

Original Research Article

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Bacterial Community Analysis of Soybean (*Glycine max*) Sprayed with Panchagavya Revealed by DGGE

Biradar Balasaheb Gunwantrao^{1*}, C.R. Patil² and Malik Ahmed Pasha¹

¹Department of Biotechnology, University of Agricultural Sciences, Dharwad, India

²Department of Agricultural Microbiology, Institute of Organic Farming, University of
Agricultural Sciences Dharwad, India

*Corresponding author

ABSTRACT

Present study was aimed to understand the bacterial community composition, diversity, richness, carrying capacity and population dynamics present on soybean leaf in response to Panchagavya sprays using DGGE (Denaturing Gradient Gel Electrophoresis) and molecular fingerprinting technique. The seeds of soybean variety JS-335 were collected, surface sterilized and used in a pot culture study. *In-vivo* pot experiment was carried out in four sets. First three sets were sprayed on 15th day after sowing (DAS), 30th DAS and at both 15th and 30th DAS respectively and the fourth set was kept as control without spraying throughout the experiment period. The metagenomic DNA was obtained in the range of 800 to 1214 ng / μ l. The Shannon diversity, Range Weighted Richness and Pielou's evenness values clearly indicated that spraying of Panchagavya twice at 15th and 30th DAS increased bacterial diversity and microbial carrying capacity and even distribution of species in phyllosphere as compared to single time spraying and control samples without spraying. Pareto Lorenz (PL) curve values obtained revealed that more than 80 % of bacterial diversity was represented by a specialized community present in phyllosphere. The bacterial dynamics in sprayed samples were higher than their unsprayed counterparts. From this study it was found that the indices of bacterial diversity, richness and evenness were found to be higher in phyllosphere receiving two sprays (on 15th and 30th) of Panchagavya as compared to single spray and control samples.

Keywords

Phyllosphere,
Panchagavya,
Metagenomics,
Diversity,
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Introduction

The aerial part of plants colonized by the microbes is termed as the phyllosphere. Phyllosphere represents the interface between the above-ground parts of plants and the air. Most work on phyllosphere microbiology has focused on leaves that are normally colonized by a variety of microorganism including bacteria, yeasts and fungi. The most abundant phyllosphere colonizers are bacteria; conservative estimates indicated that roughly one billion square kilometres of worldwide

leaf surface host more than 10^{26} bacteria (Delmotte *et al.*, 2009). A number of environmental factors such as temperature, rainfall, wind and solar radiations are known to play an important role in determining patterns of bacterial phyllosphere colonization (Kinkel, 1997). Also, plant species (Kinkel *et al.*, 2000), plant morphology, the position and height of leaves (Thompson *et al.*, 1993) and leaf age (Ercolani, 1991) are factors known to influence the number and type of phyllosphere

microbial populations. Furthermore, various leaf surface features such as epidermal cell wall junctions (Davis and Barlansky, 1991) and grooves along the veins (Mariano and Carter, 1993), stomata and the base of trichomes also play an important role in the distribution of phyllosphere bacteria (Mew *et al.*, 1985). But, very limited work is done to study effect of spraying liquid organic manure such as Panchagavya on phyllosphere microbial community of crop.

Panchagavya is the blend of five products obtained from cow. The three are direct constituents dung, urine and milk and the two derived products are curd and ghee. When the above five products of the cow are suitably mixed and used, these have miraculous positive influence on crops (Swaminathan *et al.*, 2007). Existing scientific knowledge clearly suggest that these five constitutes contains nutrients and growth promoting substances that accrue during various stages of preparation and hence when applied to crops they are known to improve growth and yield of crops. An attempt was made to understand the effect of spraying Panchagavya on native phyllosphere microbial community of soybean grown under green house conditions using culture independent method. The method has been used to understand the composition of the phyllosphere micro-biota (Lambais *et al.*, 2006 and Yang *et al.*, 2001) and found very useful.

Materials and Methods

Preparation of pots

Red sandy loam soil was collected from Main Agricultural Research Station (MARS), UAS, Dharwad. The soil was sieved through 0.4 mm mesh. Further, it was autoclaved and three kg sterilized soil was filled into 15 cm diameter plastic pots.

