CERTIFICATE

is hereby awarded to

Yunus Effendi

for valuable contribution as ORAL PRESENTER with the paper entitled

Metagenomic analysis of soil bacteria around rhizosphere area of *Canna edulis* kerr

at the 4th INTERNATIONAL BIOLOGY CONFERENCE - 2018

'From Gene to Ecosystem for Human Welfare'

October 13th, 2018

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Metagenomics Analysis of Soil Around Rhizosphere Area of Canna edulis Kerr Root System

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Abstract. Canna edulis is one of the underutilized crops that commonly consumes many Indonesian peoples. Due to its advantage characters, Canna sp is planted frequently traditionally in many areas in Indonesia. Canna sp may used also as intercropping plants under industrial plantation estate such as Rubber plantation. The aim of intercropping system was enrichment of novel soil microbial p (153opulation in the soil. Diversity, abundance and richness of rhizosphere soil microbial under rubber-*canna* agroforestry system were analyzed by using metagenomic analysis of 16S rRNA gene of soil rhizosphere bacteria. The research was carried out by collecting soil samples from two different soil conditions (soils with *Canna* (G) and soils without *Canna* (TG)). Two 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. Microbial diversity based on class taxa was found 84 types (153 total microbe with Canna (G) and 76 without Canna (TG). There are 3 classes that are mostly found in G and TG, namely Ktedonobateria, Acidobacteria and Planctomycetia. Microbial diversity in family level mostly found in G and TG, Namely Koribacteraceae, Gemmataceae, Synobacteraceae, Hyphomicrobiaceae.

Keywords: Metagenomic, *Canna edulis*., Rhizosphere bacteria

1. INTRODUCTION

Rubber plant (*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 [23]. Planting trees under rubber stands is already done which is called intercropping. The intercrop is usually used are rice, sweet potatoes, taro, canna and potatoes. Ganyong (*Canna* sp.) is a tuber plant cultivated in the tropics and subtropics as a source of easily grown carbohydrate on all types of soil, drought-resistant and shade resistant. *Canna* as a intercrop can change the presence of bacteria on soil initiated by plants with secreting root exudates so that they invite microbes to come to the rhizosphere [12]. Microbial identification in agroforestry systems still uses conventional methods. The conventional method is possible to only detect one or two types of microbes that have the same phenotype and are identified as the same species, whereas genetically it does not have similarities [30]. 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 [37] which provides a great opportunity in the discovery of new enzyme diversity because we can explore the microbial genome directly from the environment of its habitat [42,39].

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. MATERIAL AND METODS

2.1 Sampling

Soil Samples were collected from two different soil conditions (soil with *Canna* (G) and soil without *Canna* (TG)) with soil depth conditions 20 cm and 40 cm. Soils were collected from PTPN VIII in Subang, West Java, Indonesia. Each soil samples were consisted of 500 g soil samples which collected from 3 different sites nearby rhizosphere areas of *Canna* plants (G) or from 3 different sites nearby rhizosphere areas of *Rubber* plants without *Canna* plants nearby. Then the soil sample is taken about 50gr for DNA isolation and labelled according to depth and location. The soils samples were kept in 4^0 C until used.

2.2 Isolated DNA

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 result checked its quality using gel electrophoresis. The DNA was used as template for PCR. A µL of DNA was added to 12.5 µL PCR mix (*Fast Master Mix*), 8.5 µL added Free-nuclease water and 1 µL of each forward and reverse 16s rRNA primer. Reaction was performed 35 cycles which consisted of 30 sec at 94^0C and continued with 57^0C of annealing for 20 sec, followed with 2 min 72^0C 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^{st} BASE-Malaysia) after the PCR products were normalizad in equimolar amounts.

2.3 Sequences Analysis and Data Analysis

Raw sequence data generated from Illumina Miseq platform were processed in QIIME or OptiClust. 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 inspected 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) where as 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. RESULT AND DISCUSSION

3.1 Result of microbial abundance analysis

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

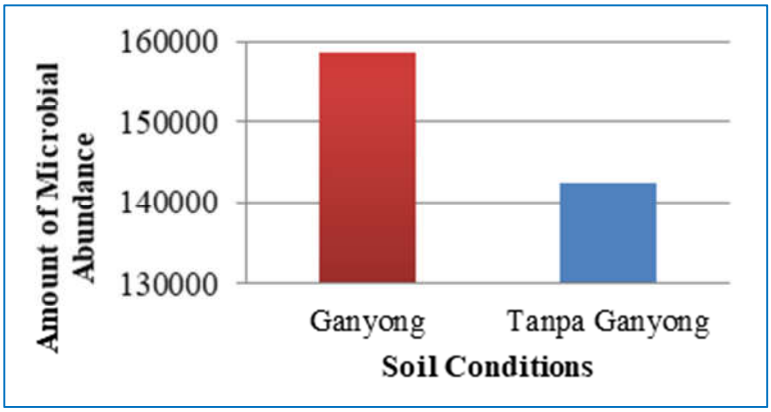


Figure 1. Total microbial abundance in soil Ganyong (G) and Without Ganyong (TG)

