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*nif*H Composition Affecting Nitrogen Fixation in Soil: Study in Coastal Saline Region

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The impact of change in salinity during different seasons in coastal saline soils of the Sundarbans, West Bengal (20º 15′N and 80º 40′E), India on PCR-RFLP of *nif*H gene pool was studied. PCR-RFLP of *nif*H gene pool was carried out by digestion of *nif*H amplicon from community DNA with HaeIII. Soils were collected during monsoon, winter and summer seasons of 2011-2012 from four different mono-cropped rice plots having variable salinity. Seasonal change in salinity was prominent and it detrimentally influenced the *nif*H gene pool both within the soil as well as the season. Temporal change in *nif*H composition maximally influenced dinitrogen fixation in soil as revealed by canonical correlation analysis (CCA). Dendogram generated from the RFLP pattern of *nif*H gene pool showed that community structure during monsoon was distinctly different from that of winter and summer. Pattern of the later two seasons belonged to the same cluster.

***(Key words**: Coastal soil, nifH, soil DNA, PCR-RFLP, HaeIII)*

Plethora of reports is available on ecology of free-living diazotrophs in non problematic soil but scarcely in problematic soils in general and coastal saline soil in particular. Since saline habitats are nitrogen poor (Sprent and Sprent, 1990), biological nitrogen fixation can dramatically increase the crop production. Ecology of diazotrophic microbial communities in coastal saline soils warrants thorough investigation.

Salinity in coastal region is temporal in nature (Tripathi *et al.,* 2007). Microbial biomass in such soils varies with change in salinity in different seasons (Tripathi *et al.*, 2006). Barua *et al.* (2011) also observed that non-symbiotic diazotrophs in coastal saline soils also change with seasonal change in salinity. Microbial biomass represents whole undifferentiated microbial community in soil. Traditional enumeration of microorganisms in soils represents a fraction of total microbial population (Rondon *et al.,* 2000). For better resolution of diazotrophic community structure in soil, Widmer *et al.* (1999) adopted PCR-RFLP of *nif*H gene. Currently the *nifH* database constitutes one of the largest gene database (Zehr *et al.*, 2003), with little information from saline soils (Chelius and Lepo 1999).

We hypothesized that PCR-RFLP of *nifH* gene could detect change in diazotrophic community structure with change in salinity. We herein studied: (1) change in *nif*H community structure in arable soils of variable salinity level in three different seasons by PCR-RFLP technique; (2) correlation of diversity index with selected physico-chemical and microbiological properties of soils; (3) determining the factor that maximally affects nitrogen fixation in soil; (4) clustering of *nif*H gene pool in different soils under study.

MATERIALS AND METHODS

Study site and soil sampling

Soils (0-15 cm) in triplicate, from each of four different mono cropped rice (*Oryza sativa*) plots having variable salinity were collected from ICAR-CSSRI, RRS, Canning Town (20º 15′ N and 80º 40′ E), West Bengal, India during summer, monsoon and winter seasons of 2011-2012. The soils of the experimental farm were fine, mixed, hyperthermic, Typic Endoaquepts (Soil Survey Staff, 1992; Bandyopadhyay *et al.*, 2003). The field moist soils were divided into two parts. With one part soil DNA analysis was carried out and with the other part physico-chemical properties of soil was determined. DNA analysis was carried out with pooled samples from each plot (Uroz *et al.*, 2010).

Determination of diazotrophic population, dinitrogen fixation and physico-chemical properties of soil

Aerobic heterotrophic non symbiotic diazotrophic population count were determined by

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soil dilution plate technique using mineral salt yeast extract agar media. The same media was used for determining dinitogen fixation of soil (Barua *et al.*, 2011). The pH (1:2.5 soil water), electrical conductivity of the soil saturation extract (EC_e), total organic carbon (OC) and total nitrogen (TN) of the soils was determined by the standard methods.

