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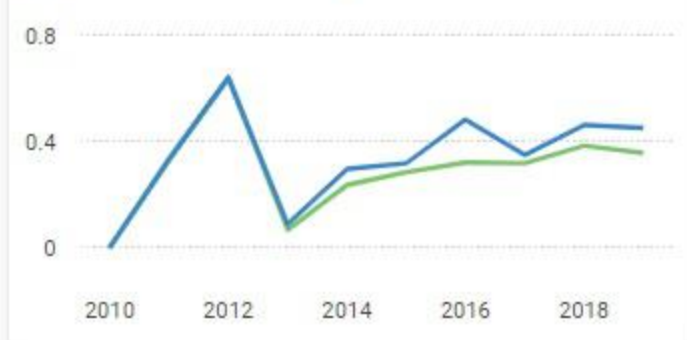
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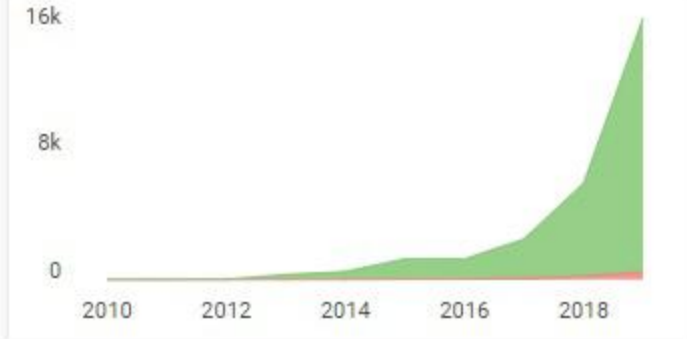
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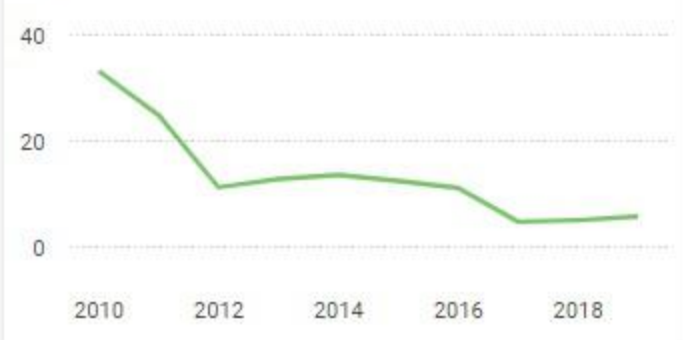
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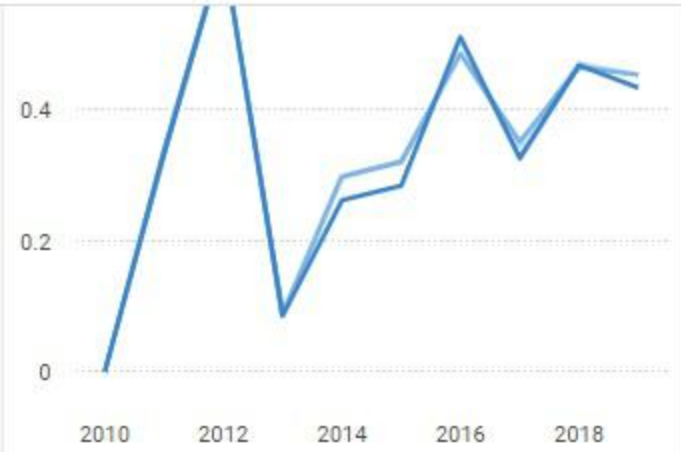
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# Metagenomics analysis of soil microbial communities in plant agroforestry system rubber tree (*Hevea brasiliensis*) – Ganyong (*Canna* sp.)

