Academia Journal of Biotechnology 8(1): 001-007, January 2020
DOI: 10.15413/ajb.2019.0121
ISSN 2315-7747

Research Paper

Pyrosequencing reveals bacterial communities in *Bacillus thuringiensis* Bollgard II cotton rhizosphere soil – A metagenomics approach

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Accepted 29th December, 2020

ABSTRACT

Bacillus thuringiensis(Bt) cotton expresses crystal toxins to kill insects and simultaneously enters the soil through root exudates. However, transformed Cry1Ac and Cry2Ab proteins from *Bt* crops may interact with other organisms and induce rhizosphere environments changes, which has been assessed over *Bt*cotton rhizosphere soil after harvesting. In this study, the bacterial diversity was investigated using high through put sequencing (454 pyrosequencing) of metagenomic DNA from rhizosphere soil. We identified simulated communities of *Bt* cotton metagenome as 28,923 sequences totaling 6,357,310 base pairs with an average length of 565 bps and 23,378 as identified rRNA features, respectively. On total bacterial phyla, *Acidobacterium* were dominant species, followed by *Chloroflexus*, *Rhodomicrobium, Levilinea*, *Geobacter* and *Derxia.* In comparative metagenomics, 130 rhizobacterial communities survived between *Bt* and non-*Bt* and the control. This is the first assessment of rhizobacterial communities conducted on *Bt*cotton rhizosphere soil using 454 pyrosequencing platform.

Key words: BT cotton, *Bacillus thuringiensis,* crystal proteins, bacterial communities, rhizosphere soil, pyrosequencing.

INTRODUCTION

Rhizosphere soil is multifaceted region in the environment with various imperative functions, since it is influenced by root secretions linked with diverse group of soil microorganisms. Soil fertility depends upon the microorganisms because it oxidized organic matter and promotes the biogeochemical cycles. Moreover, soil enzyme activities are involved in nutrient dynamics and also catalyze the conversion of nutrient from unavailable to available forms. This act as a direct mediator for biological catabolism of soil organic and mineral components, which is also often closely related to organic matter, physical properties and microbial activity that changes rapidly than other parameters, though it indicate changes in soil health (Dick et al., 1996). Ecological stress or other environmental changes in soil ecosystem can be decided in advance through some soil profile indicators. The quality of soil is affected by various physical, chemical and biological properties and it is extended to productivity (Khattak and Hussain, 2007).

The cultivation of genetically modified (GM) crops alter the microbial communities in soil which can influence soil functioning systems, such as nutrient cycling and decomposition of organic wastes (Giovannetti et al., 2005). Among these GM crops, the *B. thuringiensis* cotton harbours the crystal toxic protein, rendering the crop resistant to the attack of *lepidopterae* and *spordopterae.* The existence of crystal toxic protein potentially changes the release of root exudates of the GM crops and also influences primarily the activity of microbes. The presence of cry proteins also depend on the soil type, season, crop variety and other environmental factors that vary with location and climatic nature.

The persistence and potential effects of the products on the soils and other habitats have to be thoroughly evaluated. Hence, new intensive molecular technologies have evolved to push out the real facts and impacts of all

knowledge required for a proper research. Advanced tool such as metagenomics could help to discover and characterize the remarkable number of uncultured microbes; in addition, it perfect analytical tool to study the genome heterogeneity, providing access to microbial diversity and impacts of rhizosphere soil (Handelsman, 2004). Moreover, the genomes of most microbial group could not be revealed because more than half of the known bacterial phylum was uncultured (Tringe, 2005). For the past few years, pyrosequencing is being used to study the uncultivable microorganisms in the environment. We assessed the potential effects of crystal proteins on the rhizosphere soil and their rhizobacterial communities changes through pyrosequencing and the treatments were compared. To the best our knowledge, this is the first study conducted on *Bt* cotton bacterial communities in rhizosphere region using pyrosequencing.