Sterilization and pre-germination of soybean seeds

The seeds of soybean (JS-335) used in this study were obtained from, the All India Co-ordinate Research Project on Soybean, Dharwad, Centre, UAS Dharwad. Undamaged, uniform colour, sized and good quality soybean seeds were selected. The seeds were surface sterilized by immersing them in 0.1% of mercuric chloride solution for 1 min followed by washing in sterile distilled water. Surface sterilized seeds were soaked in sterile distilled water for one hour and 10 such seeds were transferred aseptically on 0.8 % water agar plates. The plates were incubated in dark condition at 28 °C for 24-36 hr for germination. Five good, germinated seeds were sown in each pot and kept in green house. The moisture level in pots was maintained near field capacity throughout the study period.

In-vivo pot experiment

A pot culture experiment was conducted in the green house to assess the bacterial changes in phyllosphere of soybean plant in response to application of Panchagavya. This experiment was carried out in four sets, each set replicated four times. First set of four pots were sprayed on 15th days after sowing (DAS), the second set were sprayed on 30th (DAS), the third set was sprayed on both 15th and 30th (DAS) all sets sprayed with 3 per cent freshly prepared Panchagavya. A fourth set was kept as control without spraying throughout the experiment. Fifty leaf samples from sprayed and control plants were collected separately at 24 hr, 48 hr and 72 hr after each spraying.

Extraction of DNA from phyllosphere

Fully developed leaves from each set of soybean plants were collected in sterile

polyethylene bag at each stage and stored immediately at 4°C and subsequently used them for isolating bacterial community DNA from phyllosphere using the combination of different protocols given by Delmotte *et al.*, (2009); Knief *et al.*, (2008) and Kim *et al.*, (2010). Collected leaf samples were gently rinsed with sterile distilled water to remove dust particles adhering to leaf surface. Ten leaves were placed in a sterile polyethylene tube (Tarsons) containing 10 ml of T₁₀E₁ buffer (pH 7) and kept for gentle shaking at 60 rpm for 30 min. After shaking the leaves were removed and the buffer used for washing was taken in another sterile polyethylene tube. This mixture was centrifuged at 18,000 rpm for 15 min, the supernatant was discarded and the pellet was collected in 2 ml Eppendorf tube. To this tube one ml of DNA extraction buffer [containing 100 mM Tris- HCl (pH-8), 100 mM Sodium phosphate buffer (pH-8), 100 mM EDTA (pH-8) and 1.5 M NaCl along with 50 µl of 10 % SDS], was added and mixed gently. Further contents were properly mixed by vortexing for 30 seconds followed by agitation at 1400 rpm at 60°C for 30 min. Vortexed tubes were centrifuged at 13200 rpm for 10 min at 4°C. The clear supernatant was collected and transferred to a fresh 2 ml micro-centrifuge tube. Purification of DNA was done by using organic solvents. Equal volume of chloroform: isoamyl alcohol (24:1 v/v) was added to the supernatant, to remove protein and other impurities. This homogenous mixture was thoroughly mixed and centrifuged at 13000 rpm for 20 min at room temperature. The clear supernatant was collected and transferred to a fresh 1.5 ml micro centrifuge tube. To this supernatant, equal volume of ice-chilled isopropanol was added for precipitating DNA. The mixture obtained was kept at -20 °C overnight and centrifuged at 13200 rpm for 10 min at 4°C and the supernatant was discarded recovering the pellet. The pellet was collected and

washed with 70 % ethanol further, air-dried to remove traces of ethanol and dried pellet was dissolved in 100 µl of T₁₀E₁ buffer and stored at -20 °C until further use. The concentration of extracted DNA was quantified using Nano Drop ND 1000 Spectrophotometer.

