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Evidence of Diazotrophic Community Structure in Cotton Agroecosystem

KEYWORDS: diazotroph, soil, root nodulation assay, nifH gene, cloning-sequencing

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ABSTRACT *This study was undertaken to examine the existence of diazotrophic community structure across the cropping period of cotton under the routine agricultural conditions. The traditional root nodulation assay confirmed the presence of root nodulating bacteria throughout the cropping period. Profuse nodulation was seen in tap and adventitious roots of the plants inoculated with the soil samples (control showing no nodulation). The nifH gene was amplified in all the soils and 0 day (day of sowing) as well as 150 day (period of harvesting) soils was considered for cloning-sequencing analysis. NifH gene pools were prescreened by RFLP and the selected clones were subsequently sequenced that revealed high levels of diversity in the soils. Different restriction patterns were recorded for the reamplified nifH fragments from the nifH clones with HaeIII and MspI. RFLP analysis resulted in 14 and 13 restriction patterns respectively, from DNA of 0 and 150 day soil. As a conclusion, we got enough evidence to state the presence of free living diazotrophs at the experimental site across the cropping period of cotton.*

Summary. With the help of traditional as well as molecular tools we investigated whether the routine agricultural practices as well as the growing phenological stages of the crop was causing any significant impact on the existence of diazotrophic community structure. The present study aimed to compare nifH (one of the structural genes encoding component II of nitrogenase enzyme complex involved in biological Nitrogen fixation) of the soil sampled across the cropping period of cotton. The root nodulation assay as well as detection of nifH gene and cloning sequencing analysis gave enough evidence of the existence of diazotrophs at the experimental site. Different restriction patterns were recorded for the reamplified nifH fragments from the nifH clones with HaeIII and MspI. RFLP analysis resulted in 14 and 13 restriction patterns respectively, from DNA of 0 and 150 day soil. The study of diazotrophic community structure, diversity as well as its functional significance should be the next part of the multidimensional approach.

Introduction

Soil is an integrated ecosystem constituting a number of interdependent physical, chemical as well as biological factors which affect the environment and in turn, get affected by it. The soil microbial communities are integral part of soil ecosystem which is organized in complex food webs and stabilizes various soil processes including the biogeochemical processes so that, their biomass, activities as well as diversity can directly indicate how well an ecosystem is functioning (Atlas and Bartha, 1998; Kennedy, 1999; Roose-Amsaleg et al., 2001). The cycling of nitrogen is one of the important biogeochemical processes as nitrogen is indispensable micronutrient for all living organisms. That's why; nitrogen is the limiting nutrient for crop plant growth in most developing countries (Diallo et al., 2004) including India.

Biological Nitrogen fixation is crucial as it catalyses the reduction of atmospheric nitrogen (N_2) gas 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 Bacteria and Archea either living as free forms or in symbiotic/associative relationships (Izquierdo and Nusslein, 2006). BNF is achieved by microorganisms with an evolutionary conserved nitrogenase protein complex (Howard and Reece, 1996) that is composed of two multisubunit metallo-proteins encoded by nifH, nifD and nifK genes. The nifH database is one of the largest non-ribosomal datasets which is rapidly expanding and its phylogeny is largely congruent with that of the small-subunit (SSU) rRNA gene.

Cotton is an important cash crop of India giving employment to around 60 million people involved in agriculture, ginning and textile industries. Additionally, it is a crucial export item in agricultural economy of India, making ~800-900 billion export business. It is a risky crop for the poor farmers as it has the maximum pesticide consumption of around 54%-55% of the total pesticides used. So, it is very important as well as interesting to study microbial ecology of the rhizosphere of cotton crop along its cropping period. Our aim in the present study is to find evidence of the presence of free living diazotrophs throughout the cropping period of cotton crop and to find out their functional significance.

Materials and Methods

Experimental site and soil sampling

The study site was the experimental fields of Indian Agricultural Research Institute, Pusa, New Delhi (Latitude-28°38'23'' N and Longitude-77°09'27''E), India. The fields have been the sites for extensive research studies related to various agriculturally important food as well as cash crops. Gossypium hirsutum Pusa8-6 variety was used and harvested with the crop yield as 22.8 quintal/hectare. Soil was sampled as previously described (Rai et al., 2010). Soil was collected seven times on 0 day (the day of sowing), 15 day (seedling stage), 30 day (Square formation), 60 day (Flowering), 90 day (Ball formation/development), 120 day (Ball development/opening/harvesting) and 150 day (Ball harvesting).