MATERIALS AND METHODS

Sample collection

Bacillus thuringiensis cotton Bollgard II seed, expressing Cry 1Ac and Cry2Ab protein (Monsanto company, U.S.A) were grown in each pot with ~9 kg of soils (red, sandy and clay soils and cow dung) in 1:1:1:1 ratio. The non-*Bt* and *Bt* plant roots adhered soils were collected approximately 2 mm in diameter to separate them from bulk soil at end of the growth period from the experimental pots. The collected soils were stored at -20°C before extraction of metagenomic DNA.

Metagenomic DNA extraction

Metagenomic DNA was extracted using the method of Yeates et al. (1998). Extraction buffer [1 ml of 100 mMTris-HCl (pH 8.0), 100 mM sodium EDTA (pH 8.0), 1.5 M NaCl] was mixed with 1 g of experimental rhizosphere soil. Glass beads were added with sample in vortex at maximum speed of 3 min. Sodium dodecyl sulphate (SDS) was added (100 µl; 20%) and vortex at maximum speed and continued for further 45 s. The sample was incubated at 65°C for 1 h and centrifuged at 6000 g for 10 min. The supernatant was collected and the soil pellet was re-extracted with further extraction buffer (1 ml), incubation at 65°C for 10 min and again centrifuged as above. Supernatants were transferred to centrifuge tubes (15 ml) containing a half-volume of polyethylene glycol (30%)/sodium chloride (1.6 M) and incubated at room temperature for 2 h. Samples were centrifuged (10000 g for 20 min) and the partially purified nucleic acid pellet resuspended in 50 µl of TE buffer (10 mMTrisHCl, 1mM sodium EDTA, pH 8.0). Potassium acetate (7.5 M) was added to a final concentration of 0.5 M. Samples were transferred to ice for 5 min, then centrifuged (16000 g, 30 min) at 4°C to precipitate proteins and polysaccharides. The aqueous phase was extracted with phenol/chloroform and chloroform/isoamyl alcohol and DNA was precipitated by adding 0.6 volume isopropanol. Mixtures were incubated at -20°C for 2 h. After 2 h, DNA was pelleted by centrifugation (16000 g for 30 min) and resuspended in TE buffer (50 µl). Extracted metagenomic DNA (1 µl) of each was quantified using Nanodrop (Wilmington DE, Nanodrop technologies).

PCR amplification and pyrosequencing

Rhizosphere soil metagenomic DNA was pyrosequenced in Roche 454 Life Sciences sequencer in Research and Testing Laboratory, Texas, U.S.A. The 16S rRNA gene of bacteria was amplified with 28F (5'-adaptor-tag-G AGT TTG ATC NTG GCT CAG-3') and 519R(5'-adaptor-GTN TTA CNG CGG CKG CTG -3') primers. Each sample was differentiated individually on the 454 sequencer using "tag". These unique sequence identifiers were attached to the forward primer in the pyrosequencing PCR. The thermal cycling conditions were 95°C for 5 min, 35 cycles of denaturing at 95°C for 30 s, annealing at 54°C for 40sec and extension at 72°C for 1 min, followed by another extension of 10 min at 72°C. Amplification of each could be observed by running each sample on a FlashGel. The amplification band strength is proportional to the amount of species contained in each sample. All amplified samples were pooled in equal amounts and purified using RapidTip PCR purification tips and Ampure beads (Agencourt/Beckman Coulter). The purified samples were pyrosequenced (Nacke et al., 2011). The obtained sequences were deposited in the NCBI metagenome bioproject sequence reads archives (Accession Nos.:SRX171212, SRX157291, SRX153140).

Pyrosequencing data analysis and comparative metagenomics

For the identification of unique taxonomic distribution, sequences were clustered into operational taxonomic unit (OUT) clusters with 96.5% identity (3.5% divergence) using USEARCH (Edgar, 2010). For each cluster, the seed sequences were merged into a FASTA formatted sequence file. Those files were queried against a database of high quality sequences from NCBI using a NET algorithm that utilizes BLASTN+. Using these NET and C# analysis pipelines, the resulting BLASTN+ outputs were assembled (Andreotti et al., 2011).