Extraction and purification of DNA from soil samples

DNA was isolated according to Tsai and Olson (1992), with slight modification. Briefly, 5 g soil samples were mixed with 13.5 ml of DNA extraction buffer (100 mM Phosphate Buffer [pH 5.6], 100 mM sodium EDTA [pH 8.0], 1.5 M NaCl and 1% CTAB) and 100 µl of Lysozyme (10 mg ml^{-1}) in Oakridge tubes by horizontal shaking at 225 rpm for 30 min at 37°C. After adding 1.5 ml of 20% SDS, the samples were incubated in 65°C water bath for 2 h with gentle end-over-end inversions every 15 to 20 min. The supernatants were collected in 50 ml oakridge tubes after centrifugation at 6,000 g for 10 min at room temperature, mixed with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1, vol/vol; pH 7.2), followed by centrifugation at 10,000 g for 10 min. The aqueous phase was collected and mixed with its half-volume of polyethylene glycol (30% w/v)/ NaCl (1.6 M), and incubated at room temperature for 2 h. The sample was centrifuged (10000 g for 20 min) and the partially purified nucleic acid pellet was resuspended in 0.5 ml of TE (10 mMTris-HCl, 1 mM sodium EDTA, pH 8.0). DNA was re-precipitated with 0.6 volume of isopropanol at room temperature for 1h. The pellet of crude nucleic acids was obtained by centrifugation at 16,000 g for 20 min at room temperature, washed with cold 70% ethanol, and resupended in 50μl of TE buffer (pH 8.0). DNA samples were washed using Himedia Gel Extraction Kit (MB511) using manufacturer's protocol, to reduce the humic acid content of the isolated DNA.

The isolated DNA, were quantified both spectroscopically and on Agarose gel, stained with 1 μg ml^{-1} Ethidium Bromide. Spectroscopic estimation of DNA both before and after column purification was also carried out by determining absorption of the samples at 230 and 260nm. The DNA samples were concentrated by sodium acetate-ehanol precipitation for better detection of *nif*H gene pool.

PCR amplification of the *nif*H gene fragment

PCR amplification of *nif*H gene fragment in soil DNA samples was carried out by degenerate *nif*H specific forward primer, PolF (5'-TGCGAYCCSAA RGCBGACTC-3') and reverse primer, PolR (5'-ATSGCCATCATYTCRCCGGA-3') based on *Azotobacter vinelandii nif*H coding sequence [M20568] to generate a 360bp region (Poly *et al.*, 2001a,b). 100ng soil DNA was used as a template. The PCR mix consisted of dNTPs at 200mM each, 0.25μM of each primer, 2.5mM $MgCl_2$, 1x PCR buffer and 2U of Taq DNA polymerase (Promega, USA). The cycling conditions were: 94°C for 3min, followed by 29 cycles of 94°C for 30s, 55°C for 30s and 72°C for 30s, and a ûnal extension step at 72°C for 5min before the samples were maintained at 4°C. The PCR products were run in 1% agarose gel stained with ethidium bromide.

RFLP analyses of *nifH* amplicons from soil

Precipitation of the PCR products was carried out by sodium-acetate and ethanol. The pellet was resuspended in 20μl of nuclease free water. RFLP was carried out by the method of Poly *et al.* (2001a). 10 μL of each PCR product was directly used for restriction enzyme cleavage. The reaction enzyme mixture contained 1X restriction enzyme buffer and 1.25 U of any one of the three different restriction endonuclese (*Taq*I, *Hae*III, and *Nde*II). *Hae*III was found to give most satisfactory result. The PCR products were digested overnight. Digested DNA samples were analyzed by electrophoresis in a ethidium bromide stained 2% agarose gel. The electrophoresis conditions were 1h at 70 V in 0.5X Tris-borate-EDTA buffer.

Statistical Analyses

Analysis of variance was carried out by completely randomized design (CRD), assigning soil and season as factors. The least significant (LSD) difference test was applied to evaluate the significance of difference between individual treatments using Duncan Multiple Range Test at 5% probability level.

Relatedness of microbial communities was determined using similarity coefficients of bands common to two samples. Our working definition was that two bands are common if they migrated the same distance on a gel. First, the total number of different bands was determined for the samples being compared. Then each sample was scored based on the presence or absence of each band in its profile when compared to the profile of each of the other samples. Sorensen's index of similarity [Cs = 2j/ (a + b)] was used to make pair wise calculations of band sharing between samples (Sørensen 1948). In the equation, a is the number

of bands in Sample A, b is the number of bands in Sample B, and j is the number of bands common to A and B. Canonical correspondence analysis (CCA) was used to analyze the degree of effect of different physicochemical and microbiological parameters on nitrogen fixation in soils. CCA calculations were done with the XLSTAT 2008.4.01software package (Addinsoft, NY, USA).