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**Abstract.** Rubber tree (*Hevea brasiliensis*) is one of important industrial plants in Indonesia. It is planted widely in some provinces in Indonesia, one of which is in West Java under management of the PTPN VIII plantation, Subang Regency. Intercropping system using underutilized crops *Canna* sp. (Ganyong) had been introduced on Rubber plantation. The aim of this research is to analyze the diversity, abundance and richness of rhizosphere soil microbial under rubber-*canna* agroforestry system using metagenomic analysis of 16S rRNA gene of soil rhizosphere bacteria. The research was carried out by collecting soil samples from 2 different soil conditions (soils with *Canna* (G) and soils without *Canna* (TG)). 2 different soil depths conditions (20 and 40 cm) were also investigated. The results showed that number of microbial in G soils was found more abundance than in TG soils, while based on soil depth there was no significant effect on soil microbial abundance. Based on class level was found 84 Classes. There were 3 classes that are mostly found in G and TG, namely *Ktedonobacteria*, *Acidobacteria* and *Planctomycetia*. Microbial diversity in family level mostly found in G and TG, Namely *Koribacteraceae*, *Gemmatataceae*, *Synobacteraceae*, *Hyphomicrobiaceae*.

## 1. Introduction

Rubber tree (*Hevea brasiliensis*) is one of important industrial plants in Indonesia with an area of 3.5 million ha, of which 2.9 million ha are managed by people or small-scale farmers, and the rest are managed by the state or private [1]. Planting trees under rubber stands is already done which is called intercropping. The intercrop is usually used are rice, potatoes, sweet potatoes, taro, and canna. Ganyong (*Canna* sp.) is a rhizome plant cultivated in the tropics and subtropics as a source of carbohydrate on all types of soil, drought-resistant and shade resistant. *Canna* as an intercrop can change the presence of bacteria on soil initiated by plants with secreting root exudates so that they invite microbes to colonize the rhizosphere [2]. Although the relationship between microbial diversity with functioning of soil remains debatable, it has been proved that the presence of some functional group of soil microbes are important in determining soil fertility and productivity [3, 4]. Hence, preservation of microbial diversity in soil may be relevant with sustainability of land agriculture. Most microbial identification in agroforestry systems still uses conventional methods by culturing on growth media. The conventional method is possible to only detect one or 2 types of microbes that have the same phenotype and are identified as the same species, whereas genetically it does not have



similarities [5]. Currently, new alternative method was developed, namely metagenomic, has been frequently used for studying microbial diversity. Metagenomic is a method of microbial DNA isolation directly from the environment [6, 7] which provides a great opportunity in the discovery of new microbial genome that are directly from the environment of its habitat [7, 8, 9].

In this study, analysis the diversity and abundance of soil microbial communities in agroforestry systems of rubber-*canna* plants by metagenomic methods was performed. Metagenomic analysis was performed by sequencing of 16S rRNA gene using Next Generation Sequencing Illumina platform.

## 2. Methods

### 2.1. Sampling

Soil Samples were collected from PTPN VIII in Subang, West Java, Indonesia. The different soil conditions were collected from intercropping between rubber with *Canna* (G) and without *Canna* (TG). The samples were collected from depth conditions 0-20 cm and 20-40 cm. 500 g soil samples of each sample were collected from 3 different sites nearby rhizosphere areas of *Canna* plants (G) or from 3 different sites nearby rhizosphere areas of Rubber tree without *Canna*. 50 g of each sample was subjected for DNA isolation and labelled according to depth and location. The soils samples were kept in ice-cold contained until used.

### 2.2. DNA extraction

DNA extraction of genomic DNA was extracted using PowerSoil DNA kit (MoBio). DNA extraction was done according 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 result was checked its quality using gel electrophoresis. The DNA was used as template for PCR. A  $\mu\text{L}$  of DNA was added to 12.5  $\mu\text{L}$  PCR mix (*Fast Master Mix*), 8.5  $\mu\text{L}$  added Free-nuclease water and 1  $\mu\text{L}$  of each forward and reverse 16S rRNA primer. Reaction was performed 35 cycles which consisted of 30 sec at 94<sup>0</sup>C and continued with 57<sup>0</sup>C of annealing for 20 sec, followed with 2 min 72<sup>0</sup>C for elongation. A 16S rRNA gene was amplified with primer 63F (5'-CAGGCCTAACACATGCAAGTC-3') and 1387R (5'-GGGCGGWGTGTACAAGGC-3'). The PCR products were purified and subjected for automated Illumina Miseq platform (1<sup>st</sup> BASE-Malaysia) after the PCR products were normalized in equimolar amounts.