The comparison of metagenomes was analysed in the Metagenomics RAST server (MG-RAST version 3.2.0) to quantify the bacterial populations. The metadata were analysed against M5NR using a maximum e-value of 1e-5, a minimum identity of 60% and minimum alignment length of 15. Pyrosequence reads (454 sff format) were executed

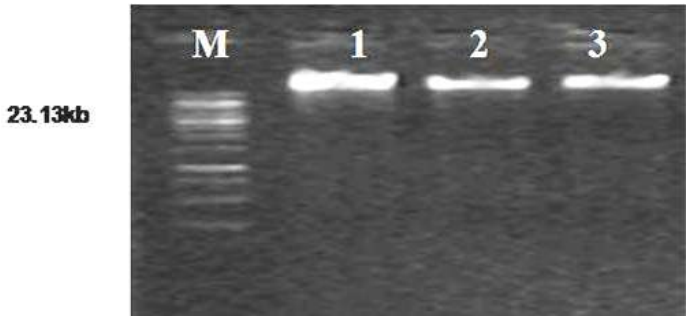

Figure 1: Metagenomic DNA of rhizosphere soils.
Lane M: Lambda DNA digested with *Hind* III(2.03Kb -23.13Kb);Lane 1: control soil; Lane 2: non-*Bt* cotton rhizospheresoil; Lane 3: *Bt* cotton rhizosphere soil.

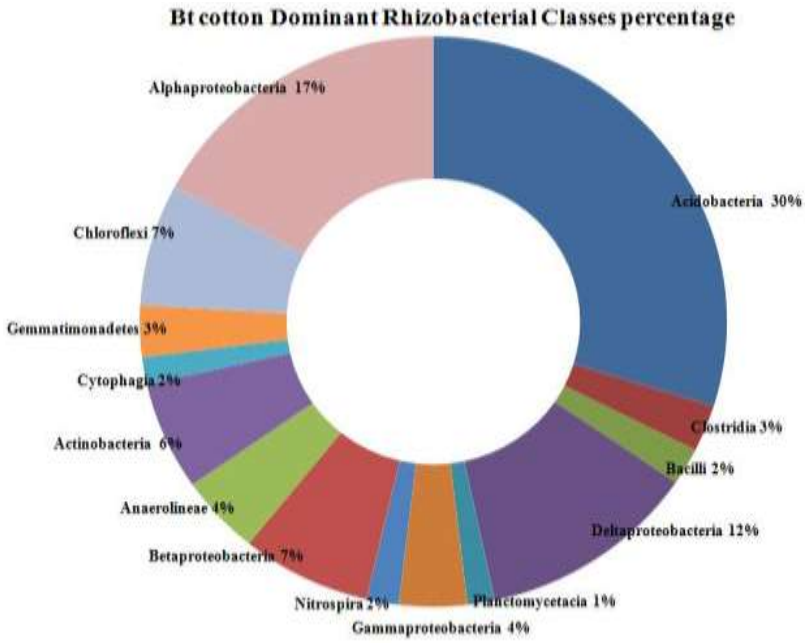

Figure 2: Dominant rhizobacterial classes percentage in *Bt*cotton.

in the process with a normalization step, all duplicate sequences and noise were removed for alpha diversity, domain distribution, taxon abundances and GC content. Heat map and rarefaction curve were also performed to compare between the *Bt* and non-*Bt* cotton rhizosphere soils (Meyer et al., 2008).