Clustering of *nif*H gene regions for pairs of samples was estimated from the proportion of common restriction fragments. Presence-absence matrices were used to construct a dendogram with cluster analysis based on Euclidean distances and Ward clustering according to Blackwood *et al.* (2003).

RESULT AND DISCUSSION

Physicochemical and microbiological properties properties of soil

Data on physico-chemical properties of the studied soils (Table 1) revealed that the soil salinity change significantly in different season with the highest salinity during summer (14.74 dS m^{-1}) followed by winter (10.35 dS m^{-1}) and monsoon (4.09 dS m^{-1}). Averaged over season the soil could be ranked as low (S1), medium (S2) and high (S3, S4) saline. Other studied parameters did not vary distinctly either in the season or in the soils. Coastal soils have distinct variability in salinity (Bandyopadhya *et al.*, 2003). The critical value of ECe for saline soils according to the classification of USDA (1954) is 4.0 dS m^{-1}. Thus, out of four soils under study, two were distinctly saline. Soil salinity is seldom constant with time or uniform in space in the area under the study. The fluctuating trends in soil salinity in this coastal area were also reported by Bandyopadhya *et al.* (1982). There exists a brackish ground water table at shallow depth in the coastal region, the water from the same moves upward to soil surface through capillary flow following evaporation loss of moisture from soil surface during dry seasons (winter and summer). As a result the salts get deposited in the surface of soil and the soil salinity increases. However, in a study conducted on coastal arable soils of Canning Town, West Bengal, India, no significant variation in average soil pH between the seasons but significant variation of soil pH between different sites, similar to our findings was previously reported (Bandyopadhya *et al.*, 2003).

The diazotrophic population varied during different periods of sampling, with the counts observed in the winter (6.89) and the monsoon (6.93) seasons were much higher than that observed in the summer (5.09) season (Table 1). Considerable variation in their diazotrophic populations between different sites were also evident. S3 had the highest diazotrophic population (5.86) followed by S2 (5.55), S1 (5.47), and S4 (4.61). Soils also varied significantly with respect to nitrogen fixation between sites as well as seasons (Table 1). Highest fixation was observed in monsoon (9.48) followed by winter (7.48) and least in summer (5.24). As per Tripathi *et al.*, (2007), salinity severely affects microbial biomass in coastal saline soils with significantly lowest biomass in summer. Present study also demonstrates a subpopulation of the total biomass, i.e. diazotrophs, are strongly affected similarly. Significant decrease in the diazotrophic count during summer corresponded to rise in salinity and depletion of available soil moisture (Iswaran and

Table 1. *Seasonal dynamics of physico-chemical properties, nitrogen fixing population and nitrogen fixation of studied soils*

Season	pH 1:2.5	ECe (dS m^{-1})	Organic carbon (g kg^{-1})	Total nitrogen (g kg^{-1})	Population (log colony forming unit)	Nitrogen fixation (mg nitrogen fixed $50ml^{-1}$ culture media)
Monsoon	6.25 a	3.63 c	8.86 b	0.72 b	6.93 a	9.48 a
Winter	6.29 a	12.85 b	9.22 a	0.76 a	6.89 a	7.48 b
Summer	6.25 a	13.46 a	8.46 c	0.70 c	5.09 b	5.24 c
Soil code						
S1	7.08 a	2.02 d	12.05 a	0.94 a	5.47 b	4.52 a
S2	5.67 c	3.89 c	8.81 c	0.8 b	5.55 b	3.76 b
S3	6.65 b	14.45 b	4.68 d	0.44 c	5.86 a	3.77 b
S4	5.66 c	19.58 a	9.86 b	0.78 b	4.61 c	2.74 c

Figures denoted by same alphabets are statistically similar at 5 % probability level by DMRT

Sen,1958; El-Shinnawi and Frankenberger, 1988). The soils, collected from different sites, showed considerable variation in their diazotrophic populations. These variations seemed to be related to the physico-chemical characteristics of soils, the inherent type and diversity of microorganisms occurring in these soils (Broadbent and Nakashima, 1971; Laura 1974; El-Shinnawi and Seifert,1975). It was interesting to note that inspite of rise in salinity to the peak in summer, the soils supported considerable diazotrophic populations. This indicated that certain nitrogen-fixing micro-organisms are highly adapted to the environment (Zahran, 1997) of the soils were basically salt tolerant, requiring certain amount of salts for their optimal growth.