PCR Amplification of 16S rRNA gene using universal primer pair

Purified DNA samples were taken for PCR amplification. Hypervariable (V₃) region of 16S rDNA was amplified using PRBA338 with GC clamp and PRUN518 primers (Nakatsu *et al.*, 2000) (Fig. 2). The reaction mixture was prepared for final volume of 10 µl which contained 0.25 pico moles each of forward and reverse primers, 0.1 mM each of dNTP's, 1X Taq buffer A containing 1.5 mM MgCl₂ (Merck Bioscience, India) and 1 unit of Taq DNA polymerase (Merck Bioscience, India). The PCR was performed in an automated thermal cycler (Eppendorf master cycler, Germany) with following PCR programme, initial denaturation of seven minutes at 95 °C followed by denaturation at 94 °C for 45 seconds, annealing at 55 °C for 45 seconds and 45 seconds primer extension for 32 cycles, followed by 10 minutes final extension at 72 °C. PCR reaction was carried out for all the samples along with their respective control at regular intervals after completion of PCR, amplified products were analysed using 1 % agarose gel.

Denaturing Gradient Gel Electrophoresis (DGGE) analysis

The PCR products were further analyzed by performing Denaturant Gradient Gel Electrophoresis (DGGE) as per the protocol given by Muyzer (1999). PCR product was separated in 12 % polyacrylamide gel containing 30 % to 80 % denaturant (40 % formamide and 7M urea corresponds to 100 % denaturant) (Muyzer *et al.*, 1999). A 100

bp ladder was also loaded in each gel. The gel was run in 1X TAE buffer for 18 hours in IngenyPhorU unit at 170 volts. After completion of run, gels were carefully removed and stained by using silver staining protocol (Torsvik and Ovreas, 2002). After staining, the gel was dried sufficiently and the documentation was done using Syngene G box gel documentation unit and processed by Gene Tools software (Syngene). Bands were scored in the Gene Tools, by giving lowest score to the least intense band in the gel. On the basis of this scored data, Sorenson's similarity coefficient, Shannon's diversity index (Shannon, 1948), Range weighted richness (Marzorati *et al.*, 2008), Pielou's evenness index (Pielou, 1966), Pareto Lorenz curve (Lorenz, 1905), Moving window analysis (Wittebolle *et al.*, 2008) were calculated to analyze respectively the similarity, diversity and carrying capacity, species evenness, functional organization and microbial dynamics of bacterial species present during the stages of Panchagavya.

Result and Discussion

Metagenomic DNA isolation and PCR amplification of control and panchagavya sprayed phyllosphere samples

The metagenomic DNA extracted from sprayed and control soybean leaves using this protocol proved to be very good as substantial quantity with optimum concentration and with no visual shearing on 0.8 % agarose gel electrophoresis was obtained. The average yield of DNA obtained was in the range of 800 to 1214 ng / μ l with a purity ratio of 1.75-1.84 at 260/280 using Nano Drop ND 1000 Spectrophotometer (Fig. 1). The primer used in this study also resulted in amplification of their targeted site with expected size of 180 bp indicating that primers targeted the exact region of 16S rRNA. There was no amplification in the negative control

indicating no contamination in the PCR components.

Analysis of DGGE profile

The DGGE profiles revealed several bands in all the sample lanes, each band representing an operational taxonomic unit (OTU) (Fig. 3). Higher differences were observed in DGGE profiles of control and panchagavya sprayed samples, which were reproducible. The DGGE profile of Panchagavya sprayed and unsprayed samples were compared and some of the OTUs were with medium and low intensity and few were prominent. Some OTU's were unique to stages of crop, like OTU No. 6, 7, 8, 9, 10, 11 (Fig. 3) were observed only in plant leaves sprayed on 30th DAS with Panchagavya while in unsprayed samples these bands were not observed. Similarly, band No. 1, 2, 3, 4, 5 (Fig. 3) were observed in plant leaves sprayed on 15th DAS with panchagavya. More numbers of bands were observed in both control and sprayed samples indicating that the phyllosphere represented a very high bacterial populations. As compared to control samples the sprayed samples recorded higher number of bands. Thus, clearly indicating that the spraying of Panchagavya lead to the increase in bacterial population in phyllosphere. Two sprays of Panchagavya increased number of bacterial population as compared to single spray which was evident from the band number and intensity of the two samples as revealed on DGGE.