RESULTS

Metagenomic DNA was isolated from the rhizosphere soil samples, which is subjected to agarosegel electrophoresis and the metaDNA of the samples were at 23.1 kb range of molecular weight respectively (Figure 1). *B. thuringiensis* cotton, non-*Bt*cotton and control (no crop) soil pyrosequences were analysed, and insignificant differences in statistics, bacterial diversity, taxon abundance, shannon diversity, phylogenetic expression (heat map) and species richness (rarefaction curve) were observed. In general, the dominant phyla in the soils were Proteobacteria, Acidobacteria, Actinobacteria, Firmucutes, Bacteroidetes and Chloroflexi. A total of 35,881 sequences with 6,357,310 bp, sequences count (28,923), mean sequence length (565 ± 41 bp), mean GC percent (58 ± 3 %) and identified rRNA features (23,378) were analysed from the rhizosphere of *Bt*cotton. From the assigned sequences, dominant bacterial classes were diversified with Acidobacteria 30%, Alphaproteobacteria 17%, Deltaproteobacteria 12%, Betaproteobacteria and Chloroflexi 7%, Actinobacteria 6%, Gammaproteobacteria and Anaerolimea 4%, Gemmatimonadetes and Clostridia 3%, Cytophagia and Bacilli 2% and Plantomycetacia 1% (Figure 2). The average

number of different species annotations for subsamples of the complete dataset was up to 1614 species count with 35875 numbers of reads. Shannon diversity as 79.836 species was diversified and total of 10,765 unculturable rhizobacteria were identified, respectively. The 12,839 total sequences with 5,050,434 bp, count (9,196), mean length (549 ± 33 bp), mean GC percent (57 ± 3 %) and rRNA features (7,437) were identified from non-*Bt*cotton rhizosphere soil metagenome.

A very similar distribution of 95.6% taxa and Alphaproteobacteria (39.0%), Acidobacteria (19.0%), Gammaproteobacteria (6.0%),Betaproteobacteria (6.0%), Deltaproteobacteria (4.0%), Gemmatimonadetes(4.0%), Opitutae (4.0%), Cytophagia (3.0%), Chloroflexi(3.0%) and Flavobacteria (2.0%) were the dominant bacterial classes. The taxon abundance represented the taxonomic richness and evenness of the uncultured bacterium which was above the range of 2690 organisms and Shannon diversity was 92.300 of annotated species in the soil. In the control (no crop), soil metagenome contains 11,664 sequences, with a total of 4,591,797 basepairs. The statistics of the sequence count (8,324), mean length (551 ± 40 bp), mean GC percent (58 ± 2%) and identified rRNA features (7,129), the total relative abundances 95.8% bacterial domain were identified and predominant bacterial classes were Alphaproteobacteria (28.0%), Acidobacteria (12.0%), Betaproteobacteria (12.0%), Actinobacteria (10.0%), Gammaproteobacteria (8.0%), Deltaproteobacteria (6.0%), Bacilli (5.0%), Sphingobacteria (3.0%) and Cytophagia (3.0%) in the soil. The taxon abundance revealed the uncultured bacterium above the range of 2380 and Shannon diversity as 148.872 species abundance weighted average of the logarithm of the relative abundances of annotated species.

The comparative metagenomics of the three treatments between species were compared. The distribution of bacteria, eukaryota and unassigned organism domains was analysed. Thus bacteria was found to dominantly survive than eukaryote and unassigned organism. The *Bt*cotton soil bacteria showed maximum normalized value as 1, non-*Bt*cotton soil as very near to 0.9, and control soil as 0.778 normalized value. Moreover, the both cotton soils have unassigned organism of 0.408 normalized value. These analyses showed that *Bt*cotton rhizosphere metagenome clustered more closely to without crop soils and also to non-*Bt*cotton rhizosphere soil. Three treated soils rhizobacterial community were compared through venn diagram. It was shown that 112, 65 and 108 bacterial species specifically survived only on *Bt*, non-*Bt*cotton and without crop treated soils.