Extraction of DNA from soil

Culture based techniques are known for their selectivity and are not considered representative of the extent of the bacterial community. The proportion of cells which can be cultured is estimated to be 0.1% or at most 10% of the total population (Amann *et al.*, 1995) and few data are available concerning how closely they reûect the actual composition of these communities. Recent advances in the field of molecular biology (extraction of nucleic acids, polymerase chain reaction (PCR) ampliûcation, DNA cloning, DNA sequencing) have made it possible to develop techniques which no longer require the isolation and culture of bacteria and thus reduce the bias associated with it (Ranjard *et al.*, 2000).

Spectroscopic examinations herein revealed that DNA extracted from 5g soils in different seasons varied from 0.3-0.72 mg g^{-1}, which on purification yielded 0.24-0.61 mg g^{-1}. Extracted DNA from saline soil was less than those isolated from normal soils (Yeates *et al.*, 1997). The A_{260}/A_{230} ratio (Yeates *et al.*, 1998) of the DNA samples ranged from 2.2-2.9 (data not shown) indicating the samples were suitable for PCR analysis.

The primary requirement for culture independent study of bacterial community in soil is isolation of bulk DNA directly from soil. Different authors obtained different amounts of DNA from different quantity of soils. Steffan *et al.*, 1988 obtained 9 μg DNA g^{-1} soil from 100g soil while from the same amount of soil Yeates *et al.*, 1997 recovered 15 to 23.5 μg g^{-1} soil. The recoveries relates to normal arable soils, while our soils were saline, which possibly resulted in lesser recovery of DNA due to lesser number of microbes in such soils. In our study, the quantity of DNA extracted varied with the soils as well as the season, which could be related to change in various physico-chemical parameters between soils as well as between seasons. This could be explained by significant negative co-relation between EC_e of the soils and amount of DNA extracted from the respective soils. Humic acid is a potential inhibitor of PCR amplification of soil DNA, it chelates out Mg^{2+} ion necessary for the activity of Taq polymerase (Tsai and Olson, 1992). Hence, for successful PCR amplification, it seems mandatory to remove humic substances from the DNA samples as much as possible.

PCR amplification of the *nif*H gene fragment and RFLP analysis

Enumeration of dinitrogen fixing populations represents a fraction of total microbial population in soil (Sekiguchi, 2006) and fails to throw light on diversity of diazotrophic population in soil. Diazotrophic populations herein were determined by culture based technique, which eliminated unculturable diazotrophs. Nitrogen fixation in soil is contributed by both culturable and unculturable diazotrophs. Hence significant positive correlation between nitrogen fixation, but no significant correlation between culturable diazotrophic population count in particular soil and the quantity of DNA extracted from the respective soil was observed.

Amplification of all the DNA samples yielded single product bands of 360 bp at the expected *nif*H gene fragment size (Fig. 1). RFLP pattern of *Hae*III digested *nif*H amplicons is shown in Fig. 2. Smearing appearance of all the samples was due to degeneracy of *nif*H primers. RFLP pattern distinctly demonstrated difference in *nif*H composition in soils collected in different seasons. A decrease in the number of dominant diazotrophic bacterial types, detectable by *nif*H PCR-RFLP patterns, was observed from monsoon to summer with a consistent presence of few bands in all the seasons. Soil samples collected in monsoon exhibited more *nif*H PCR-RFLP products compared to the other two seasons.

Based on the presence and absence of bands in each sample, Sorenson's similarity coefficients (Cs) were determined from the RFLP profiles generated (Table 2). A value of 1.0 indicates all bands are shared and 0.0 indicates no bands are in common.

