Diazotrophic Community Structure and Functioning in the Soil of Crop Plants (Legumes Versus Non-Legumes) at Two Depths

Zoology

KEYWORDS : biological nitrogen fixation, soil, nifH, nitrogenase, diazotrophic community structure, T-RFLP

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ABSTRACT

This study was undertaken to compare the diazotrophic community structure and functioning (nitrogenase activity) in the crop soil of gram, pea, mustard and wheat sampled at two depths, 0-15 cm and 15-30 cm. The nitrogenase activity was higher in the soil of leguminous crop plants (1236.93 ± 23.4 ηmole C2H4 g-1 soil h-1 and 199.97 ± 33.7 ηmole C2H4 g-1 soil h-1 in 0-15 cm depth soil of gram and Pea respectively) compared to that of the non-legumes and significantly decreased along the depth in all the crops. The diazotrophic community structure of different soils was compared using Terminal Restriction Fragment Length Polymorphism (T-RFLP) analysis. Each crop soil provided distinct T-RFLP profile of its own which showed comparatively lesser deviation with increase in depth. Abundance of certain nifH genotypes is more pronounced in legumes while in non-leguminous crops, the T-RFLP profile showed more number of TRFs but could not be correlated to the nitrogenase activity. Considerable functional redundancy of nifH gene pool was found at the experimental site due to lack of correlation between the diazotrophic community structure and functioning.

Summary. The present study aimed to compare nifH (one of the structural genes encoding Component II of nitrogenase enzyme complex involved in biological Nitrogen fixation) as well as nitrogenase activity of plant soil of four crop plants (legumes vs non-legumes) sampled at two depths (0-15 cm and 15-30 cm). Spatial analysis of diazotrophic community functioning of the rhizospheric soil of various crop plants revealed higher activity in legumes than non-legumes which decreased significantly along the depth. Each crop provided a specific HaeIII-TRFLP profile of its own and depth-wise profiles showed lesser deviations from each other. Profound effect of crop type was observed on nifH community structure as the dominant T-RFs of legumes rhizospheric soil indicated functional nitrogen fixers. Considerable functional redundancy of nifH gene pool was found at the experimental site due to lack of correlation between the diazotrophic community structure and functioning.

Introduction

Biological Nitrogen Fixation (BNF) is crucial as it catalyses the reduction of atmospheric nitrogen (N_2) to biologically acceptable ammonium which relieves the N limitation of the ecosystem (Zehr et al., 2003). BNF is an exclusive characteristic of many phylogenetically diverse groups of prokaryotes belonging to *Bacteria* and the *Archea* either living as free-living forms or in symbiotic/associative relationships (Young, 1992; Postgate, 1998; Izquierdo & Nusslein, 2006). BNF is achieved by microorganisms with an evolutionary conserved nitrogenase protein complex (Howard & Reece, 1996) that is composed of two multisubunit metallo-proteins encoded by *nifH, nifD* and *nifK* genes. Considerable sequence variability in *nifH* gene has led to the *nifH* database to be one of the largest non-ribosomal datasets which is rapidly expanding. On the other hand, relatively few sequence availability of *nifD* and *nifK* limits the usage of these genes for phylogenetic analysis (Zehr et al., 2003).

Diazotrophic community structure was studied using Terminal Restriction Fragment Length Polymorphism (T-RFLP) analysis which is a representation of the abundance of the gene sequences. **T-RFLP** analysis of PCR-amplicons of environmental gene pool provides a rapid and sensitive technique of microbial ecology (Marsh, 1999; Tiedje et al., 1999; Dunbar et al., 2001**)** that bypasses the necessity of cultivating the microbes. The key issue in microbial ecology is to test the intensity of link between the microbial community structure and functioning of the ecosystem (Patra et al., 2006). In this study, we tried to investigate the genetic structure and activity of diazotrophic community in soils collected at two different depths from the plant soil of agriculturally important crops; two leguminous: *Cicer arientinum* (gram) and *Pisum sativum* (Pea) and two non-leguminous: *Triticum aestivum* (wheat) and *Brassica campestris* (mustard). **We tried to find out whether crop type (legumes versus non-legumes) and soil sampling depth has any significant effect on diazotrophic community structure and functioning. We aimed to find functional significanc**e/redundancy of *nifH* gene structure at the experimental site in order to understand its ecological significance.