Moreover, there were 56 common bacterial species that survived between *Bt* cotton and without crop soils, 33 bacterial species between *Bt* cotton and non *Bt* cotton soils, also 20 bacterial species were common between non-*Bt* cotton and without crop soils. Overall, 130 bacterial species *Anaeromyxobacter* sp., *Acidobacterium* sp., *Ramlibacter* sp., *Nitrospira* sp., *Byssovorax* sp., *Smithella* sp., *Opitutus* sp., *Derxia* sp., *Gemmatimonas* sp., *Patulibacter* sp., *Polyangium* sp., *Methylococcus* sp., *Bacillus* sp., *Chloroflexus* sp., *Caldilinea* sp., *TM7 uncultured, Opitutus terrae, Steroidobacter* sp., *Levilinea* sp., *Actinomadura* sp., *Rhizobium* sp., *Eubacterium* sp., *Rhodovibrio* sp., *Rhodomicrobium* sp., *Schlegelella* sp., *Geothermobacter* sp., *Clostridium* sp., *Geobacter* sp., *Desulforhabdus* sp., *Beggiatoa* sp., *Halothermothrix* sp., *Conexibacter* sp., *Nitrosovibrio* sp., *Inquilinus* sp., *Desulforegula* sp., *Holophaga* sp., *Haliangiumtepidum, Koribacter* sp., *Longilinea* sp., *Pirellula* sp., *Streptomyces* sp., *Oleomonas* sp., *Flexibacter* sp., *WS3 uncultured, Bradyrhizobium* sp., *Sphingomonas* sp., *Dietzia* sp., *Bosea* sp., *Kaistobacter* sp., *Roseiflexus* sp., *Mesorhizobium* sp., *Altererythrobacter* sp., *Novos*p*hingobium* sp., *Pedomicrobium* sp., *Parvularcula* sp., *Hyphomicrobium* sp., *Defluviicoccus* sp., *Prosthecomicrobium* sp., *Rhodocista* sp., *Runella* sp., *Stella* sp., *Verrucomicrobium* sp., *Rhodoplanes* sp., *Deferribacter* sp., *Mycobacterium* sp., *Oscillochloris* sp., *Sterolibacterium* sp., *Ochrobactrum* sp., *Amycolatopsis* sp., *Flavobacterium* sp., *Novos*p*hingobium pentaromativorans, Rhodobium* sp., *Thermoanaerobacter* sp., *Brevibacillus* sp., *Brevundimonas* sp., *Parvibaculum* sp., *Methylocapsa* sp., *Pseudomonas* sp., *Flavisolibacter* sp., *Cellvibrio* sp., *Rhodoblastus* sp., *Nitroso*sp*ira* sp., *Solirubrobacter* sp., *Caulobacter* sp., *Burkholderia* sp., *Lysobacter* sp., *Iso*sp*haera* sp., *Geopsychrobacter* sp., *Pseudaminobacter* sp., *Wolinella* sp., *Alkaliflexus* sp., *Desulfofustis* sp., *Acidovorax* sp., *Salicola* sp., *Aquiflexum* sp., *Lysobacteryangp yeongensis, Iamiamajanohamensis, Actinomyces* sp., *Microvirga* sp., *Blastochloris* sp., *Methylosinus* sp., *Niastella* sp., *Methylobacterium* sp., *Azos*p*irillum* sp., *Arcicella* sp., *Janthinobacterium* sp., *Cytophaga* sp., *Roseomonas* sp., *Kouleothrixaurantiaca, Rhodopseudomonas* sp., *Thermodesulforhabdus* sp., *Sphingobacterium* sp., *Phenylobacterium* sp., *Ralstonia* sp., *Filomicrobiumfusiforme, Herbas*p*irillum* sp., *Bacillus niacin, Gemmata* sp., *Paracoccus* sp., *Thiorhodospira* sp., *Thermanaeromonas* sp., *Pseudomonas indica, Flavisolibacter ginsengisoli, Bacteroides* sp., *Terrimonas* sp., *Leptothrix* sp., *Balneimonas* sp., *Bacillus funiculus, Ammoniphilus* sp., and *Arthrobacter* sp. were commonly similar between all the three treated soils (Figure 3).