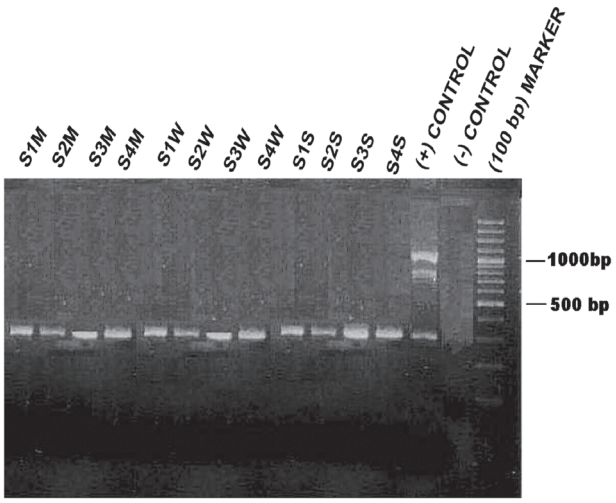

Fig. 1. *nif*H (PolF/PolR) amplicon of DNA isolated from different soils of variable salinity in different seasons. S1 Monsoon (S1M), S2 Monsoon (S2M), S3 Monsoon (S3M), S4 Monsoon (S4M), S1 Winter (S1W), S2 Winter (S2W), S3 Winter (S3W), S4 Winter (S4W), S1 Summer (S1S), S2 Summer (S2S), S3 Summer (S3S), S4 Summer (S4S), Lane 13 positive control (DNA from *Azotobacter vinelandii* used as template), Lane 14: negative control, Lane 15: 100 marker DNA.

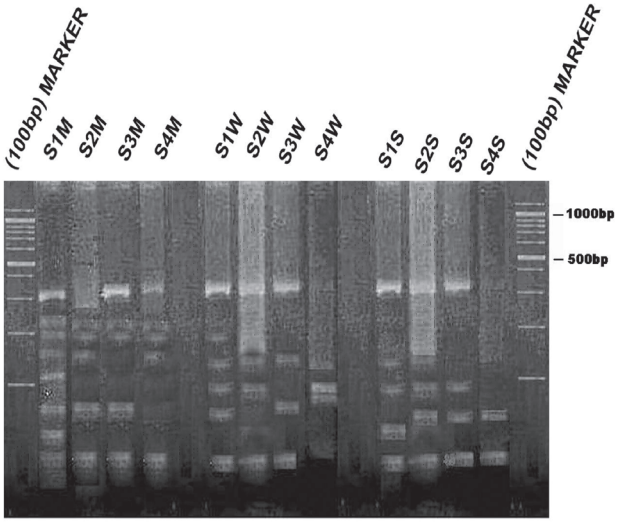

Fig. 2. Lane 2-5, 7-10 and 12-15 represented Hae III digested product of *nif*H PolF/PolR amplified DNA extracted from different soils of variable salinity level in different seasons. S1 Monsoon (S1M), S2 Monsoon (S2M), S3 Monsoon (S3M), S4 Monsoon (S4M), S1 Winter (S1W), S2 Winter (S2W), S3 Winter (S3W), S4 Winter (S4W), S1 Summer (S1S), S2 Summer (S2S), S3 Summer (S3S), S4 Summer (S4S), Lane 16 *nif*H of *Azotobacter vinelandii* (MTCC 2460). Lane 1 and Lane 17: 100 bp marker.

Averaged over four sites, no significant variation in mean Cs was observed between monsoon (0.52) and winter (0.49) but significantly lesser value was observed in summer (0.42). Irrespective of seasons, S1 and S2 soils exhibited statistically similar Cs value, which significantly decreased in S3 and S4 soils.

Linerar correlation coefficients between of *nif*H in soil and different physic-chemical and microbiological properties revealed (-0.833**) that only EC_e exhibited significant negative correlation. Nitrogen-fixing bacteria have *nif* genes, essential for N_2-fixation. Molecular approaches that study the diversity of diazotrophic organisms are primarily based on PCR amplification of a marker gene for N_2 fixation, which is generally the *nif*H gene (Zehr and Capone, 1996). Changes in the structure of *nif*H gene pools may affect nitrogen fixing activity in soil. PCR-RFLP technique was also efficiently used to study variation in *nif*H gene pool in soil and litter at a forest site (Widmer *et al.*, 1999). PCR-RFLP of *nif*H gene has been used to compare the community structure of N_2-fixing bacteria from different soil sources (Chelius and Lepo, 2010; Singh *et al.*, 2010).