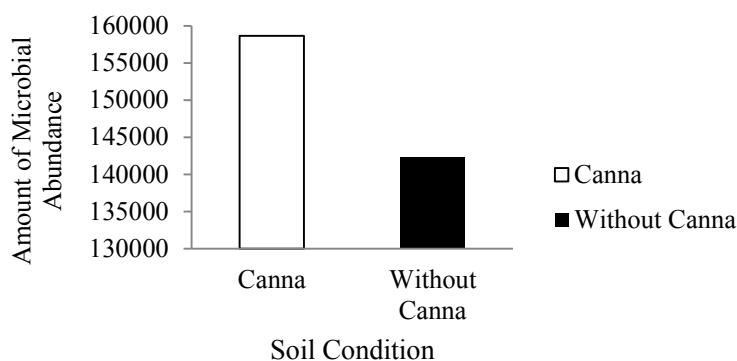
### 2.3. Sequences analysis and data analysis

Raw sequence data generated from Illumina Miseq platform were filtered and assessed of its quality using MultiQC software. Then the result was processed in QIIME2. All sequences are shorter than 150 bp or longer than 600 bp are removed from downstream processing. Read were then aligned with 16S rRNA, and followed inspection for chimeric errors. The Operational Taxonomy Unit (OTU) grouping is carried out with 97% similarity based on "Species-level" analysis. Rare OTUs with only 1 (singleton) or 2 (doubleton) that is fake are deleted from downstream processing. Taxonomic analysis is performed using RDP Classifier. Alpha and Beta diversity were performed using Excel (Microsoft) whereas statistically data was calculated with T-Test. Venn diagrams were mad with Excel (Microsoft) to compare the abundance of microbes in sample G and TG. Diversity analysis uses Shannon-Winner index and Simpson index.

## 3. Results and discussion

### 3.1. Microbial abundance

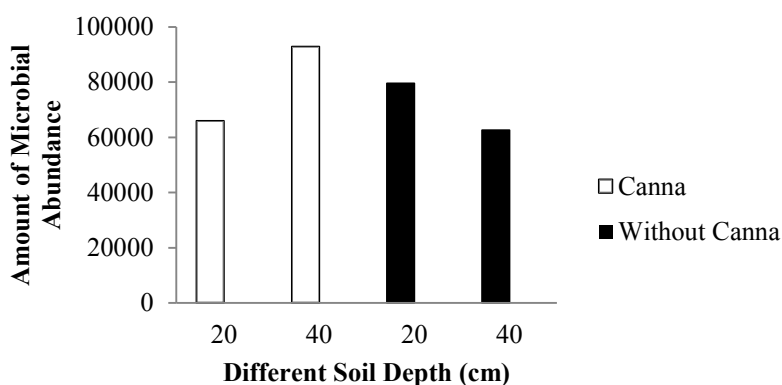
The result of the OTUs analysis showed that the number of microbial abundances in soil G (158.636) was higher than that of TG (142.292) (Fig. 1). This microbial abundance is allegedly because plants especially the roots release metabolites (Exudates) which are beneficial for soil microbes.



**Figure 1.** Total microbial abundance in rubber plantation soil intercropped (G) and without intercropped (TG) with *Canna*.

The microbial community actively influences the composition of the rhizosphere [10]. This is supported by previous report [11], that plants will actively select and attract specific microbes so that they change the composition of microbes by securing certain exudate compounds into the rhizosphere. Results of the independent T-Test, microbial abundance in the G and TG samples had a significant value (sig <0.05). This shows that there are significant differences in microbial abundance in samples G and TG. The growth of rubber plants planted with rice intercrops showed a better effect than monoculture [12]. This is caused by intensive maintenance of intercropping so that rubber plants obtain additional nutrients and avoid weed disturbances.

Microbial abundance based on depth (20 cm and 40 cm) shown varied results. The microbial abundance on TG20 was higher than that on TG40 (Fig. 2). In general, microbial abundance would decrease with increasing soil depth. This was caused by soil conditions that are increasingly dense and the availability of oxygen is diminishing, making space for bacterial growth to decrease and nutrients will be difficult to absorb [13].