The data were furthermore compared using a maximum e-value of 1e-5, a minimum identity of 60%, and a minimum alignment length of 15 measured in amino acid for protein and RNA base pair databases. The species richness were calculated and constructed for three soils through rarefaction curve. The curve was plotted based on the number of species as a function on the number of samples. A reasonable number of individual samples were taken and more intensive sampling yield a very few additional number of species in *Bt* than non-*Bt*cotton and the steep slope indicates in a control that a large fraction of the species diversity remains the same (Figure 4). Heat map combination with dendogram showed relative abundances

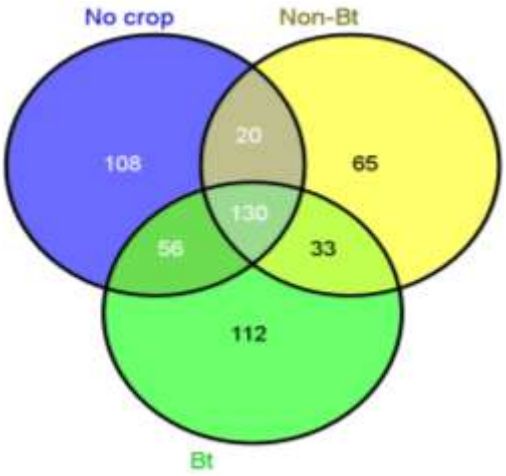

Figure 3: Venn diagram of rhizobacterial communities between treated soils

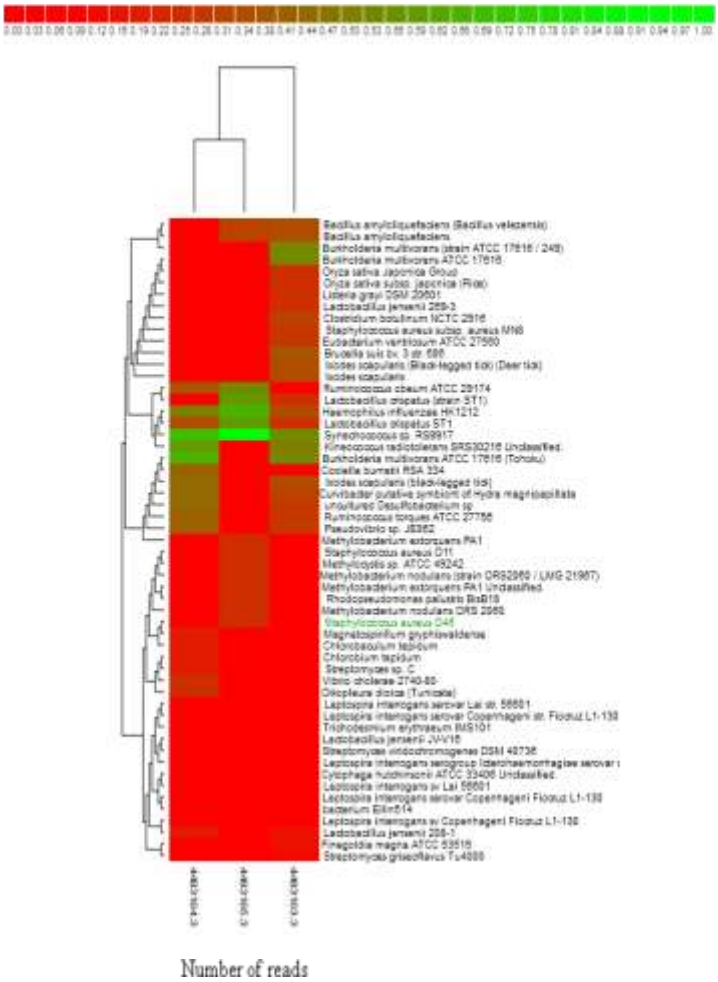

Figure 4: Heat map forthree treated soils.
Double hierarchal dendogram to assess bacterial strains distribution between the treatments using the weighted pair group clustering method *Bt* and non-*Bt*cotton rhizosphere soil compared with control, 4493163.3-*Bt* cotton (3), 4493165.3 (2) -Control, 4493164.3 (1)-non-*Bt*cotton.

of top 55 subsystem with percentage cutoff near 0.0 to 1.00. According to the cutoff level, the microbes are placed in to different groups with color indication. Red implies absence of expression, light green was partially expressed and dark green completely expressed specifically group of microbes in the sample.