Experimental

Materials and methods

Experimental site description. The study site was Indian Agricultural Research Institute, New Delhi (Latitude-28°38'23'' N and Longitude-77°09'27''E). We chose four crops: two leguminous: *Cicer arientinum* (gram) and *Pisum sativum* (pea) and two non-leguminous: *Triticum aestivum* (wheat) and *Brassica campestris* (mustard). Our research field was divided into four plots each of size 2500 sqm and subsequently the four crops were grown separately in each plot.

Soil Sampling and storage. Soil was sampled from the fields having full-grown plant stage which has completed all the phenological stages of growth and development. After the removal of the litter layer, soil was sampled using rectangular sampler (4 x 4 x 10 cm) for each of the four crops at each depth (4 crops x 2 depths) Each soil sample constituted eight separate subsamples collected (randomized block design) from which plant materials and other debris were removed by hand, the soil was passed through a sterile sieve (2-mm mesh size) and stored at -70ºC for further analyses. Soil physical and chemical properties were analyzed following standard protocols.

Acetylene reduction assay. Nitrogen-fixation rates of the soil samples were analyzed through acetylene reduction assay {ARA} (Stewart et al., 1967; Hardy et al., 1973) on the same day of sampling. 15 grams soil (free of any plant materials or nodules) was collected in glass vials (55x150x20 mm) and sprinkled with water (5 ml) to prevent from desiccation during incubation that could limit ARA. ARA was done in replicate from each one of the eight soil subsamples of one crop at a particular depth (8x2 replicate per plant per depth). Similar vials with 20 ml of water in triplicate served as control. The vials were tightly sealed and injected with enough acetylene to create 10% acetylene atmosphere followed by incubation under field conditions for 4 hrs. Ethylene produced was measured by withdrawing 1 ml of gas sample from the incubated vial and injecting into a Nucon Gas chromatograph packed with Porapak N (80-100 mesh). Nitrogen gas flowing at the rate of 30 ml/min was used as the carrier, while hydrogen and air were used to produce the flame for the Flame Ionization Detector. After measuring the acetylene reduction, the soil was air-dried and final ARA values were expressed per unit dry weight of soil. The data was statistically analyzed using MSTAT-C package.

Community DNA extraction and *nifH* PCR. Soil DNA was extracted using the Mobio Ultraclean™ soil DNA kit (Carslbad, CA, USA) following the manufacturer's instructions except for few minor modifications from equal mass aliquots of each eight soil subsamples in triplicate and later on all the 24 DNA samples were pooled as a composite DNA sample for each sampling (4 crops x 2 depths) and quantified in triplicate (Table 2).
Community DNA was subjected to PCR amplification targeting ~370-bp fragment of the *nifH* gene. Amplification reactions were carried out in six replicate using nested PCR protocol and degenerate primers (Yeager et al., 2004).

NifH T-RFLP analysis. 6-carboxyfluorescein was used to label *nifH*11 primer in the second round of PCR. Fluorescent *nifH* fragments were amplified by the nested PCR protocol in six replicate. Amplicons were pooled, concentrated and gel purified. 100 ng of amplicons were digested with 5U *Hae*III at 37ºC for 4 hrs and the restriction digests were heated at 95ºC for 20 min in order to deactivate the enzyme. The samples were sent to Macrogen Inc, South Korea for fragment sequencing (genescan sequencing). Peak Scanner Software version 1.0 (Applied Biosystems) was used to analyze fragment sizes and peak fluorescence intensities. To establish the relative abundance of TRFs, peak areas for each sample were converted to a percentage of the total peak area (total fluorescence) for that sample (Yeager et al., 2004, Walker et al., 2008). Peaks representing 0.5% or more of the total peak area were found significant for analysis.