The microbial community actively influences the composition of the rhizosphere[40]. This is supported by Broeckling *et al.* (2008)[6] plants will actively select and attract specific microbes so that they change the composition of microbes for the purpose association 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) (**Figure 2**). This shows that there are significant differences in microbial abundance in samples G and TG. Rasyid *et al.* (2004)[38] stated that the growth of rubber plants planted with intercrops showed a better effect than monoculture. This is caused by intensive maintenance of intercropping so that rubber plants obtain additional nutrients and avoid weed disturbances.

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
bakteri	Equal variances assumed	21.201	.000	-3.007	40058	.003	-2.192	.729	-3.621	-.763
	Equal variances not assumed			-3.007	33422.550	.003	-2.192	.729	-3.621	-.763

Figure 2. Independent Sample T-Test in the soil G and TG based on Plant

Microbial abundance based on depth (20 cm and 40 cm) has varied results. The G40 sample has an abundance lower than the G20. While the microbial abundance on TG20 is higher than that on TG40 (**Figure 3**). In general, microbial abundance will decrease with increasing soil depth. This is 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[20].

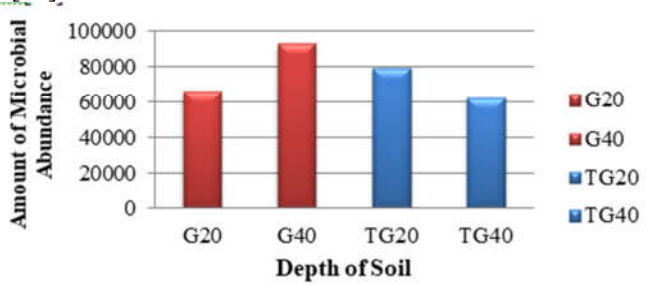


Figure 3. Total microbial abundance in soil G and TG from different soil depth conditions.

Many microbial abundances in G40 are thought to be due to *canna* plants emitting root exudates which can alter microbial populations on the soil. Microbes present in the soil show the need for abiotic conditions and nutrients available 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 [29]. While the microbial abundance on the TG20 more than 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 [3]. Based on the results of the T-Test between the depth of microbial abundance showed that there were no significant differences (sig> 0.05) (**figure 4**). This shows that the depth does not affect the abundance of microbes in the soil. This is supported by Winarso et al. (2015) [45] 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.

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
bakteri	Equal variances assumed	3.226	.072	-1.144	40058	.253	-.834	.729	-2.263	.595
	Equal variances not assumed			-1.144	36216.861	.253	-.834	.729	-2.263	.595

Figure 4. Independent T-Test test for soil samples G and TG based on differences in depth

3.2 Result of analysis of Microbial Diversity

3.2.1 Analysis of microbial Diversity by Shannon-Winner(H') index and Simpson (H2) index

Analysis of microbial Diversity based on Shannon-Winner(H') index it was found 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 Fanani *et al.* (2013)[15] show that the calculation results are determined based on the high and low H' values where H'≤1 indicate low microbial diversity, 1≤H'≤3 indicates moderate microbial diversity, and H'≥3 means high diversity.

Table 1. 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)
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 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)[9] states that 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[32]. 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 [19]. Microbial diversity is also influenced by the physical chemistry of soil[8]. This is in accordance with the research of Hill *et al.* (2003)[21] 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 (H2) in Table 1 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). Simpson Index value is determined by the dominance index value 0 <D <0.5, so there are no dominating microbes, and an index value of 0.5 <D <1, there is a dominating microbe [33]. This is

reinforced by the statement Basmi (2000) in Pirzan (2008)[36] that the index value is close to a value of 1 then in a community there is a dominating genus, on the contrary if the index value approaches 0 then 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 four individual depths does not dominate. This condition shows that the microbial and ecological communities are still in a stable state[41].

3.2.2 Analysis of Microbial Diversity

Comparison of bacteria abundance between soil with Canna and soil without Canna, clearly showed that in phylum level, there is different in abundance of bacteria (Figure 5). Soil with Canna indicated had more abundance in WP3 phyla in comparison to soil without Canna.