Shannon diversity index

Shannon diversity index values were obtained from DGGE profile of each lane and the average values for sprayed and unsprayed samples at each stage are compiled in table 1. The Shannon diversity analysis values clearly indicated that spraying of Panchagavya increased bacterial diversity in phyllosphere

at all stages viz., 15th day, 15th + 30th day and 30th day against their respective unsprayed control samples. The phyllosphere diversity in control (unsprayed) plants also, increased with age of plant even though control plants were sprayed only with water (Table 1). The Shannon diversity index provides important information about rarity and commonness of species in a community. When both diversities and richness increase Shannon diversity index value also increases. The changing values of Shannon diversity index in Panchagavya sprayed samples (Table 1) drawn at different intervals clearly suggested that both number of species and also number of individuals within species changed over time due to spraying which clearly demonstrated that Panchagavya spraying was responsible for increased bacterial population of phyllosphere. Panchagavya is good source of bio-fertilizer strains such as *Azospirillum*, *Azotobacter*, *Phosphobacteria* and *Pseudomonas* besides ammonia and nitrite oxidizers were found to colonize the leaves and increased the ammonia uptake and total N supply of crop Solaiappan (2002) this may be the probable reason for increase in Shannon the diversity.

Range weighted richness (Rr)

The range weighted richness values for both sprayed and unsprayed phyllosphere at different intervals were found to be higher (all are more than 30) (Table 1). However, the values for control (without Panchagavya sprayed) were much lower than those observed with Panchagavya sprayed phyllosphere sample irrespective of the time and number of sprays (Table 1). The critically analyzed bands were used to calculate species richness and expressed as range weighted richness (Rr). The Rr value of more than 30 implied that the phyllosphere is a typical and very habitable environment with broad carrying capacity, high microbial diversity

and high range weighted richness. Similarly, the values of range weighted richness for all samples were higher in phyllosphere irrespective of spray schedule. However, higher richness value was documented in phyllosphere sprayed at two stages as compared to those without spraying. This clearly suggested that Panchagavya which by itself is a rich source of bacteria (Biradar, *et al.*, 2015) when sprayed to phyllosphere selectively increased richness of bacterial species. Panchagavya was prepared from cow dung, cow urine, milk, ghee and curd which are good sources of native microorganisms. Although, these microorganisms may provide enough nutrients in the area of application, but they help in quick build up of beneficial microorganism for the enhancement of activity of micro-flora and fauna in sprayed area (Yadav *et al.*, 2005).

Pielou's evenness index

The Pielou's evenness index of bacterial population in phyllosphere (Table 1) appeared to be moderately even between (0.60 - 0.80) during different stages of Soybean growth. Pielou's evenness index value 1 indicated that species distribution in sample was highly even while, an index value of 0 indicated highly uneven distribution of species present in sample (Pielou, 1966). Panchagavya sprayed samples showed higher even species distribution as compared to unsprayed control samples. Spraying Panchagavya at 15th and 30th days resulted in higher even distribution of species as compared to control and Panchagavya sprayed at only one time (Table 1). This is possibly because spraying of Panchagavya second time (15th day followed by 30th day) could have added similar species of microbes on phyllosphere and thus contributing to greater evenness of species (Rasche *et al.*, 2006). It is well known that the phyllosphere bacteria have agricultural and environmental importance as they affect plant

growth and suppress the colonization and infection of tissues by plant pathogens (Lindow and Brandl, 2003). Hence, increased bacterial diversity in the phyllosphere due to spraying of Panchagavya as observed in this study could significantly influence soybean plants both agriculturally and environmentally. These may be the probable reasons for increase in yield of crop plants with Panchagavya application was due to enhancement in the biological efficiency of crop plants phyllosphere Natarajan (2002).