Meteorological conditions during the experimental tenure

The monthly meteorological conditions were recorded during the experimental tenure (Fig.1). The maximum temperature during the cropping period ranged from 28.2°C to 38.2°C and the minimum temperature ranged from 10.6°C to 27.2°C. RH I and II indicated relative humidity recorded in the morning and evening respectively. RH I ranged between 66% and 91% while RH II ranged between 41% and 74%. Therefore the mean RH ranged from 55% to 80%. The maximum and minimum rainfall was 216.8 mm and 0mm, respectively. Time duration in hours of sunshine in the experimental field was minimum in the month of November as 3.7 hrs and maximum in the month of May and October as 7.9 hrs.

Volume : 4 | Issue : 2 | Feb 2014 | ISSN - 2249-555X

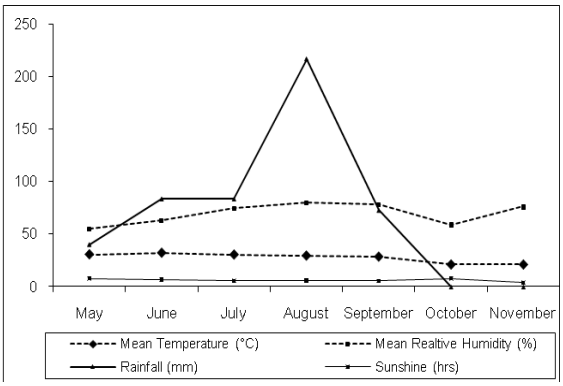


Fig. 1. Meterological conditions recorded during the cropping period of cotton in the experimental field (May- November).

Soil characteristics

Soil characteristics were determined by analysis of physical and chemical properties of soil sampled on the first and last day in eight replicate (one from each subsamples) following the standardized protocols and presented in Table 1. Analysis of chemical properties of soil: 1) pH: saturated soil solution (1:2.5::soil:water) was prepared and pH was determined using a glass electrode pH-meter. 2) Organic carbon: It was calculated by titration protocol of Walkley and Black, 1934. 4) Available Potassium: It was calculated by flame photometer. 5) Available Nitrogen: It is present in the form of NH_4^+, NO_3^- and NO_2^- (nitrite doesn't contribute significantly) in the soil and can be extracted and measured spectrophotometrically (Keeney and Nelson, 1982). 6) Available Phosphorus: It was calculated colorimetrically (Olsen and Sommers, 1982). Analysis of physical properties of soil: Dry matter and water content: Determined by the weight loss method (Schichting and Blume, 1966). Maximum water holding capacity: 50g moist soil samples were saturated with water. From each cylinder, 25 grams of soil was taken in porcelain dishes, dried to constant weight at 105°C for 3 hours, cooled in desiccator and weighed. It is expressed in terms of gram water x 100. % WHC = (saturated soil-dried soil)/ dried soil x 100. Particle Size Distribution: Percentage was calculated using the following formula. Sand fraction % = Weight (g) of fraction on sieve/25 x 100; Silt fraction % = Weight (g) of fraction under sieve/25 x 100; Clay fraction % = Oven dried soil weight (g)/25 x 100.

Table 1. Physical and Chemical properties of the soil samples.

Soil sample	pH	Organic Carbon (%)	Available Nitrogen (g N/m²)	Available Phosphorus (g P/ m²)	Available Potassium (g K/ m²)	Water Holding capacity (%)	Soil Moisture (%)	Particle size distribution			Soil Type
								% Sand	% Silt	% Clay	
C0	7.93±0.33	0.34± 0.06	21.9±1.2	4.47±0.4	41.3±1.2	51.2±2.7	36±2.0	59±8	28±3	13±3	Loam soil
C150	7.93±0.55	0.68±0.09	23.6±1.7	4.52±0.1	38.0±1.3	50.9±2.8	34±1.9	58±7	29±1	13±2	Loam soil

(Standard Error is calculated: n=8).

Root Nodulation Assay

Green bean seeds were sterilized with 0.1% Mercuric Chloride and sowed in the autoclaved pots (4 seeds /pot; 3 pots/ soil sample) containing sterilized sand. Triplicate of control (without inoculum) were also planted. During seed germination, the soil inoculum (0.5g) was put to the plumule of each seed. Plants were watered with 40 times diluted Jenson's medium and grown in aseptic condition for 20-25 days. After 25 days, plants were uprooted and root nodulation was checked (Vincent, 1970).

Community DNA extraction and quantification

Soil microbial community DNA of each of the eight soil subsamples was extracted in triplicates using direct lysis based on the method of Zhou et al., 1996 and later on the entire 24 DNA samples were pooled as a composite DNA sample for each sampling day. The quality of DNA was checked by agarose gel electrophoresis and immediately photographed using Gel Documentation Systems. The DNA was quantified by Nanodrop Spectrophotometer ND-1000 prior to any further analysis.

