprints across different time intervals for year 2013–2014 using canonical correspondence analysis (CCA)

nitrogen fixing bacteria population in soil samples. Presence of sufficient amount of mineral nitrogen in form of fertilizers should reduce fixation of nitrogen by bacteria, as this process requires lots of energy than the uptake of mineral nitrogen [23]. Ammonium concentration influences the ratio of carbon and nitrogen therefore shifting the soil bacterial community [24]. In this study soil pH was poor predictor of bacterial diversity but it an important factor which strongly affects the availability of other abiotic factors like carbon, nutrients and metals solubility for the growth of microbes in soil [25].

Strong temporal variation was reported in the diazotrophic population in two land-use types. This temporal variability in the diazotrophic community was higher in agricultural land than land not in use for agriculture purpose. Unique clustering of agricultural soil was reported due to high abundance of bacterial taxas belonging to the *Azospirillum* species, *Ensifer adharenes*, *Frankia, Pseudoacidovarix* and *Ideonella* phyla. *Azospirillum* taxon was commonly and abundantly present across the analysis. Its population varied with temporal changes. *Azospirillum* species are broadly distributed ecologically and are associated with vast varieties of plants including wheat, rice, maize and several non- leguminous crops [26] *Ensifer adharenes* was reported during the year 2011–2012 but this taxon did not appear again in rest of the study and previously reported in wheat cultivated soil samples [27]. Similarly, *Frankia* is a filamentous bacteria found in symbiotic association with non-leguminous crop, observed during the second year of study [28]. Indole acetic acid producing bacteria *Ideonella* sp. and *Pseudacidovorax* sp. were also observed in samples [29]. All the taxas reported during the study, belong to proteobacteria phyla. There were some bands showing similarity with unidentified

uncultivable nitrogen fixing bacteria. Bacterial taxas reported in agriculture land were distinct from taxas of other agricultural lands according to earlier studies. However, the specific types of bacterial communities responsible for variation between these lands are not necessarily responsible for these changes in other studies. Presence of distinct community may be due to the differences in the study types, soil type and environmental parameters [30]. Temporal variations in these samples could be due to the difference in the strategies of bacterial life history and their relationship with soil properties [31]. Cruz-Martinez et al. [32] reported that these variations may eventually be specific for soil type or climatic change.

A hierarchically clustered heatmap was created which showed the relative percentage of bacterial species (relative front) on Y-axis and soil samples on X-axis (Fig. 7). Color intensity with the legend indicated the relative abundance for bacterial species. Bray Curtis similarity coefficient was calculated between the agricultural land and fallow land samples (Table 2). Here, 0 means dissimilar composition of bacterial communities whereas 1 means presence of same communities in the soil samples. Value of coefficient

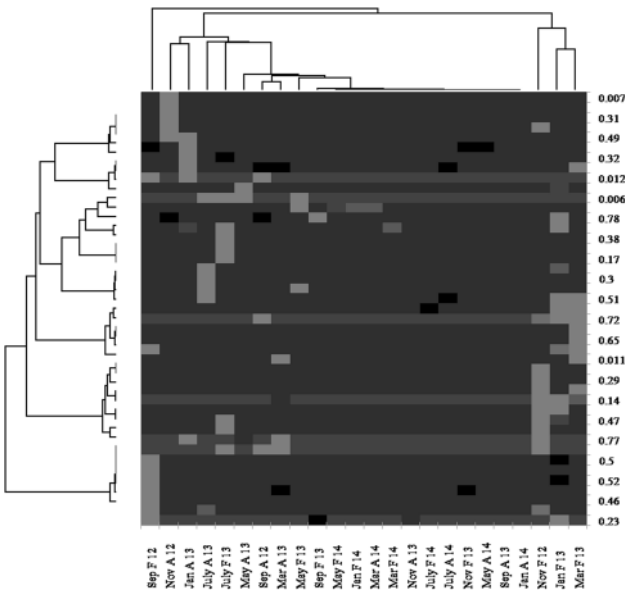

Fig. 7 Heat map analysis of the diazotrophic communities detected through DGGE gel. Relative front representing bacterial species on Y axis and soil samples on X axis. The *color code* indicates relative abundance, ranging from *dark grey* (low abundance) to *black* and *light grey* (high abundance)

was close to 0 in all samples except the samples of July month during third year of study. This showed the presence of different diazotrophic communities in agricultural soil samples as compared to fallow land samples. Coefficient values were 0 for agricultural samples of Jan and March during the third year which means presence of unique diazotrophic communities as compared to fallow land samples. Agricultural and fallow land samples harbor different bacterial communities.