DISCUSSION

The cotton yield depends on its soil fertility and it reacts unfavorably to any shift and also changes in periods of sowing until harvest. Soil fertility was closely related to organic matters which influence the physical, chemical and biological properties of the soil. When the rhizosphere soil is maintained properly, then only the whole ecosystem health could be preserved. So, the release of substances from root as exudates was potentially risk to the soil environment. In the present study, it was found five major dominant bacterial groups in all the rhizosphere soils such as Acidobacteria, Alphaproteobacteria, Deltaproteobacteria, Betaproteobacteria and Actinobacteria. These bacterial groups are involved in all the soil enrichment and plant growth. Barriuso et al. (2012) reported through next generation sequencing that Acidobacteria, Proteobacteria and Actinobacteria survived dominantly in the *Bt*maize rhizosphere soil, and this is in agreement with the finding of the present study. Proteobacteria were involved in the major cycle of the soil such as carbon, sulfur and nitrogen (Kersters et al., 2006).

In *Bt*cotton, *Burkholderia multivorams* strains were expressed individually and certain groups of bacteria were similarly expressed in non-*Bt*cotton soil than the control soil. The control and non-*Bt*cotton soils were commonly evolved and expressed phylogenetically from *Bt*cotton. Acidobacteria commonly survived in soils (Kielak, 2010) and all these members of this phylum are possible to play a related role in soil ecosystems. In summary, the bacterial phylum was significantly common among *Bt*, non-*Bt* and control, but in the distribution differed between each other in all the rhizosphere soils. Based on the results obtained, no major effects were identified in *Bt*cotton in all three different experimental soils. This finding is in line with previous report that observed significant variations in bacterial phyla and proteobacterial class abundances in Actinobacteria, Firmicutes, Verrucomicrobia, Cyanobacteria, Gemmatimonadetes and Alphaproteobacteria (Nacke et al., 2011). Moreover, previous study have reported predominant phylums of Proteobacteria, Actinobacteria and Gemmatimonadetes in the soil of livestock cotton production system (Martinez et al., 2010). The rhizosphere bacterial community structure variations were possibly due to soil profiles including enzyme levels and release of *Bt*genes.

In rhizosphere soil, the crystal proteins are not expressed and the protease level also increased. This may be the reason for increase and decrease of rhizosphere bacterial communities. Every pyrosequence readings have a unique identifier for each community individual to assess the bacterial diversity (Quince et al., 2009). The unclassified sequences showed maximum number owing to the increase in bacterial species in the rhizosphere soils. Due to increase in bacterial population in the *Bt*cotton rhizosphere soil, both cultivable and uncultivable bacteria were increased in the soil. This may be due to enzyme profile changes and release of minute crystal proteins from plant to soil through root exudates. This is in line with previous report on root exudation; enzyme activities and shed matters to the root surface were decreased in microbial population (Yang et al., 2011). As compared with the control, the microbial classes in the rhizosphere showed variation due to physico-chemical and biological characteristics (Grayston et al., 1988).

Roesch (2007) expected 0% dissimilarity in sequences, but it was dramatic in the estimation of species in the soil revealed by rarefaction. Rarefaction curves showed the effect of dissimilarity on the number of species count (Martinez et al., 2008). So, we found the dissimilarity of bacterial diversity between *Bt*, non-*Bt*cotton and control with minimum identity of 60%, indicating that species richness was high in *Bt*cotton in soil. Thus, few bacterial species were significantly and phylogenetically analogous in the taxonomic heat map with *Bt,* non-*Bt*cotton and control. The *Bt*cotton was taxonomically as well as phylogenetically analogues to each other with non-*Bt*cotton, indicating that similar bacterial species survived, but the numbers differed between each other. Finally, it was observed absence of differences between *Bt*and non-*Bt*cotton in all soils.

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Cite this article as:

Ebenezer KS, Antony AR, Velu RK (2020). Pyrosequencing reveals bacterial communities in *Bacillus thuringiensis* Bollgard II cotton rhizosphere soil – A metagenomics approach. Acad. J. Biotechnol. 8(1): 001-007.

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