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Metagenomic analysis of diversity and composition of soil bacteria under intercropping system *Hevea brasiliensis* and *Canna indica*

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Abstract. Composition of soil microbiomes plays important ecological roles and vital ecosystem processes in nature of soil. Indeed, interaction between plant root, microbes, and soil influences significantly biologically, physically and chemically of soil properties. In this study, high throughput sequencing of 16S rRNA gene of soil bacteria using Illumina platform was performed for analyzing composition, richness and biodiversity of soil bacteria in two different soil conditions. Two soil samples from Rubber tree – Canna indica intercropping areas and two soil samples from area without C. indica were collected and analyzed the diversity and richness of soil bacterial communities. Bioinformatic data analysis showed in average more than 5500 bacterial OTU were identified in each soil samples. Relative abundance of individual OTU between soil with and without C. indica showed statistically no difference. From 30 phyla that have been identified in both soil conditions, only 5 phyla (BH180.139, Cyanobacteria, Proteobacteria, TM6, and WS3) showed a statistically different ($p < 0.05$) in their abundance. A total 270 and 355 Familia and Genus respectively were identified. There is no statistically difference in richness, abundance and diversity of species between both soil conditions. However, intercropping soil with C. indica showed lower species abundance in comparison with soil without C. indica (ACE index 112 and 125,6 respectively). In contrast, soil with C. indica has more taxa richness in comparison with soil without C. indica (Fisher index 278 and 230,4, respectively)..

Keywords: 16S rRNA, metagenomic analysis, Rhizosphere bacteria, Intercropping system, Canna indica

1. Introduction

Rubber tree (*Hevea brasiliensis*) is one of economically important crop plants in some countries, especially in developing countries. With about 3.5 million hectare rubber tree plantation and total production about 2.7 tones raw rubber (Statistik Perkebunan, 2010), Indonesia is one of a major rubber producer in the world. Mostly rubber tree plantations in Indonesia are managed by traditional farmers (84.66%) and the rest by private corporates and Government (BPS, 2009). However, for the last couple years world rubber price is showing a declining trend. It makes rubber plantation is not any more interesting for most of traditional farmers. Increasing production cost especially fertilizer and labor costs that is not followed by an increase in rubber price is major problem in rubber tree

production. As the results, the agronomy and management system of rubber plantations belonged traditional farmers and Indonesian government are no longer ideal. Thus, low productivity and quality of raw rubber are theconsequents faced by raw rubber production.

Some efforts have been proposed to resolving this problems, one of them is using intercropping system rubber tree with other suitable plants. Intercropping system is a practice of growing a crop with other crops in close proximity in the same growing season. Basically, intercropping system is application of ecological principle and an efficiency effort of natural resources usage (Duan et al, 2011).

On the rubber plantation, usage of arable land under rubber tree canopy is a challenging idea. Since the rubber canopy is quite dense allowing little solar radiation through understorey. It has been reported only about 20% of incoming solar radiation is available under 4-5 years old rubber canopy (Ibrahim, 1991), thus determining of properly plants for intercropping system with rubber tree is critical factor for success intercropping system. Indeed, intercropping system has been shown could enhance soil nutrient contents such as N and P, soil enzyme activities and crop yield (Zhou et al, 2011; Ghosh et al, 2006).

Another important factor which appears as the result of intercropping system is change of soil microbial community. Several studies showed that structure of microbial community and metabolic capabilities could reveal soil quality conditions (Yao et al, 2006). Microbial community has important role in determining of the soil ecosystem and the conversion of soil nutrient cycling. Nitrogen, sulfur and phosphorus cycles, as well as ecosystem functioning are some crucial role of soil microbes. Later on it could change soil structure formation, organic matter decomposition, and toxin removal (Acosta-Martinez *et al.*, 2010; Karlen *et al.*, 1997; Gomes *et al.*, 2003). Indeed, soil microbial diversity is important for maintaining good quality of agricultural soil (Acosta-Martinez *et al.*, 2010; Garbeva *et al.*, 2004; Janvier *et al.*, 2007).

Since soil bacteria is huge not only in diversity but also in abundance, and most of bacteria are unculturable (Nihorimbere et al. 2011), thus using of standard culturing method will not enough to describe the richness of soil bacteria. Using high throughput sequencing technique (Next Generation Sequencing/NGS) of specific conserved genes or genomic fragments such as 16S rRNA, this information become accessible (Riesenfeld et al. 2004; Effendi et al, 2019; Ghosh et al. 2019). Using metagenomic analysis of 16S rRNA gene of soil microbial that isolated around rhizosphere area of *C. indica* root system, this research was aimed to investigate impact of intercropping of *C. indica* in the rubber plantation on diversity and richness of soil microbial.