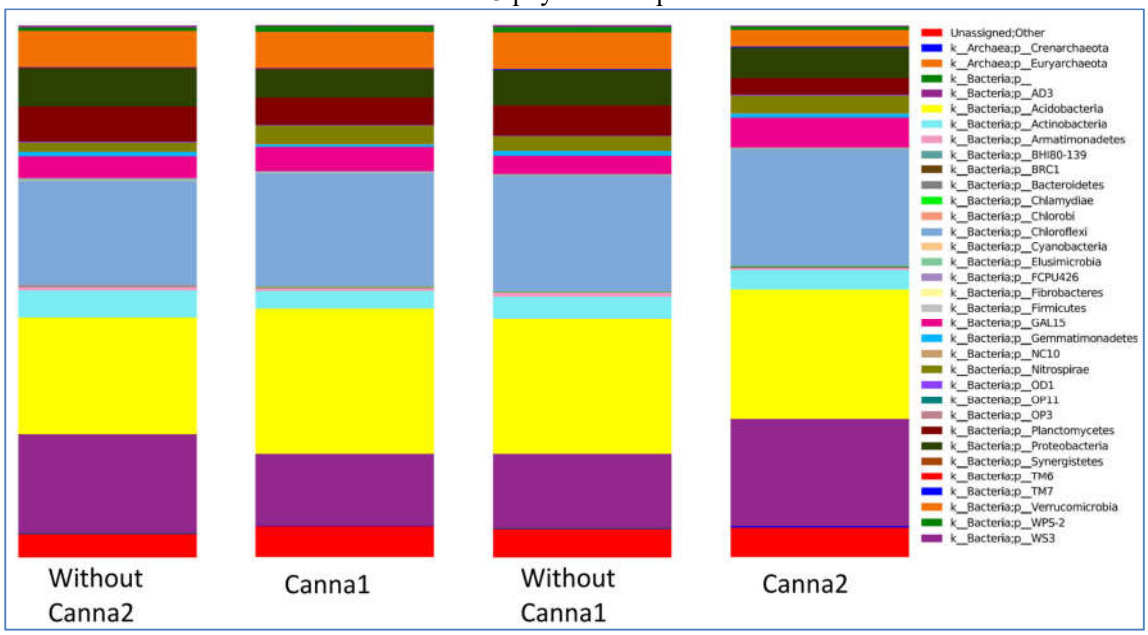
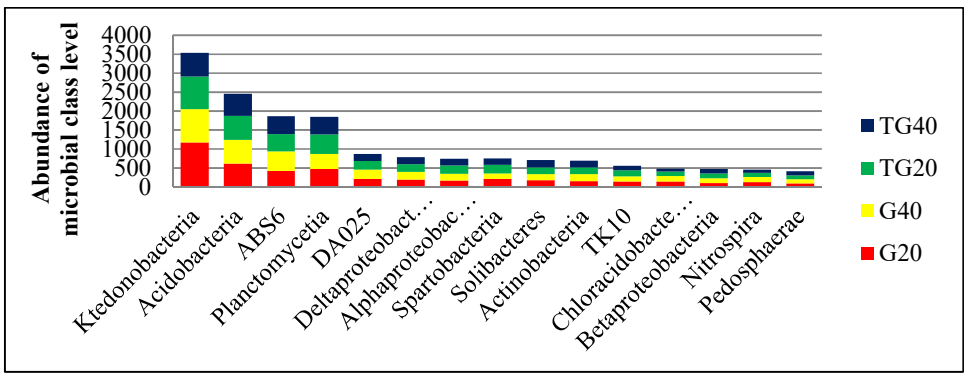


Figure 5. Bacterial abundance in phyla level.

Moreover, the results of the OTUs analysis of microbial diversity in the class found 87 species of bacteria (154 on G and 152 on TG), there were 3 types of bacteria that were mostly found, namely ktedonobacteria, acidobacteria and planctomycetia (**Figure 6**). The abundance of these bacteria is thought to be due the availability of nutrients from tillage so that supporting several microorganisms can grow well[44] and plant factors have an important role in the presence of soil microbes in relation to the important role of root exudates[5].