**Figure 2.** Total microbial abundance in rubber plantation soil intercropped (G) and without intercropped (TG) with *Canna* from different soil depth conditions.

The higher microbial abundances in G40 are thought to be due to *canna* plants emitting root exudates which can alter microbial populations on the soil. The presence of microbes in the soil are needed for abiotic conditions and nutrients availability in the biosphere. A stable soil can be hypothesized that the soil can be inhabited by microbes capable of adapting to the environment which ultimately function as biochemical biocatalysts that take place in the soil which causes changes in the



soil [14]. While the microbial abundance on the TG20 was higher than of the TG40, this is because at a depth of 20 cm it is an oxidative zone that allows many types of aerobic microbes to live. Environmental conditions such as temperature, humidity, aeration, and energy sources are factors that influence the number of microorganisms in the soil [15]. Result of the T-Test between the depth of microbial abundance showed that there were no significant differences ( $\text{sig} > 0.05$ ). This shows that the depth does not affect the abundance of microbes in the soil. This is supported by previous result [16] that the similarity of the availability of water, food and supporting ecology are some of the factors that cause bacteria in both depths to not differ significantly.

### 3.2. Microbial diversity

*3.2.1. Analysis of microbial diversity by shannon-winner( $h'$ ) index and simpson ( $h_2$ ) index.* Analysis of microbial Diversity based on Shannon-Winner( $H'$ ) index indicated that the highest value occurred in the TG40 sample ( $H' = 3.0506$ ) and the lowest value at G20 ( $H' = 2.9467$ ) (Table 1). According to previous report [17] where  $H' \leq 1$ ,  $1 < H' \leq 3$  and  $H' \geq 3$  indicates moderate, means, and low microbial diversity, respectively.

**Table 1.** Shannon-Wiener ( $H'$ ) and Simpson ( $H_2$ ) diversity index values on G and TG at each soil depth conditions.

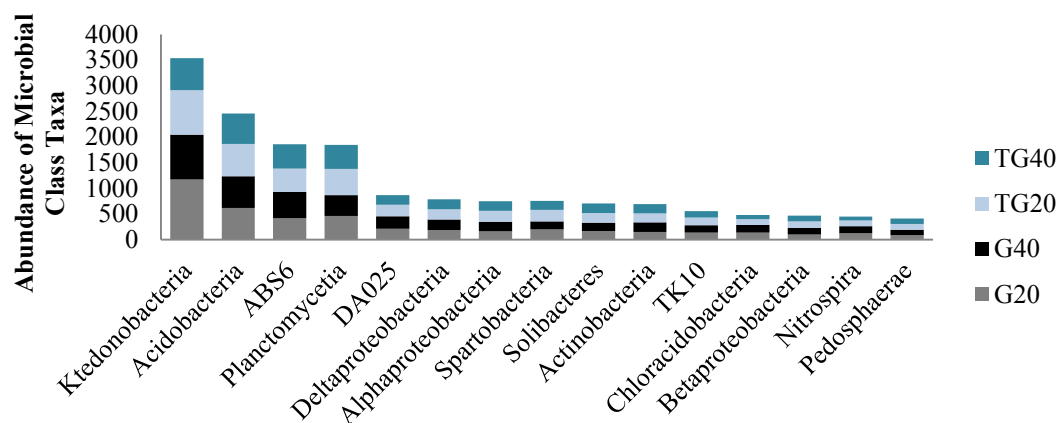
Sample	Indeks Shannon-Winner ( $H'$ )	Indeks Simpson ( $H_2$ )
G20	2.9467	0.093
G40	3.0236	0.085
TG20	3.0148	0.085
TG40	3.0506	0.078

The low  $H'$  index value for the G20 can be suspected due to the presence of certain types of microbes that dominate the G20 area, so that microbial diversity has decreased. According to [18] the value of the diversity index will be maximized when all individuals are equally represented. The higher the diversity value shows that a community has a relatively even number of individuals and no species dominates. Conversely, if the value of the diversity index is low, the number of individuals per species is different, and there are dominating species [19]. One of the values of diversity is influenced by competition between microbes due to changes in the environment and changes in time caused by the high functional abundance of soil bacteria [20]. Microbial diversity is also influenced by the physical chemistry of soil [21]. This is in accordance with the report of previous researcher [22] that the land that has undergone a diversification function will experience a decline, especially in acacia forests, oil palm plantations and rubber plantations.