Sorensen similarity index (C_s) has been efficiently used to determine microbial community level composition as well as distribution of functional genes by analyzing various community level fingerprints like PCR-RFLP (Kaldorf *et al.*, 2004), DGGE (Nakatsu *et al.*, 2000) and T-RFLP (Smalla *et al.*, 2007). This is an unique attempt to analyse seasonal variation of nifH composition in saline soils by comparing PCR-RFLP patterns of *nif*H of total soil DNA and analyzing it by Sorensen similarity index. Use of Cs is probably for the first time demonstrated by us for elucidating *nif*H variation in coastal soil. Decrease in Cs of the *nif*H gene types with increase in salinity clearly demonstrated that salinity not only affects the number of diazotrophs (Barua *et al.*, 2011), but also their types. However, for more precise analysis and to determine the effect of various parameters on nitrogen fixation in soils, canonical correspondence analysis (CCA) was carried out.

Canonical correspondence analysis (CCA)

Canonical correspondence analysis (CCA) was carried out using nitrogen fixation in soils together with environmental and microbiological variables. The first ordination CCA axis was mainly correlated to Mean Cs and EC_e of the studied soils and described a 36.97% of the total variability in nitrogen fixation in soil. The second CCA ordination axis, described 35.74% of the variability (Table 3). The unexplained fraction due to unknown factors sums up to 23.12% of the total variation. CCA1 and CCA2 were found to explain 80.23 and 97.60% of the overall variance, respectively, indicating a strong gradient in the data set. CCA biplot was shown in Fig. 3. An arrow represents each analysed variable; the length of the individual arrow indicates how much variance is explained by that particular

Table 2. *Similarity coefficient (Cs) of nifH gene in coastal soils of variable salinity level collected in different seasons*

Soil Samples	S1M*	S1W	S1S	S2M	S2W	S2S	S3M	S3W	S3S	S4M	S4W	S4S
S1M	1	0.92	0.73	0.83	0.55	0.55	0.55	0.4	0.4	0.6	0.44	0.22
S1W	0.92	1	0.6	0.8	0.6	0.4	0.6	0.44	0.44	0.67	0.25	0.25
S1S	0.73	0.6	1	0.33	0.75	0.5	0.5	0.57	0	0.29	0.57	0.33
S2M	0.83	0.8	0.33	1	0.67	0.22	0.72	0.5	0.25	0.75	0.29	0.29
S2W	0.55	0.6	0.75	0.67	1	0.5	0.25	0.37	0.57	0.57	0.33	0.33
S2S	0.55	0.4	0.5	0.22	0.5	1	0.4	0.57	0.56	0	0.67	0.33
S3M	0.55	0.6	0.5	0.72	0.25	0.4	1	0.57	0.29	0.86	0	0.33
S3W	0.4	0.44	0.57	0.5	0.37	0.57	0.57	1	0.33	0.67	0	0
S3S	0.4	0.44	0	0.25	0.57	0.86	0.29	0.33	1	0	0.8	0.4
S4M	0.6	0.67	0.29	0.75	0.57	0	0.56	0.33	0	1	0	0
S4W	0.22	0.25	0.57	0.29	0.33	0.33	0	0	0.4	0	1	0
S4S	0.44	0.25	0.33	0.29	0.33	0.67	0.33	0	0.8	0	0	1
Mean Cs	0.59	0.58	0.51	0.55	0.54	0.5	0.48	0.42	0.42	0.45	0.36	0.29
Mean Cs of sites		S1 0.57 a**			S2 0.53a			S3 0.44b			S4 0.37c	
Mean Cs of seasons		Monsoon 0.52a					Winter 0.49b				Summer 0.42c	