2. Materials and Methods

2.1. Sampling

Soil samples were collected from rubber tree plantation of PTPN VIII in Subang, West Java, Indonesia. Soil samples consisted of 500 g soil samples were collected from three different sites nearby rhizosphere areas of Rubber tree-*Canna* plants (G) intercropping area or from three different sites nearby rhizosphere areas of *Rubber* trees without *Canna* plants nearby. Each three soil samples were homogenized and taken 50 g for DNA isolation. This procedure was repeated twice for each point locations. The soil samples were labeled according to location and kept in 4 °C until used.

2.2. DNA extraction and molecular works

Whole genomic DNA was extracted using PowerSoil DNA kit (MoBio). DNA extraction was done following manual procedure of the kit. A 0.25 gr soil of each sample were used as source of the

genomic DNA. All procedures were performed aseptically for avoiding contamination. The DNA results were quantified using NanoDrop and checked its quality using gel electrophoresis. The DNA was used as a template for PCR. A 2µL of DNA was added to 10µL PCR mix (GoTaq ® Green Master mix-PROMEGA) and 1 nmol of each forward and reverse 16S rRNA primer. Reaction was performed 35 cycles which consisted of 30 sec at 94ºC and continued with 57ºC of annealing for 20 sec, followed with 2 min 72ºC for elongation. A region V4 of 16S rRNA gene was amplified with primer F515 (5'-GTGCCAGCMGCCGCGGTAA-'3) and 907R (5'-CCGTCAATTCMTTTRAGTTT-'3) (Lane 1991). The PCR products were purified and subjected for automated Illumina Miseq platform (1st BASE-Malaysia) after the PCR products were normalized in equimolar amounts.

2.3. Sequences analysis

Raw sequence data generated from Illumina Miseq platform were processed in QIIME Ver 6.0 (Caporaso et al. 2010). All sequences shorter than 150 bp or longer than 600 bp were removed from downstream processing. Read were then aligned with 16S rRNA SILVA database (www.arb-silva.de) and GRD database (metasystems.riken.jp/grd/), then followed inspected for chimeric errors. "Species-level" of OTU was used in analysis, thus reads then were clustered at 97% similarity into OTUs. In this step, rare OTUs with only one (singleton) or two reads (doubleton) were deleted from downstream processing. Taxonomic assignment was carried out with the RDP Classifier (Wang et al. 2007).

2.4. Data analysis

Alpha diversity analysis was conducted using qiime2 for evenness and Faith phylogenetic distance index, and phyloseq for Shannon and Chao1 diversity index. Beta diversity was analyzed with Bray-Curtis and Weighted Unifrac index in Qiime2 Alpha and Beta diversity analysis were performed using Explicet ver 2.10.5 software (Robertson et al. 2013). Statistical comparison of alpha diversity between samples was carried out with Excel (Microsoft) whereas statistically different (*P-value*) was calculated with T-test. Sample-specific OTUs (showing significantly different relative abundances between samples) were assessed by T-test. Heat map which showed relative abundances between samples in certain taxa level was generated with Explicet ver 2.10.5 software.

3. RESULT AND DISCUSSION

After filtering total reads based on quality control of raw read data, total of 764,951 sequences were collected from 4 soil samples. About 300,570 singleton and chimera were identified and removed. Altogether 25,243 OTUs were identified after were aligned with SILVA database and GRD database with similar proportion among the 4 soil samples (Table 1).

Table 1. Number of reads after filtering and OTUs

Samples	Raw reads	Chimera and singleton	Number of OTU
With Canna (G20)-intercropping	177219	65846	6254
With Canna (G40)-intercropping	229973	92792	6546
Without Canna (TG20)	165555	62485	5810
Without Canna (TG40)	192204	79447	6633

3.1. Diversity and Structure of bacterial community

Analysis of microbial Diversity based on Shannon-Winner (H') index showed that the highest index value was occurred in the TG40 sample (H '= 3.0506) and the lowest value at G20 (H' = 2.9467) (Table 2). According to Fanani *et al.* (2013) a low H' values (H'≤1) indicate a low microbial diversity, whereas sample with H'≥3 indicate a high diversity in microbial community. Our data indicated all

soil samples have high microbial diversity, except soil sample from intercropping area collected from 20 cm soil depth which has moderate microbial diversity.

Table 2. Shannon-Wiener (H ') and Simpson (H2) diversity index values on G and TG at each soil depth conditions.