Results

Soil characteristics, microbial biomass and PCR results. Soil was loamy with varying pH of different crop soils (soil of gram and mustard showed slight alkalinity) (Table 1). Percent soil moisture of the soils was comparable with lowest moisture content (29%) was found in the mustard soil. Low percentage of organic carbon as well as available Nitrogen was observed in all the samples. Microbial biomass is the part of organic matter that constitutes living matter. Therefore, we calculated ng DNA per gram soil as an estimate of microbial biomass. Good DNA yield as 397.8±21.5, 378.4±21.9, 395.4±28.3 and 368.2±27.3 ng DNA g soil^{-1} was obtained from the upper zone soil (0-15 cm) of gram (Gu), pea (Pu), wheat (Wu) and mustard (Mu), respectively. Lower DNA yield and hence lesser microbial biomass as 238.4±22.6 (gram), 298.6±26.5 (pea), 285.2±21.5 (wheat) and 228.6±30.3 ng DNA g soil^{-1} (mustard) were observed as the depth increased (15-30 cm). *NifH* PCR amplifications were positive in all the attempts (100%) when DNA extracted from upper zone soil (0-15 cm) was taken as template in all the crop plants. However, 75% of positive amplification results were observed in the case of lower zone soil of wheat (Wl) and mustard (Ml) (Table 2), showing negative results in the rest 25%.

Table 1. Physical and chemical properties of the experimental soils

Soil source	pH	Organic Carbon (%)	Available Nitrogen (Kg N/ha)	Available Phosphorus (Kg P/ha)	Available Potassium (Kg K/ha	E.C. (dS/m)	Water Holding capacity (%)	Soil Moisture (%)	Particle size distribution			Soil Type
									% Sand	% Silt	% Clay	
Gram field	8.71± 0.45	0.31± 0.02	199± 19	14.4±2.7	312±33	0.30±0.09	34.53±1.6	35±2.1	58±8	28±2	14±5	Loam soil
Pea field	8.61± 0.39	0.35± 0.07	201± 21	19.4±3.1	299±25	0.34±0.08	35.46±1.2	36±2.7	59±7	27±2	14±3	Loam soil
Wheat field	8.01± 0.41	0.31± 0.10	212± 17	27.9±3.9	296±45	0.45±0.09	35.17±1.9	37±3.3	57±7	29±4	14±2	Loam soil
Mustard field	8.71± 0.64	0.22± 0.09	197± 24	19.4±2.9	217±36	0.32±0.05	37.09±1.5	29±2.9	59±6	28±3	13±2	Loam soil

(Standard Error is calculated: n=8).

Table 2. Estimates of microbial biomass in terms of DNA yield (ng DNA /g soil^{-1}) and PCR results associated with the soils

Soil source	Depth analyzed (cm)	Soil sample designation	Microbial biomass (DNA extracted, ng DNA g soil^{-1})[a]	NifH amplification results [b]
Gram field	0-15	Gu	397.8±21.5	4/4
Gram field	15-30	Gl	238.4±22.6	4/4
Pea field	0-15	Pu	378.4±21.9	4/4
Pea field	15-30	Pl	298.6±26.5	4/4
Wheat field	0-15	Wu	395.4±28.3	4/4
Wheat field	15-30	Wl	285.2±21.5	¾
Mustard field	0-15	Mu	368.2±27.3	4/4
Mustard field	15-30	Ml	228.6±30.3	¾

[a] DNA yields represent the average ± standard deviation of DNA extracted in triplicate (n=3).

[b] Amplification results are presented as the number of DNA samples (soil samples) yielding the desired size amplified product divided by the number of (DNA samples) soil samples analyzed.

Acetylene reduction assay. The non-leguminous crop soil showed statistically lesser ARA activity as compared to that of the leguminous crop (P<0.05) (Fig. 1). Mean ARA rate in the gram upper zone soil (Gu) was highest as 1236.93 ± 23.4 ηmole C_2H_4 g^{-1} soil h^{-1} followed by 1199.97 ± 33.7 ηmole C_2H_4 g^{-1} soil h^{-1} in Pu (pea upper zone soil). ARA decreased significantly along the depth showing 32.56% and 31.02% reduction in the lower zone soil of gram (Gl) and pea (Pl) respectively. The ARA of wheat and mustard upper zone soil was 947.47 ± 26.2 and 823.10 ± 42.7 ηmole C_2H_4 g^{-1} soil h^{-1} respectively. With the increase in depth, the activity was reduced by 20.61% and 9.05% in lower zone soil of wheat (Wl) and mustard (Ml), respectively. Nitrogenase activity of the entire upper and lower zone soil were significantly different from each other (P<0.05).