Diversity based on the Simpson index ( $H_2$ ) in Table 1 at the depth of the G20 had a higher index value of ( $H_2=0.093$ ) while the lowest value was found in the TG40 sample ( $H_2=0.078$ ). Simpson Index value at  $0 < D < 0.5$  indicated there were no dominating microbes, and on the other hand an index value of  $0.5 < D < 1$  indicating a dominating microbe [23]. This is strengthened by the statement of [24] that the index value is close to a value of 1 means that in a community there is a dominating genus, on the contrary if the index value near to 0 indicating in a community no genus dominates. Based on the analysis results, the index value is less than 0.5 which means that each class at the 4 individual depths does not dominate. This condition shows that the microbial and ecological communities are still in a stable state [25].

*3.2.2. Analysis of microbial diversity class level.* The results of the OTUs analysis of microbial diversity in the class found 84 type of bacteria. There were 3 types of bacteria that were mostly found, namely *Ktedonobacteria*, *Acidobacteria* and *Planctomycetia* (Fig. 3). The abundance of these bacteria was thought to be due the availability of nutrients from tillage so that supporting several

microorganisms can grow well [26] and plant factors have an important role in the presence of soil microbes in relation to the important role of root exudates [27].



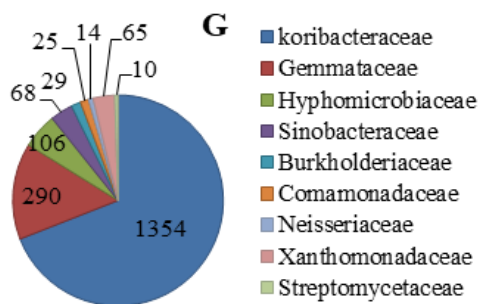
**Figure 3.** Abundance of top 15 classes taxa in G and TG soils with different soil depth.

*Ktedonobacteria* are Gram-positive bacteria, found in many acidic environments, according to Kim *et al.* [28] based on observations of the American soil ecosystem showing that *Ktedonobacteria* decreased with an increase in pH 5-7. *Ktedonobacteria* are able to hydrolyze starch, casein and produce the enzyme catalase. This was supported by similar researcher [29] that all bacterial samples isolated from rubber-canna plantations positively produced the catalase enzyme.

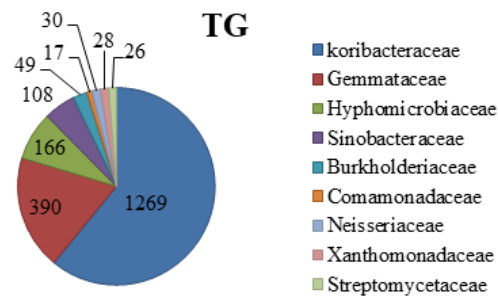
*Acidobacteria* are bacteria that are aerobic, heterophilic which are mostly distributed in environments such as soil, freshwater, sea water and polluted environments [30]. This bacterial abundance is regulated by pH, ammonia concentration, soil moisture, soil temperature, soil respiration [31]. *Acidobacteria* have the ability to fixation nitrogen [32]. This is supported by [29] that all bacteria on those isolated from rubber-canna land have the ability to fixation nitrogen.

*Planctomycetia* is a bacterium that doesn't have peptidoglycan on its cell wall, splits with buds, and its lipids resemble cells similar to eukaryotes. *Planctomycetia* is an aerobic, facultative chemoorganotrophs which specializes in carbohydrate metabolism, which can obtain energy through chemical oxidation through anaerobic ammonium oxidation [33]. The oxidation of ammonium produces hydrazine as an intermediate compound, a toxic compound that is converted to dinitrogen by hydrazine oxireductase [26]. It is also reported by [34] that analysis of the restriction fragment polymorphism of the 16S rRNA gene from paddy soil reveals that the diversity of planctomycetes is higher in anoxic soils than in the rhizosphere of oxics, indicating that changes in soil oxygen distribution affect the planctomycetes community.