**Figures denoted by same alphabets are statistically similar at 5% probability level by DMRT

*S1 Monsoon (S1M), S2 Monsoon (S2M), S3 Monsoon (S3M), S4 Monsoon (S4M), S1 Winter (S1W), S2 Winter (S2W), S3 Winter (S3W), S4 Winter (S4W), S1 Summer (S1S), S2 Summer (S2S), S3 Summer (S3S), S4 Summer (S4S)

Table 3. *Relationships between CCA ordination axes and various physico-chemical parameters of soils under study*

Variable	F1	F2
Mean Cs	**1.326**	**1.693**
ECe	**-0.674**	**1.890**
pH	0.462	0.190
Organic carbon	-0.505	-0.104
Total nitrogen	0.191	0.721
Diazotrophic population count	0.019	-0.027
Cumulative % of total variation	36.97	54.56

The factors most strongly correlated with each axis are shown in bold type.

variable. The projection of nitrogen fixation in a particular soil in a particular season on this axis indicated the level of the variable where the fixation is most abundant. It was revealed that magnitude of the effects are in the order of Mean Cs > ECe > Organic carbon > Total nitrogen content > pH > diazotrophic population count of the soils.

Although a considerable amount is known about how environmental variables affects nitrogen ûxation rates in saline ecosystems (Whiting and Morris, 1986; Holguin *et al.*, 2001; Serraj and Gyamfi, 2004), little is known about the relationship between environmental parameters and the structure of nitrogen-ûxing bacterial communities in such ecosystems. Multivariate techniques, such as principal component analysis (PCA) (Poly *et al.*, 2001a) and canonical correspondence analysis (CCA) (Zhang *et al.*, 2008) have been proven to be effective in detecting the relationship between nitogen *nif*H community composition and environmental parameters. In this study, seasonal variation in nitrogen fixation was compared by CCA with Sorensen similarity index (Cs) of *nif*H, diazotrophic population count and selected physico-chemical properties of each soils under study. Previously, it has been reported that changes in the

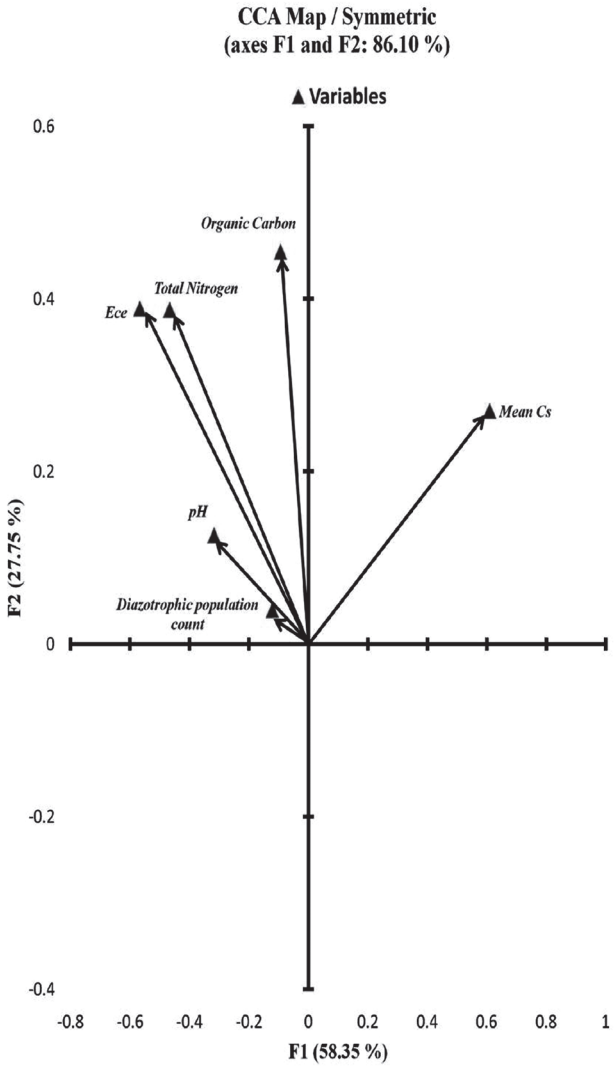

Fig. 3. Canonical correspondence analysis (CCA) ordination diagram of dinitrogen fixation in soil with Sorenson similarity index, physico-chemical factors and diazotrophic population count as arrows and nitrogen fixation at different sites in different seasons

structure of *nif*H gene pools may affect nitrogen fixing activity and the variation in N_2 fixation presumably results from the induction/inhibition of the nitrogenase and/or variation in the number of efficient N_2-fixing bacteria (Poly *et al.*, 2002). Among the parameters analyzed, Cs of nifH affected nitrogen fixation maximally while the effect of diazotrophic population count was found to be minimum, which signifies the importance of *nif*H richness in soil.