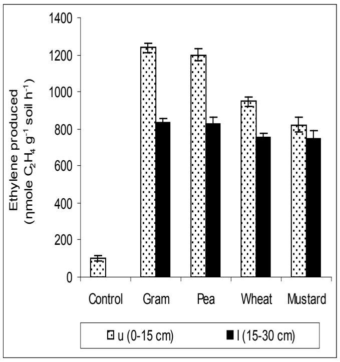

Fig. 1. Nitrogen fixation rates measured as acetylene reduction activity (η mole C_2H_4 g^{-1} soil h^{-1}). Comparison of nitrogenase activity was done with plant soils sampled from the four crops (legumes versus non-legumes) at two depths, u: 0-15 cm and l: 15-30 cm. Error bars represent standard error: n=16. Bars having the same superscripts are significantly not different (P<0.05, one way ANOVA followed by Tukey's all pairwise multiple comparison test.

Impact of crop on nifH community structure. Two terminal restriction fragments (TRFs) of 31 bp and 88 bp were the dominant nifH sequences in soils of both the depths of gram (Fig. 2). Small TRFs were more dominant in gram soil than the larger ones of 187 bp and 355 bp in length. Of the 12 TRFs, only 8 TRFs were distinct in the gram soil. The pea soil also showed the characteristic absence of larger TRFs like 108bp, 154bp, 187bp and 355bp indicating the complete digestion of the nifH sequences into small fragments. In the case of wheat soil, the TRFs of 31bp and 88bp contributed to 55.46% and 57.62% at both the upper (Wu) and lower (Wl) zone, respectively. Of the total 12 TRFs, 10 distinct TRFs were found in the wheat soil showing the presence of more diverse nifH sequences than the soil of leguminous crops. Mustard upper zone soil (Mu) was quite distinct from the others in having 50.09% relative abundance of TRF 88bp. TRF of 31bp in length was dominant in all the soils except for Mu where it contributed a small percentage (2.64%) to the overall relative abundance. Mustard upper zone (Mu) profile was different from the lower zone (Ml) soil. TRFs of 31bp and 88bp in length contributed to 52.73% of the total abundance in Mu that significantly declined to 37.88% in Ml.

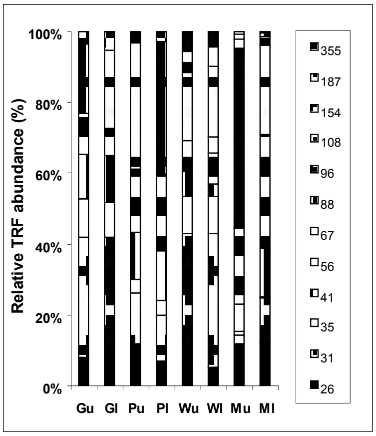

Fig. 2. Relative abundance of *nifH* TRFs generated for the comparative analysis of diazotrophic community structure in the soil collected at two depths from four crop plants. The bars represent the abundance (percentage of total fluorescence) of each TRF (classified by size) identified in the T-RFLP profiles of samples. The first letter indicates plant soil of the respective crop, that is G: gram, P: pea, W: wheat and M: mustard. The second letter denotes the depth of soil sampling, that is, u: 0-15 cm and l: 15-30 cm.

Impact of soil depth on *nifH* community structure. TRFs of 31bp and 88bp were found dominant at both the depths in gram soil (Gu and Gl) (Fig. 2). Abundance of TRFs of 26bp, 88 bp and 108bp increased with the depth while that of 31bp, 56 bp and 154bp decreased. In the pea soil, TRFs of 31bp, 41bp, 56bp and 88bp were the dominant ones at both the depths. Abundance of TRFs of 56bp and 88bp increased with the depth while that of 26 bp decreased. In the wheat soil, TRFs of 31bp and 88bp were the dominant nifH sequences that showed a slight increase in abundance with increase in depth. The abundance of TRFs of 41bp, 108 bp and 355 bp decreased along the depth while that of TRFs of 67 bp and 96bp increased. TRFs of 56bp and 88bp were the dominant one in the soil of mustard crop with relative abundance of 21.27% and 32.09% in Mu and Ml, respectively. High abundance of TRF of 88bp (50.09%) in Mu clearly put T-RFLP profile of mustard soil quite apart from rest of the crops. Although, the abundance significantly decreased along the depth to 25.35% in Ml.