Functional organization (Fo)

Phyllosphere of soybean showed that the curve values for all the samples were more than 80 per cent on Y axis (Fig. 4) at 20 per cent intercept on X- axis. This implied that a small amount of the species in each sample was dominant and all the others were present in low numbers (Lorenz, 1905). It is also likely that a specialized microbial community could exist in each sample (Fernandez *et al.*, 1999). If curve values for the samples are more than 80 per cent at 20 per cent intercept on X-axis, then the microbial community could be functionally highly organized but fragile to external changes Massimo *et al.*, (2008). Spraying may disturb the native microbial population on phyllosphere which might require longer recovery times for microbes to maintain their favourable environment Massimo *et al.*, (2008). It is evident from the results of the present study that Panchagavya which inherently has very high bacterial diversity when sprayed to soybean contributed to more diversity of bacteria in phyllosphere while repeated sprays could have contributed for increased populations of the same bacterial species which must have resulted in functionally highly organized bacterial community. Curves with 25 per cent PL range represented the community with high evenness, while those with 45 per cent PL range and the area around

it represented communities with lower evenness as compared to the 25 per cent PL curve.

In this study PL curves for all the samples showed a value of more than 80 % thus, representing a specialized community in which a small amount of the species were dominant and all the others were present in low numbers. The presence of naturally occurring, beneficially effective microorganisms (EMOs) predominantly those belonging to lactic acid bacteria (*Lactobacillus*), yeasts (*Saccharomyces*), actinomycetes (*Streptomyces*), photosynthetic bacteria and certain fungi in Panchagavya; act as bio-pesticides, provide the easy micronutrient source for the plant, may help to maintain beneficial microbes on leaf surface which ultimately increase growth and yield of crops (Rasche, 2006). Culture-independent DGGE analysis methods earlier revealed that bacteria do not occur in a uniform pattern across leaf surfaces (Weller and Saettler, 1980; Leben, 1988). In this study the phyllosphere bacterial communities were more diverse than previously detected (Yang *et al.*, 2001).

Microbial community dynamics

Microbial community dynamic of soybean leaves unsprayed and sprayed with Panchagavya clearly revealed that spraying increased community dynamics and the highest was observed with samples receiving two sprays one on 15th and another on 30th DAS. The average changes in bacterial community for unsprayed phyllosphere samples were 52 with a deviation of 11.08 while the average change in bacterial community in phyllosphere sprayed with Panchagavya was 37 with a deviation of 4.96 over 30 days (Fig. 5). The values of change in bacterial community of unsprayed leaf sample were 27 from 15th to 30th day while the values

between 15th and 30th day sample was 11. The values of bacterial community changes in leaves sprayed with panchagavya were much narrower with a value of 11 between leaves sprayed on 15th day only and those sprayed on 15th and 30th days and a value of 10 between the sample sprayed on 15th day and sprayed on 30th day only. The changes in bacterial community between the samples sprayed on both 15th and 30th days and only 30th day was

with a value of 1. Dynamics of a microbial community in a Panchagavya sprayed sample is a measure of the average rate of change in parameter and degree of change between consecutive DGGE profiles of the same community over a fixed time interval (Marzorati *et al.*, 2008). This situation was assumed to represent a medium level of bacterial population dynamics present at all stages of Panchagavya sprayed samples.

Table.1 Bacterial richness, evenness and diversity measurement in panchagavya sprayed and unsprayed samples calculated based on DGGE profile

Sampling intervals						
Treatments	15 th Day		15 th + 30 th Day		30 th Day	
	Control	Sprayed	Control	Sprayed	Control	Sprayed
Primer pairs						
Shannon diversity	2.33	2.48	2.42	2.68	2.44	2.59
Range weighted richness	98	125	108	144	111	136
Pielou's evenness	0.71	0.70	0.78	0.76	0.71	0.74

Fig.1 Metagenomic DNA isolation from control and panchagavya sprayed phyllosphere samples M - Mlu single digest ladder 1 – 15th day / 24 hr control, 2 – 15th day spray/24 hr sample, 3 – 15th day /48 hr control, 4 – 15th day spray/48 hr sample, 5 – 15th day /72 hr control, 6 – 15th day spray/72 hr sample, 7 - 15th + 30th day / 24 hr control, 8 -15th + 30th day spray/ 24 hr sample, 9 -15th + 30th day/48 hr control, 10 -15th + 30th day spray/48 hr sample, 11 - 15th + 30th day/72 hr control, 12 - 15th + 30th day spray/72 hr sample, 13 - 30th day/24 hr control, 14 - 30th day spray/24 hr sample, 15 - 30th day/48 hr control, 16 - 30th day spray/48 hr sample, 17 - 30th day /72 hr control, 18 - 30th day spray/72 hr sample