**3.2.3. Analysis of microbial diversity family level.** The results of the analysis on samples G and TG showed uniform diversity. The bacteria found in the soil was identified as *Koribacteraceae*, *Gemmataceae*, *Hyphomicrobiaceae*, *Sinobacteraceae*, *Xanthomonadaceae*, *Burkholderiaceae*, *Comamonadaceae*, *Neisseriaceae*, and *Streptomycetiaceae* (Figs. 4 and 5). In this study 4 bacteria were mostly found in the sample G and TG, namely *Koribacteraceae*, *Gemmataceae*, *Hyphomicrobiaceae*, *Sinobacteraceae*. Previous study [35] indicated that analysis at a lower taxonomic level (family or genus) showed a stronger effect because of the type of soil. Distribution of soil microbes has a close relationship with soil particles and plant roots [36].



**Figure 4.** The relative abundance of microbial at the family level in rubber plantation soil intercropped with *Canna* (soil condition G).



**Figure 5.** The relative abundance of microbial at the family level in rubber plantation soil without intercropped with *Canna* (soil condition TG).

The group of *Koribacteraceae* is one of the family of *Acidobacteria* which is heterotrophic, chemoorganotrophic, grows at acidic pH (4-5.5) and is able to oxidize carbon [37]. The results showed that the *Koribacteraceae* group was more commonly found in G samples, this is because the *Koribacteraceae* has an important role in the cycle of organic carbon originating from plant litter or root exudates [38].

*Gemmataceae* is a Gram-negative bacterium which belongs to the *Planctomycetia* class and is aerobic [39] and chemoorganotrophic [40]. This bacterium is able to grow in the accumulation of organic material from dead cells and plant litter. The results showed that the bacterium is abundant on TG; this is because in TG there are many plant litters found that support bacterial growth. This is supported by Lima [35] that *Gemmataceae* bacteria are abundant in soil with accumulation of organic matter and plant litter.

*Hyphomicrobiaceae* is Gram negative derived from *Alphaproteobacteria* class that are aerobic, chemoheterotrophic, and chemolithoautotrophic [41]. But there are some that are anaerobic by using nitrates as electron acceptors. These bacteria are now applied as biodegradation and bioremediation, biosensors and enzymes for analytical purposes, as well as biosynthesis of chemicals [42].

*Sinobacteraceae* are a family of *Gemmaproteobacteria*, including Gram negative, non-motile and obligate aerobes [43]. Researcher [44] reported that bacterium *Sinobacteraceae* acts as an oxidizing nitrate. Another researcher [45] supported that bacterial abundance is influenced by a low pH and high  $\text{NO}_3^-$  concentration. The results showed that *Sinobacteraceae* was mostly found on TG, this was thought to be due to soil composition and abiotic factors that influence the nitrification process. This is supported by others researchers [46] that the low rate of nitrification on Gintungan farmland can be influenced by the availability of ammonia [47, 48]. The rate of nitrification is also influenced by abiotic factors, one of which is the diversity and abundance of microorganisms that play a role in the process of nitrification [49, 50].

#### 4. Conclusion

The study showed that the total microbial abundance in soils with *Canna* (G) were higher than soil without *Canna* (TG), while based on soil depth conditions the microbial abundance which found in 20 cm and 40 cm were statistically not different. Diversity index (Shannon-Winner Index) showed that G20 has a lower value than G40, TG20 and TG40, while the Simpson index analysis shows that no microbes dominate not only in soil G but also in soil TG. There were 3 bacteria in class level which are found in soil G and soil TG, namely *Ktedonobacteria*, *Acidobacteria* and *Planctomycetia*. At the family level there are 4 bacteria that play a role in the soil, namely *Koribacteraceae*, *Gemmataceae*, *Hyphomicrobiaceae*, and *Sinobacteraceae*.

## Acknowledgements

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