Discussion

A comparative analysis of diazotrophic community structure and functioning in the field soil of leguminous (Gram: *Cicer arientinum* and Pea: *Pisum sativum*) and non-leguminous (Wheat: *Triticum aestivum* and Mustard: *Brassica campestris*) crops was done at two different depths. Since the experimental soils were sampled from the same location (the four plots which were receiving same agricultural inputs previously), the extraneous variation was nullified which could be the case with geographically distinct locations with different climatic conditions and soil characteristics. N-fixation rates of the soil significantly decrease under low moisture content and pH (Brouzes et al., 1969; Sindhu et al., 1989; Roper et al., 1991; Limmer & Drake, 1996). This might be the reason of low nitrogenase activity in mustard soil that was having lowest moisture content of 29%. The activity was not affected by the pH as there was no significant variation.

Maximum diazotrophic community functioning (nitrogenase activity) was found in the upper root zone which decreased along the depth. This could be due to decrease in nutrient composition as well as less favorable soil characteristics with increase in depth. Crop type (legumes versus non-legumes) had a significant effect on functionality of *nifH* community structure, as the ARA of leguminous plants was higher than that of non-leguminous that indicated abundance of functionally active diazotrophic community in the soil of legumes. It has been shown that plant species impacts enzyme activities related to nitrogen metabolism in soil as well as influences the microbial community structure (Ibekwe & Kennedy, 1998; Priha et al., 1999; Briones et al., 2002; Söderberg et al., 2002). Tan et al. (2003) reported large differences of the root associated diazotroph population structure caused due to variation in rice plant species. The diazotrophs responds differentially to Al-tolerant and -sensitive soybeans and the dominant diazotrophs varied in different growth stages of both soybean genotypes (Li et al. 2012). The diversity of nitrogen-fixing microbial community in wasteland samples varied with the physico-chemical properties of wastelands, plant species, and the development period of plant community, and was influenced by plant rhizosphere (Zhan & Sun 2012).

T-RFLP approach is very helpful in providing a semiquantitative representation of the relative abundance of sequences as both the sizes and relative signal intensities of individual T-RFs of a sample are highly reproducible (Dunbar et al., 2000; Smalla et al., 2007). Peak areas reflected the relative abundance of the *nifH* genes with larger peaks corresponding to the abundant gene fragments or TRFs. Abundance of certain *nifH* genotypes is more pronounced in legumes as revealed by the T-RFLP profile which could be correlated to selected dominant and/or abundant *nifH* community. So, diversity in terms of number of peaks is low and the *nifH* community structure consisted of few dominant and/or abundant diazotrophs. The root sphere of a particular crop plant might be selecting and supporting specific *nifH* gene community structure and this selection could be biased to dominant and/or abundant *nifH* gene community in the leguminous crop soil which are actually doing the nitrogen fixation and responsible for significantly higher values of ARA activity. The rhizosphere of legumes is not only attracting the root nodulating bacteria but also other functional (in terms of nitrogen fixation) free-living diazotrophs which are not completely known. In non-leguminous crops: wheat and mustard, the T-RFLP profile showed more number of distinct peaks but the T-RFLP profile (relative TRF abundance) could not be correlated to the lower ARA values. The root zone of non-legumes might not be favoring functionally active diazotrophs, although the populations of potential nitrogen fixers were present as revealed by T-RFLP profile. Thus, changes in the community structure could not be correlated to the community functioning.

Hsu & Buckley (2009) supported that changes in diazotrophic community structure and diversity had a direct relationship with the N-fixation rates. On the other hand, Deslippe *et al.* (2005) mentioned that nitrogenase activity and *nifH* gene community structure are controlled by different factors. A similar study conducted by Patra *et al.* (2006) emphasized a lack of correlation between the changes in enzyme activity and overall changes in genetic structure. The abundance of *nifH* was not related to land-use, with similar copy numbers observed for both managed and remnant sites at some locations (Hayden et al. 2010). As a conclusion in this study, no concrete relationship between the diazotrophic community structure and functioning was established; indicating considerable functional redundancy of *nifH* gene pool existed at the experimental site.

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