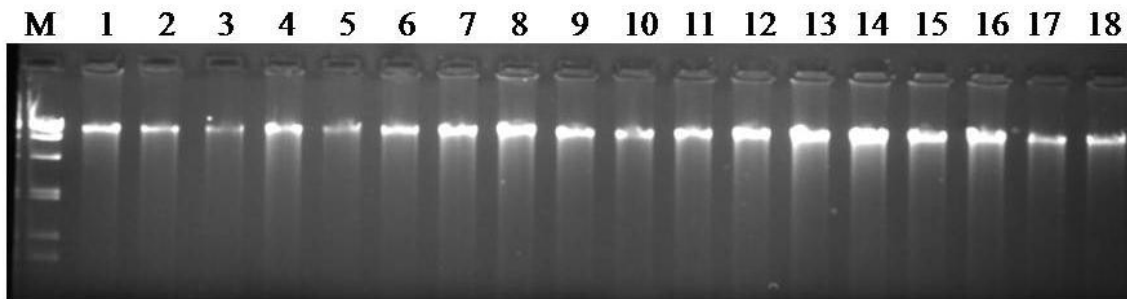


Fig.2 PCR amplification of control and panchagavya sprayed phyllosphere samples using PRBA338-PRUN518 primer pair M -100 bp ladder, N – Negative control 1 – 15th day / 24 hr control, 2 – 15th day spray/24 hr sample, 3 – 15th day /48 hr control, 4 – 15th day spray/48 hr sample, 5 – 15th day /72 hr control, 6 – 15th day spray/72 hr sample, 7 - 15th + 30th day / 24 hr control, 8 -15th + 30th day spray/ 24 hr sample, 9 -15th + 30th day/48 hr control, 10 -15th + 30th day spray/48 hr sample, 11 - 15th + 30th day/72 hr control, 12 - 15th + 30th day spray/72 hr sample, 13 - 30th day/24 hr control, 14 - 30th day spray/24 hr sample, 15 - 30th day/48 hr control, 16 - 30th day spray/48 hr sample, 17 - 30th day /72 hr control, 18 - 30th day spray/72 hr sample