NifH PCR and clone library analysis

Community DNA was subjected to PCR amplification targeting ~370-bp fragment of the nifH gene. Amplification reactions were carried out in 12 replicate using nested PCR protocol (Rai et al., 2013). The soil samples of day of sowing (0 day) and period of harvesting (150 day) were considered for cloning and sequencing studies. The pooled nifH amplicons were gel purified using QiaexII kit (Qiagen, Germany) and cloned into a pGEM-T-easy vector (Promega, Madison, WI, USA), following the manufacturer's instructions. Clones were checked by plasmid DNA isolation followed by EcoR1 enzyme restriction to detect those with the correct size insert (~370 bp). Plasmid DNA of the positive clones (clones with correct size insert) were subjected to nifH PCR amplification with the same primers and PCR conditions as mentioned above and further screened by digesting amplicons with restriction endonucleases HaeIII and MspI to identify unique restriction profiles (unique nifH sequences) in each clone library. A clone library was created for both the samples; C0 and C150 and each of the 8 libraries were screened for a total of 30 clones each. The screened clones were sent to Macrogen Inc., S. Korea for sequencing.

Results and discussion

This study was undertaken to examine the presence of free living nitrogen-fixing bacterial community structure across the cropping period of cotton under the routine agronomic and agricultural conditions being used by the common farmers. With the help of traditional as well as molecular tools we investigated whether the routine agricultural as well as the growing phenological stages of the crop was causing any significant impact on the existence of diazotrophic community structure. We chose cotton crop because it is a risky crop with high consumption statistical data in terms of chemical inputs and high economic importance as an important cash crop providing employment to millions of people. The agroecosystem is artificial and disturbed one, so, studying the temporal dynamics of the functional genes and correlating it with ecosystem functioning is an important aspect of soil microbial ecology. Because the cotton crop relies on extensive chemical inputs (fertilizers, pesticides), so we were quite doubtful of detecting nifH gene pool throughout the cropping period.

Soil characteristics were determined by analysis of physical and chemical properties of the soil sampled on 0 day and 150 day (Table 1). There is a direct impact of plant community composition and genotype on diazotrophic community structure and functioning (Tan et al., 2003; Patra et al., 2006;

Volume : 4 | Issue : 2 | Feb 2014 | ISSN - 2249-555X

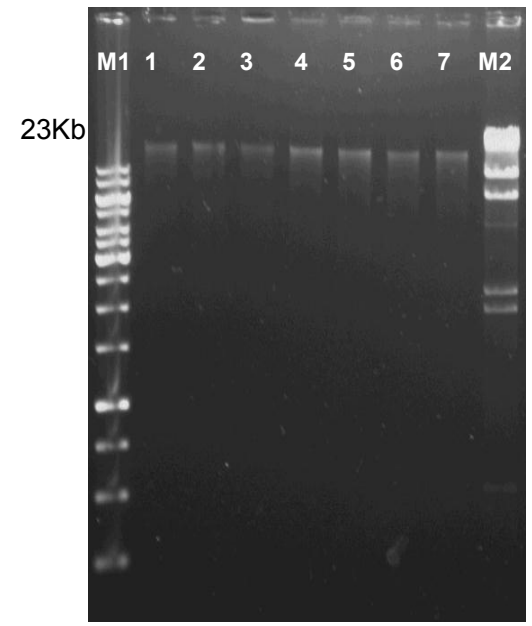
Picard et al., 2008). The soil type was loam soil with nearly normal pH of 7.93±0.33- 7.93±0.55. The experimental soil was loamy and since the plant and soil type remained the same so extraneous variation was nullified. Decreased moisture content and low pH are unfavorable component for nitrogenase activity (Brouzes et al., 1969; Sindhu et al., 1989; Roper et al., 1991; Limmer and Drake, 1996). The soil pH was near normal with moderate moisture content and water holding capacity. Percent moisture content of soil was 36%. Low percent organic matter (0.34± 0.06-0.68±0.09%) as well as available Nitrogen (21.9±1.2-23.6±1.7 g N/m^2) was observed. Significant low % organic matter and available nitrogen indicated depletion of nutrients due to long-term agricultural practices. The soil was having significantly higher available Potassium as 485 Kg K/ha at the time of sampling.

Root nodulation assay was the preliminary testing of the soil to confirm the presence of root nodulating Rhizobia sp. Rhizobium bacteria form symbiotic nitrogen-fixing nodules with host legumes. During the establishment of the symbiosis, the first signal is the induction of Rhizobium nodulation genes by compounds exuded from the host (Peters and Long, 1988). Because the cotton crop relies on extensive chemical inputs (fertilizers, pesticides), so we were quite doubtful of detecting nifH gene pool throughout the cropping period. But the traditional root nodulation assay confirmed the presence of root nodulating bacteria throughout the cropping period. In control, no nodulation was observed whereas profuse nodulation was seen in the taproot region of the plants inoculated with the soil samples (Fig. 2). Nodulation was observed in the adventitious roots also. This clearly explained presence of root nodulating bacteria present in all the soil samples giving us hint to carry out further study.

