Research Article

The Effect of Biological Control Agents on Paddy Soil Bacterial Community Structure

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ABSTRACT

Biodiversity has been defined as the range of significantly different types of organisms and their relative abundance in an assemblage of community. The aim of this research was to understand about soil bacterial community after on biological control agents (BCAs) treatments with various formulations. This research was conducted at Sleman Regency, Special Region of Yogyakarta and Faculty Agriculture of Universitas Gadjah Mada. The research conducted with culture dependent and culture independent methods to assess soil bacterial diversity. The results showed that soil bacterial diversity before and after treatment of biological control agent were different. Results from this research suggested different molecular methods regarding soil bacterial diversity based on their benefits and challenges.

Keywords: bacteria; culture dependent; culture independent; diversity; soil

INTRODUCTION

Soil microorganisms play an important role in maintaining soil fertility through biochemical processes, especially in intensive agricultural systems. Microbial diversity and activity are sensitive indicators that reflect sustainability and productivity of terrestrial agro-ecosystems (Cardinale *et al.*, 2012). Xuan (2012) analyzed soil samples collected after rice seed harvest treated with four treatments of organic compounds and revealed different bacterial communities between treatments. The incorporation of potential beneficial microorganisms that inhabit rice fields plays an essential role in protecting soil environment by reducing the chemical inputs required to maintain nutrient levels and increasing microbial diversity, which all contribute to increasing yield, hence, reducing poverty.

Biodiversity has been defined as the range of significantly different types of organisms and their relative abundance in an assemblage of community.

Diversity has also been defined according to information theory, as the amount and distribution of information in an assemblage or community (Torsvik *et al.*, 1998). Microbial diversity refers unequivocally to biological diversity at three levels: within species (genetic), species number (species) and community (ecological) diversity (Harpole, 2010). The term species diversity consists of two components; the first component is the total number of species present which can be referred to as species richness. In other words it refers to the quantitative variation among species. The second component is the distribution of individuals among species, which is referred to as evenness or equability. A problem that occur is that evenness often is unknown in bacterial systems because individual cells very seldom are identified to the species level. An attractive possibility for the measurement of biodiversity is to use divergence in molecular characters, especially the percentage of either nucleic acid homology or base sequence difference.

In the past, diversity has been determined based on taxonomic species, which may limit the scope of information and relationship obtained.

Soil quality, an indicator of the sustainability of land use and soil management practices, can be assessed through the measurement of soil physical, chemical, and biological characteristics (Yao *et al.*, 2013). Soil organisms contribute in maintaining soil quality by controlling plant and animal decomposition, biochemical cycling and formation of soil (Turco *et al.*, 1994). Soil microbial community and its diversity are important bio-indicators of soil health and quality (He *et al.*, 2008). Yang and Zhang (2014) reported that using physical, chemical, phospholipid fatty acid (PLFA), and PCR - denaturing gradient gel electrophoresis (PCR-DGGE) analyses, confirmed that the soil microbial community structure and genetic diversity both affected and were affected by soil physiochemical properties that were subject to land-use conversion and land-use history. In addition, soil moisture, organic matter, and nitrogen, which were the main nutrients for soil microbial growth, were the most important factors affecting the differences of soil microbial community structure between rice field and orchard soils. Furthermore, the identification of bands from the DGGE patterns demonstrated that soil aeration was also one of the pre-dominant driving factors governing microbial diversity. After land-use conversion from rice field to orchard farm, bacterial abundance declined significantly, and the soil microbial genetic diversity and community structure shifted over time that were primarily attributed to initial change of land use rather than the subsequent age of the orchard. In addition, land-use conversion from rice field to orchard farm has adverse effects on soil quality. In contrast, diverse soil microbial community was achieved and maintained in rice field soil systems, indicating the potential for substantial contributions to biogeochemical element cycling.