Sample	Indeks Shannon-Winner (H')	Indeks Simpson (H2)
With Canna - intercropping (G20)	2.9467	0.093
With Canna - intercropping (G40)	3.0236	0.085
Without Canna (TG20)	3.0148	0.085
Without Canna (TG40)	3.0506	0.078

The lowest H' index value for the G20 can be expected due to the presence of certain types of microbes that dominate the G20 area, so that microbial diversity has decreased. According to Cover (1991), the value of the diversity index will be maximized when all individuals are equally represented. The higher diversity value shows that a community has a relatively even number of individuals and no species domination. Microbial diversity is influenced by the physical chemistry of soil (Mejani et al, 2012; Effendi et al, 2019). Hill *et al.* (2003) reported that diversification function of a land will experience a decline, especially in acacia forests, oil palm plantations and rubber plantations.

Moreover, the Simpson index (H2) at the depth of the G20 has a higher index value of (H2=0.093) while the lowest value is found in the TG40 sample (H2=0.078). Based on the analysis results, the index value is less than 0.5 which means that each class at the four individual depths does not dominate. This condition shows that the microbial and ecological communities are still in a stable state.

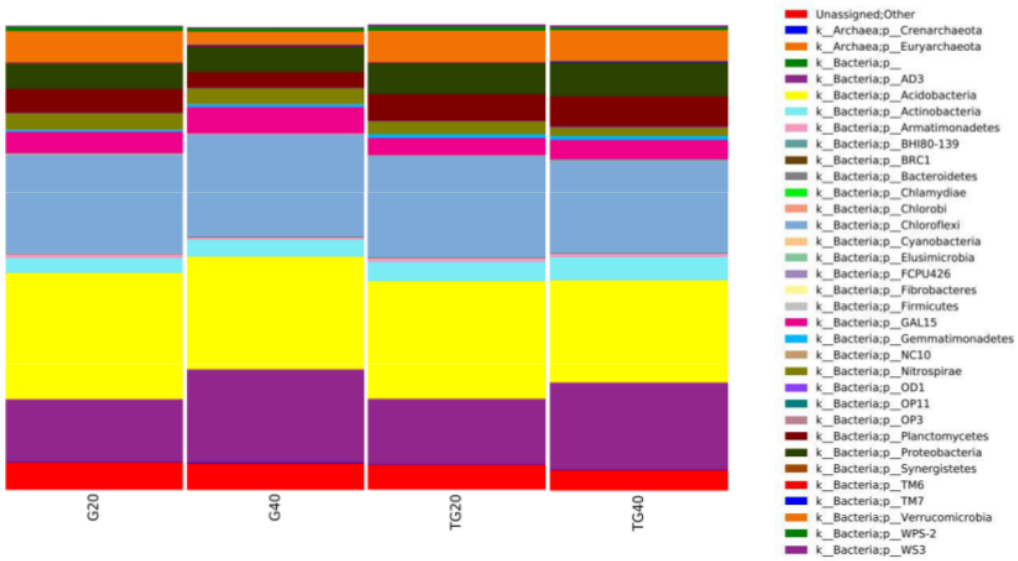

Figure 1. Relative abundance of the phyla from 4 soil samples (G20, G40, TG20, TG40).

Based on the number of observed OTUs, 97% OTUs were occupied by bacteria and the rest was Archaea (3%). Figure 1 showed relative abundance of phyla composition that identified. Although the 4 soil samples showed similar relative abundance, it is clear that some phyla abundance showed obvious variations between different soil conditions. A total 34 phyla were affiliated to bacteria phyla in database, whereas 2 remaining groups were still unclassified. The most abundant phyla of *Acidobacteria, Chloflexi* and *AD3* were found in all soil samples with relative similar proportion (25.8%, 21.6%, 16,7% respectively in sample G dan 23.5%, 20.5%, 16.2% respectively in samples TG). The next 6 phyla (*Verrucomicrobia, Protobacteria, Planctomycetes, GLA15, Nitrospirae* and *Actinobacteria*) were found in all soil samples at a relative abundance that higher than 2% but lower than 8% with obvious variations. The relative abundance of *Verrucomicrobia* were relative similar in G20, TG20 and TG40, but lowest in G20. *Protobacteria* were found more abundant in TG samples than in G samples.