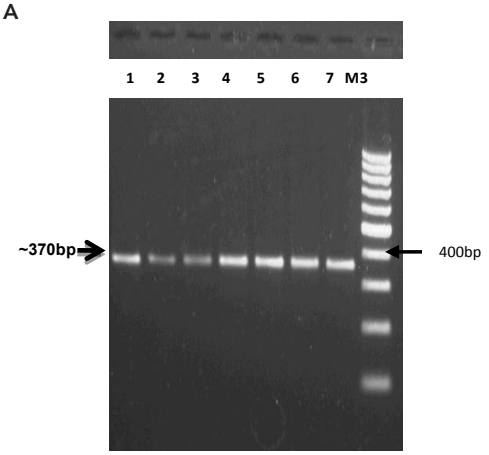
A

B

Fig. 2. Root nodulation assay. A. control (without inocula) showing no root nodulation, B. prominent root nodulation were seen when experimental soil was used as inoculum.



A



B

Fig. 3. A. Soil community DNA of all the seven samples. B. Soil community nifH gene amplification of the samples. Lane 1-7 represents soil collected on 0 day, 15 day, 30 day, 60 day, 90 day, 120 day and 150 day respectively. M1- 1 Kb DNA ladder, M2- HindIII digested lambda phage DNA, M3- 100bp DNA ladder

DNA yield was more or less same showing no significant variation among the samples (Fig. 3A). NifH amplification results were also positive in all the attempts clearly proving the marked presence of nitrogen fixing genes (Fig. 3B). The soil samples of day of sowing (0 day) and period of harvesting (150 day) were considered for cloning and sequencing studies. The clone libraries for each soil were constructed to understand the level of complexity and diversity of the two soils. NifH gene pools were prescreened by RFLP and the selected clones were subsequently sequenced that revealed high levels of diversity in the soils (Fig. 4). A clone library was created for both the samples; 0 day as well as 150 day soil and each of the 8 libraries were screened for a total of 30 clones each. The white clones with nifH insert clones from each soil were picked up and screened for the presence of nifH inserts by reamplification of the nifH fragments from E. coli transformants. Different restriction patterns were recorded for the reamplified nifH fragments from the nifH

Volume : 4 | Issue : 2 | Feb 2014 | ISSN - 2249-555X

clones with HaeIII and MspI. RFLP analysis resulted in 14 and 13 restriction patterns respectively, from DNA of 0 and 150 day soil. As a conclusion, we got enough evidence to state the presence of free living diazotrophs at the experimental site across the cropping period of cotton. The root nodulation assay as well as detection of nifH gene and cloning sequencing analysis gave enough evidence of the existence of diazotrophs at the experimental site. The study of diazotrophic community structure, diversity as well as its functional significance should be the next part of the multidimensional approach in order to understand the concept of microbial ecology clearly.

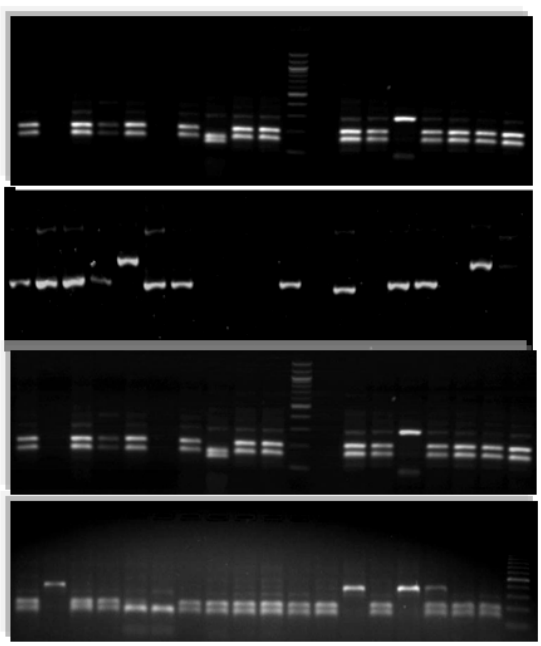


Fig. 4. Screening of clone libraries to select clones for sequence analysis. The DNA ladder represents 100 bp DNA molecular marker and rest of the lanes represent restricted profiles of nifH amplicons of positive clones of the clone library analysis to identify unique restriction patterns.

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