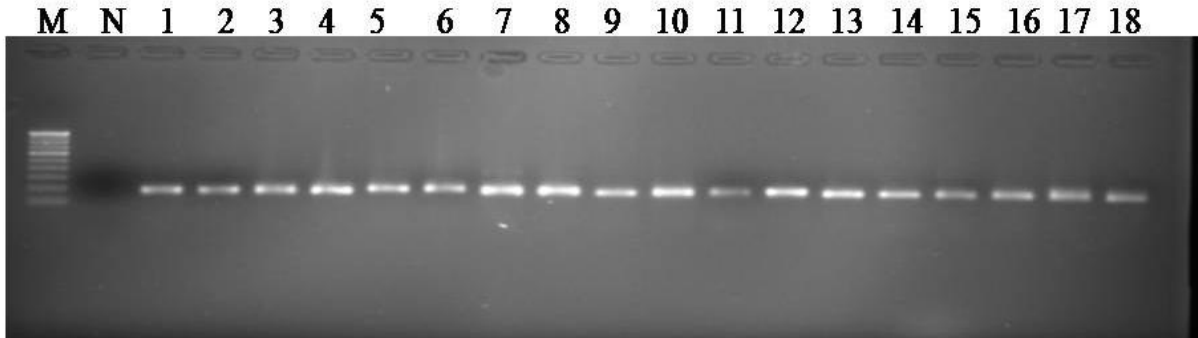


Fig.3 PCR-DGGE profile of control and panchagavya sprayed phyllosphere samples. 16S rDNA amplified by PRBA338GC and PRUN518 primers was separated in 12 % polyacrylamide gel containing 30%-70% denaturant and silver stained. M -100 bp ladder, 1 – 15th day / 24 hr control, 2 – 15th day spray/24 hr sample, 3 – 15th day /48 hr control, 4 – 15th day spray/48 hr sample, 5 – 15th day /72 hr control, 6 – 15th day spray/72 hr sample, 7 - 15th + 30th day / 24 hr control, 8 -15th + 30th day spray/ 24 hr sample, 9 -15th + 30th day/48 hr control, 10 -15th + 30th day spray/48 hr sample, 11 - 15th + 30th day/72 hr control, 12 - 15th + 30th day spray/72 hr sample, 13 - 30th day/24 hr control, 14 - 30th day spray/24 hr sample, 15 - 30th day/48 hr control, 16 - 30th day spray/48 hr sample, 17 - 30th day /72 hr control, 18 - 30th day spray/72 hr sample

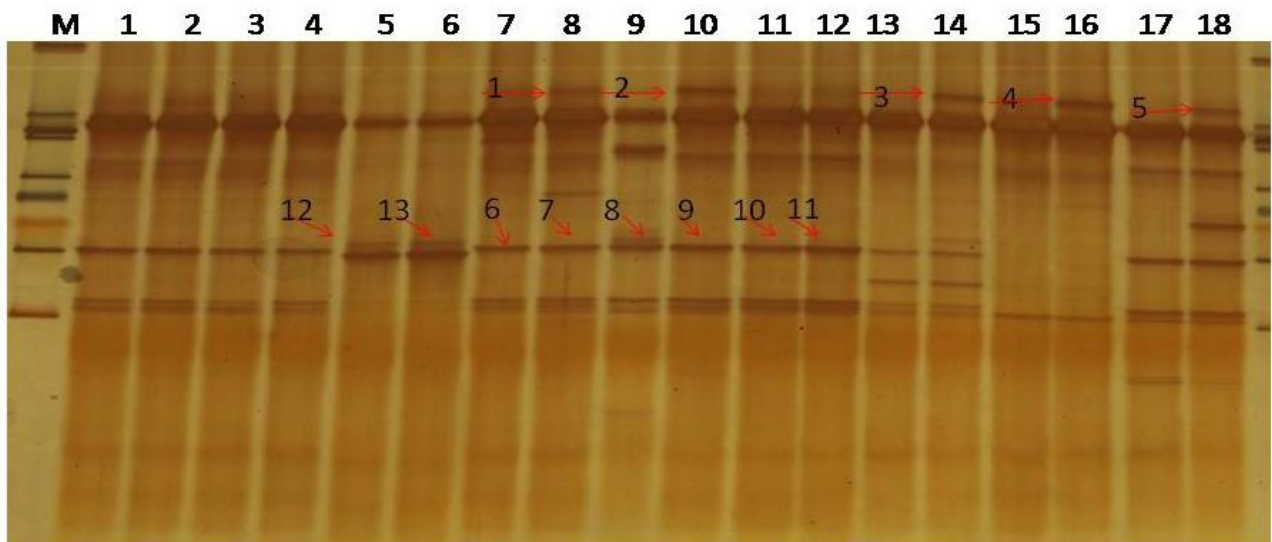


Fig.4 Pareto–Lorenz (PL) curves derived from the DGGE pattern of six different stages of Panchagavya sprayed and unsprayed samples. Large number of individuals belongs to only few species reflecting these panchagavya bacteria are highly functionally organised.