Most of the phyla have similar abundance in the soil samples, but some phyla were identified different in abundance. The phyla of BHI80.139 ($p<0.00$), *Proteobacteria* ($p<0.02$), *Cyanobacteria* ($p<0.03$), TM6 ($P<0.03$), and WS3 ($p<0.02$) were found significantly different in their abundances. The complete data is shown in table 3. *Proteobacteria* and *Cyanobacteria* were found more abundant in TG samples. Both phyla were known involving in biogeochemical cycle particularly in nitrogen fixation (Vijayan and Ray, 2015). The dominant phylum of *Proteobacteria* was also reported in some publications in various soil types (Sul et al, 2013, Chu et al, 2010; Nacke et al, 2011). The bacterial community composition and diversity were largely affected by environment factors, soil pH and plant species (Wei et al, 2017). The soil pH was the key factor influencing bacterial diversity, with lower pH associated with less diverse communities (Wei et al, 2017). However, in this study, soil conditions and pH were similar. Soil analysis of TG and G samples indicated pH of 4.3 and 4.6, respectively. Moreover, their C organic contents were also almost same 1.54% and 1.95%, respectively. It indicates that the presence of Canna plant may contribute significant in abundance of *Proteobacteria* and *Cyanobacteria*.

Vijayan and Ray (2015) indicated that there is positive correlation on the total number of *Cyanobacteria* inhabiting tropical paddy wetland to total nitrogen in these soils. However, our data showed that the N total in the TG and G samples has no significant differences (0.45% and 0.36% respectively). According to soil analysis data, both soils conditions indicated low fertile soil condition.

Table 3. Signification of abundance of the phyla group between Canna samples (Cs) and without Canna samples (TGs)

Phylum	***Canna indica* (G)**	**Without *C. indica* (TG)**	***p-value***
AD3	0.33397 ±0.03336	0.32471 ±0.02294	0.46
Acidobacteria	0.51663 ±0.01456	0.47099 ±0.01643	0.20
Actinobacteria	0.06958 ±0.00217	0.09389 ±0.00446	0.07
Armatimonadetes	0.01092 ±0.00145	0.01410 ±0.00092	0.23
BHI80.139	0.00003 ±0.00002	0.00000 ±0.00000	0.00
BRC1	0.00006 ±0.00000	0.00000 ±0.00000	0.09
Bacteroidetes	0.00342 ±0.00081	0.00392 ±0.00078	0.42
Chlamydiae	0.00139 ±0.00023	0.00159 ±0.00017	0.38
Chlorobi	0.00018 ±0.00002	0.00010 ±0.00001	0.09
Chloroflexi	0.43239 ±0.00326	0.41125 ±0.01023	0.21
Cyanobacteria	0.00109 ±0.00006	0.00169 ±0.00005	0.03

Elusimicrobia	0.00227 ±0.00022	0.00316 ±0.00032	0.18
FCPU426	0.00283 ±0.00012	0.00311 ±0.00017	0.28
Fibrobacteres	0.00002 ±0.00001	0.00003 ±0.00002	0.41
Firmicutes	0.00069 ±0.00003	0.00125 ±0.00019	0.15
GAL15	0.10245 ±0.00512	0.07792 ±0.00306	0.09
Gemmatimonadetes	0.01192 ±0.00116	0.01736 ±0.00005	0.07
NC10	0.00148 ±0.00025	0.00052 ±0.00013	0.12
Nitrospirae	0.06793 ±0.00064	0.04387 ±0.00494	0.06
OD1	0.00286 ±0.00012	0.00392 ±0.00055	0.22
OP11	0.00003 ±0.00002	0.00000 ±0.00000	0.21
OP3	0.00005 ±0.00003	0.00003 ±0.00002	0.38
Planctomycetes	0.08480 ±0.00910	0.12221 ±0.00413	0.10
Proteobacteria	0.11121 ±0.00074	0.14108 ±0.00336	0.02
Synergistetes	0.00000 ±0.00000	0.00004 ±0.00002	0.21
TM6	0.00034 ±0.00005	0.00029 ±0.00008	0.03
TM7	0.00301 ±0.00032	0.00254 ±0.00010	0.28
Verrucomicrobia	0.09866 ±0.01891	0.13636 ±0.00072	0.21
WPS.2	0.01723 ±0.00170	0.01625 ±0.00148	0.42
WS3	0.00294 ±0.00033	0.00679 ±0.00022	0.02

CONCLUSION

This study showed that intercropping of *Hevea brasiliensis* with *Canna indica* may contribute in the status of diversity and abundance of soil bacteria. Most of the phyla members were not changed significantly after intercropping of *Canna indica*. However, *Proteobacteria* and *Cyanobacteria* were found lower abundance in the soil without *Canna indica* (TG samples), whereas both phylum has been reported positively correlated with biogeochemical cycles in the soil particularly in determining of nitrogen content in the soil.

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