Figur 5. Abundance of Microbial class level in soil condition G and TG on different dept

Ktedonobacteria are gram-positive bacteria, found in many acidic environments, according to Kim *et al.* (2015)[25] based on observations of the American soil ecosystem showing that ktedonobacteria decreased with an increase in pH 5-7. Ktedonbacteria are able to hydrolyze starch, casein and produce the enzyme catalase. This was supported by Yulianti research (2019)[48] 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 [22]. This bacterial abundance is regulated by pH, ammonia concentration, soil moisture, soil temperature, soil respiration [16]. Acidobacteria have the ability to fixtion nitrogen [24]. This is supported by Yulianti research (2019) [48] 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 in euariot. Planctomycetia is an aerobic, facultative cemoorganototrof which specializes in carbohydrate metabolism, which can obtain energy through chemical oxidation through anaerobic ammonium oxidation[7]. The oxidation of ammonium produces hydrazine as an intermediate compound, a toxic compound that is converted to dinitrogen by hydrazine oxireductase[43]. It is also reported by Derakshani (2011)[11] that analysis of the restriction fragment polymorphism of the 16s rRNA gene from paddy soil reveals that the diversity of planctomycetes is more 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 based on KRONA on samples G and TG have uniform diversity, some bacteria found include Koribacteraceae, gemmataceae, hyphomicrobiaceae, sinobacteraceae, xanthomonadaceae, burkholderiaceae, comamonadaceae, neisseriaceae, streptomycetiaceae (**Figures 7 and 8**). In this study 4 bacteria were mostly found in the sample G and TG, namely Koribacteraceae, gemmataceae, hyphomicrobiaceae, sinobacteraceae. According to Lima *et al.* (2015)[28] that analysis at a lower taxonomic level (family or genus) shows a stronger effect because of the type of soil, this is supported by abiotic data showing that soil types G and TG are clay. Distribution of soil microbes has a close relationship with soil particles and plant roots[1].

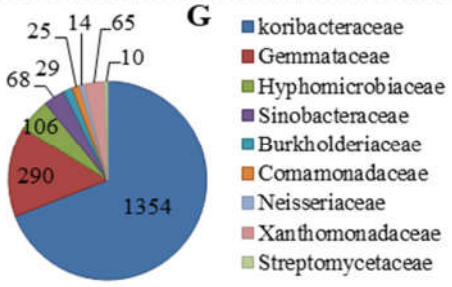


Figure 7. The relative abundance of microbial at the family level in soil Condition G

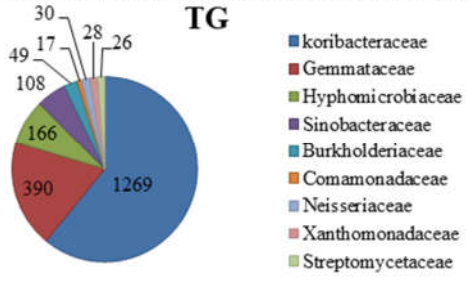


Figure 8. The relative abundance of microbial at the family level in soil Condition TG

The group of koribacteraceae including bacteria is one of the family of acidobacteria which is heterotrophic, chemoorganotrophic, grows at acidic pH (4-5.5) and is able to oxidize carbon[14]. The results showed that the koribacteraceae group was more commonly found in G

samples, this is because the coribacteraceae have an important role in the cycle of organic carbon originating from plant litter or root exudates[10].

Gemmataceae is a gram negative bacterium which belongs to the planctomycetia class and is aerobic[26] and chemoorganotrophic[2]. This bacterium is able to grow in the accumulation of organic material from dead cells and plant litter. The results showed that this bacterium is abundant on TG, this is because in TG there are many plant litter found that support bacterial growth. This is supported by Lima (2015)[28] 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 [17]. 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 [18].

Sinobacteraceae are a family of gemmaproteobacteria, including gram negative, non-motile and obligate aerobes [49]. According to Mannisto (2016) [31] states that bacterium sinobacteraceae acts as an oxidizing nitrate, this is supported by Kuramae (2012) [27] that bacterial abundance is characterized by a low pH and high 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 Nugroho and Meitiniarti (2012) [34] research that the low rate of nitrification on gintungan farmland can be influenced by the availability of ammonia as found in Peng et al. (2012) [35] and Yamamoto et al. (2012) [47] the rate of nitrification is influenced by abiotic factors, one of which is the diversity and abundance of microorganisms that play a role in the process of nitrification [4,13].

In conclusion, the results of this study showed that the total microbial abundance in soils with *Canna* (G) were more higher than soil without *Canna* (TG), based on the T-Test it was found that there were significant differences between soil samples G and TG, while based on soil depth conditions the microbial abundance which found in 20 cm and 40 cm were statistically not different.did no show significant results. Diversity index baseusing the (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 the land 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 Koribacteracee, Gemmataceae, Hyphomicrobiaceae, and Sinobacteraceae.

Acknowledgements : This research had been supported financially by research grant of PDUPT 2018 from The Ministry of Research Technology and Higher Education-Republic of Indonesia.

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