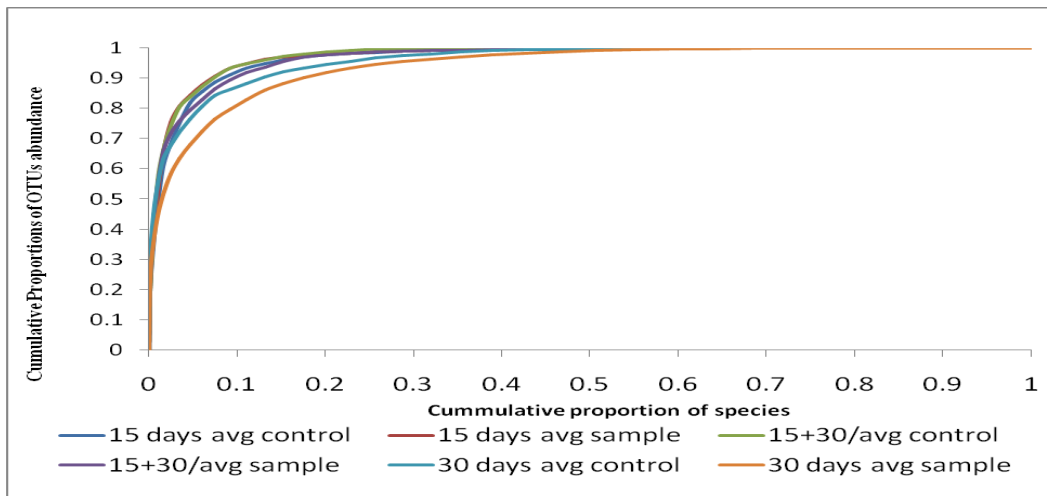
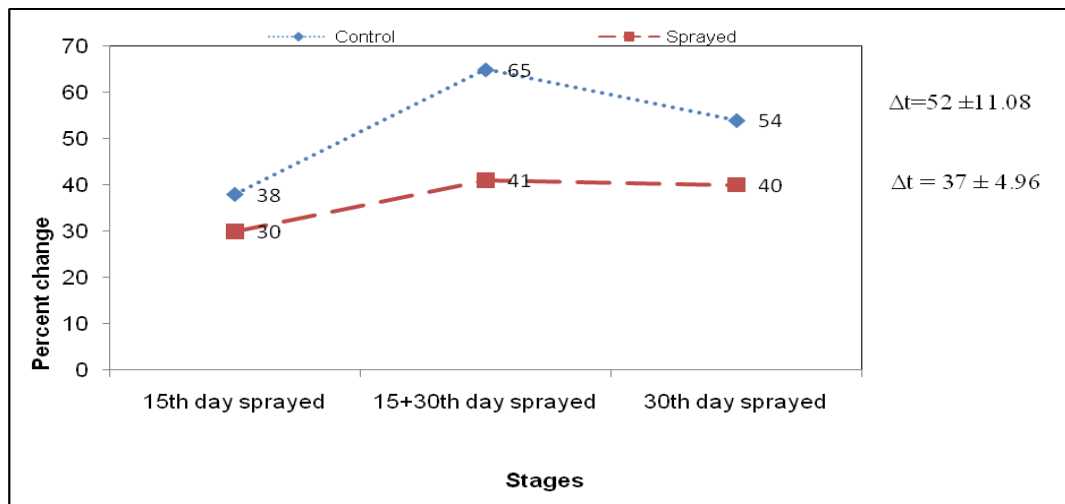


Fig.5 Moving window analysis for phyllosphere samples using PRBA338-PRUN518 primer pair simultaneously



Further, it could imply that in panchagavya spraying is responsible to increase new species bacterial population into pre existing bacterial community but these new species cannot interfere with the functionality of the pre-existing population as observed in this study.

In conclusion, the present research was mainly focused on studying the effect of

Panchagavya on phyllosphere bacterial community of soybean crop using a culture independent approach such as the DGGE analysis. In Sanskrit, Panchagavya means the blend of five products obtained from cow and the product is more widely used as a traditional agricultural practice. Panchagavya spraying has shown beneficial effects on a variety of crops to safeguard plants, soil microorganisms and to increase plant

productivity (Swaminathan *et al.*, 2007). The bacterial diversity, richness and evenness at all the stages were found to be higher in phyllosphere sprayed twice on 15th and 30th days as compared to leaves receiving only single spray of Panchagavya. Much less is understood about the identity or properties of the numerous non-pathogenic microbes that are present in Panchagavya; such microbes apparently play important roles in modulating population sizes of beneficial microbes in the phyllosphere, which are being exploited as biological control agents for disease and pest control. This study clearly showed that Panchagavya when sprayed to soybean leaves increased the microbial diversity and richness in the phyllosphere. The introduced microbial diversity comprised of more even community of bacteria inherently present in Panchagavya.

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