*nif*H gene transcription analysis determines the abundance of bacteria capable of nitrogen fixation. Similar to fluctuation in diazotrophs population, *nif*H transcripts level in soil samples also exhibited substantial variability with time interval. It did not exhibit identical trend year after year. Significantly large number *nif*H transcripts (0.51–0.72 folds upregulation, $p < 0.05$) were reported in agricultural land during the early stages of the crop growth for first (2011–2012) and third year (2013–2014) of study whereas for the second year (2012–2013) soil samples, *nif*H transcripts level elevated during the end of cropping period ($p < 0.05$) (Fig. 8). Uniform correlation between the *nif*H gene abundance with environmental factors was not identified. First (2011–2012) and third (2013–2014) year of agricultural soil, *nif*H transcripts level showed positive correlation with the soil nitrogen, ammonium and nitrate content ($r = 0.53$, 0.45 $r = 0.966$, 0.67 and $r = 0.08$, 0.76 respectively, $p < 0.05$) and for second year (2012–2013) samples were positively correlated with temperature and soil nitrate ($r = 0.40$ and $r = 0.25$, $p < 0.05$).

Thus, our observation indicates that not only land-use type significantly influence the composition of diazotrophs but the sampling time, environmental parameters, agricultural practices also influenced the diazotrophic abundance. Similar dynamics in *nif*H gene abundance of different land-use types was not observed even though both fields were near to each other and were under the exposure of similar environmental conditions. This observation proposed that land-use type, agricultural practices and vegetation influence might have a significant input in regulation of changes in diazotrophic communities. This distribution of diazotrophic communities helps to utilize BNF to enhance the nitrogen content of soil and may lead to less usage of fertilizers. Variation in the abundance of *nif*H gene throughout the samples could be due to changes in the environmental factors [33]. High level of ammonium and

Table 2 Bray–Curtis similarity coefficient calculated by comparing agricultural land and fallow land soil samples

Sampling period	Sep	Nov	Jan	Mar	May	July
2011–2012	0.3125	0.277778	0.25641	0.263158	0.333333	0.137931
2012–2013	0.16	0.193548	0.15	0.171429	0.153846	0.1
2013–2014	0.181818	0.3	0	0	0.3125	1

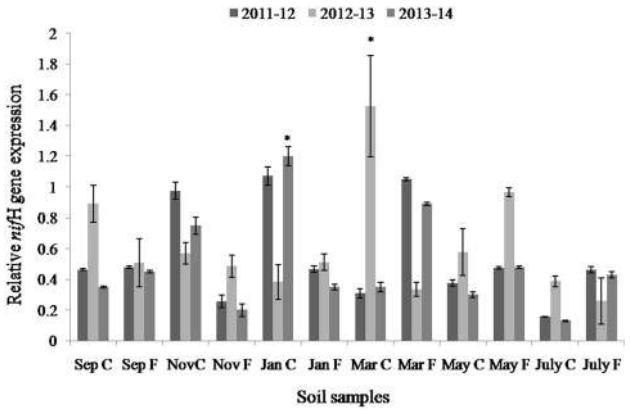

Fig. 8 Quantification of *nif*H gene of the diazotrophs in both land types throughout the period of 3 years. Agricultural land is represented by C while fallow land by F. Each data point represents mean and *error bar* indicates the standard error. Significant level is marked with *asterik*

nitrate concentration in soil negatively influences the abundance of *nif*H gene as earlier reported in managed ecosystems. In 2009 Orr et al. have shown contrary result of soil nitrate on the *nif*H gene transcription, where *nif*H gene abundance increased with high nitrate level in soil.

To anticipate the potential of nitrogen fixing bacteria to fix nitrogen in an ecosystem, *nif*H gene diversity appears to be an appropriate marker. This might show that *nif*H gene diversity could reveal the working redundancy of N fixing bacteria. Numerous environmental factors are essential to fix the biological nitrogen fixation. Different diazotrophs need unique fixed and ideal environmental conditions to fix atmospheric nitrogen.

Conclusion

Present work is first to report the temporal variation in the diazotrophs population and *nif*H gene transcription in the Rajasthan soil. Diversity of diazotrophs and *nif*H gene expression varies over the time with samples having diverse communities changing from month to month. Both lands under same climatic conditions could not reveal similar changing pattern in the bacterial diversity. Unique environmental factors may be responsible for the temporal fluctuations in diazotroph population and *nif*H gene expression. Presence of crop may also have a role in modulating the temporal variations. Present research help to understand the factors influencing diazotroph communities and BNF will improve N demand of crops. More research has to be done to investigate the specific factors for temporal variability in bacterial community and their functional ability.

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