Among the soil physic-chemical factors, ECe, a major indicator of soil salinity, varied significantly between seasons, thereby maximally affecting temporal change of dinitrogen fixation in studied soils, which corroborated with previous findings (El-Shinnawi and Frankenberger, 1988). pH of the soils remained significantly same between different seasons, minimally affected dinitrogen fixation in soil under study.

Cluster analysis of *nif*H

Dendograms constructed out of environmental *nif*H restriction patterns have been efficiently used to determine the similarity and/or variation in *nif*H composition between different soil samples (Poly *et al.*, 2002; Singh *et al.*, 2010). In this study, *nifH* RFLP complexity is represented in a dendrogram of distance between the 12 soil samples collected over the seasons (Fig. 4). The clustering patterns clearly indicated the existence of two clusters, one containing patterns obtained by RFLP of *nifH* in soils in the monsoon season and the other one encompassing the summer and winter seasons. Based on the dendogram, it could be inferred that the diazotrophic community structure in the studied soils during monsoon is different from those in summer and winter.

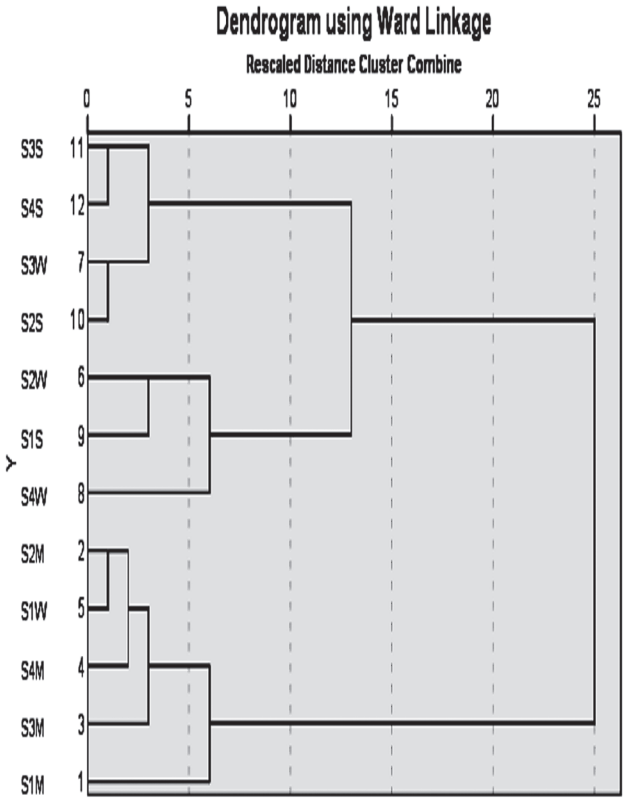

Fig. 4. Dendrogram deduced from the comparison of *nif*H-PCR-RFLP profiles generated from samples of DNA extracted from soils under study. S1 Monsoon (S1M), S2 Monsoon (S2M), S3 Monsoon (S3M), S4 Monsoon (S4M), S1 Winter (S1W), S2 Winter (S2W), S3 Winter (S3W), S4 Winter (S4W), S1Summer (S1S), S2 Summer (S2S), S3 Summer (S3S), S4 Summer (S4S)

Considering the dendogram generated in this study, it could be inferred that the abundant diazotrophic population in monsoon in all the soils under study might be different from that in summer or winter. This seems to be related to higher salinity of soil in winter and summer than that of monsoon.

CONCLUSION

The present study is a sequence independent approach for studying the comparative alteration of *nif*H composition in saline soils in different seasons. The structure of the *nif*H gene pool in monsoon was totally different from winter and summer. Increase in salinity seems to have detrimental effect on types of diazotrophs in coastal saline soils.

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