It is well known that soil chemical and physical properties such as soil nutrient, pH, moisture, temperature, and ventilation, have influences on the diversity of soil microbial community (Yang & Crowley, 2000; Marschner *et al.*, 2004). Song *et al.* (2014) reported that no obvious differences in community composition and abundance of bacteria in rice field soil between cry1Ac/cpti transgenic rice and non-cryAc/cpti rice may be attributed to the consistency in soil nutrient of N, P, and K, soil pH, and soil organic C between the rice field soils with transgenic and non-transgenic rice. The cultivation of cry1Ac/cpti transgenic rice had no effect on composition and abundance of bacterial community in rice field during study period.

Another study reported microbial biomass and number of heterotrophic bacteria in rice field soil applied with synthetic fertilizers alone was not significantly different from rice field soil under conventional farming (synthetic fertilizers and pesticides application) (Sheng *et al.*, 2005). Agricultural management practices were observed by Lopes *et al.* (2011) to influence the bacterial community structure. Organically farmed soil showed temporal variations in the functional and bacterial community structures. Conventionally farmed soil showed similar temporal variations in the functional, but not in bacterial community structure. The objective of this study was to identify soil bacterial community based on biological control agents treatments with various formulations.

MATERIALS AND METHODS

Soil Sampling

Soil samples were taken from the experimental fields before (-B) and after (-A) treatments. The experimental field was a paddy field with regosol soil (*Bappeda Daerah Istimewa Yogyakarta*, 2021), planted with IR64 variety from seedling to harvest. Rice seedlings were planted in each plot (6 m × 8 m) with a distance of 25 cm×30 cm. Plant maintenance followed land owner practices by applying fertilizers of 300 kg/ha organic fertilizer before replanting and 4th week after replanting. Treatments were added using three different formulation types (liquid [L]; wettable powder [WP]; granule [G]) and five different active ingredients of BCA, the list of BCA and the application of the treatments are shown in Table 1. Fifty grams of soil samples with 15 cm of depth were taken diagonally at ten spots for each treatment. Soil samples were transferred immediately after collection to the laboratory of Plant Disease Clinic, Faculty of Agriculture, Universitas Gadjah Mada at room temperature for DNA isolation.

Table 1. Composition of treatment for soil samples

Treatment	Active ingredients		Formulation type	Application
L1	Mycorrhiza mix	> 10 propagules/ml	Liquid 1	Seed Treatment (1 time before seed planting)
	Trichoderma spp.	> 1 × 10⁷ CFU/ml		
	Bacillus spp.	> 1 × 10⁷ CFU/ml		
L2	Mycorrhiza mix	> 10 propagules/ml	Liquid 2	Seed Treatment (1 time before seed planting)
	Trichoderma spp.	> 1 × 10⁷ CFU/ml		
	Bacillus spp.	> 1 × 10⁷ CFU/ml		
	Total Nitrogen	> 4%		
	Potassium (K₂O)	> 4%		
L3	Mycorrhiza mix	> 10 propagules/ml	Liquid 3	Seed Treatment (1 time before seed planting)
	Trichoderma spp.	> 1 × 10⁷ CFU/ml		
	Bacillus spp.	> 1 × 10⁷ CFU/ml		
	Total Nitrogen	> 15%		
	Potassium (K₂O)	> 4%		
WP	Mycorrhiza mix	> 100 propagules/g	Wettable Powder	Foliar Spray (2 times) 1. at transplantation or within 2 weeks 2. before panicle initiation
	Trichoderma spp.	> 1 × 10⁸ CFU/g		
	Bacillus spp.	> 1 × 10⁸ CFU/g		
G	Mycorrhiza mix	> 10 propagules/g	Granule	Broadcasting (2 times) 1. at transplantation or within 2 weeks 2. before panicle initiation
	Trichoderma spp.	> 1 × 10⁷ CFU/g		
	Bacteria spp. of the rhizosphere	> 1 × 10⁷ CFU/g		
C1	Control 1		With organic fertilizer	
C2	Control 2		Without fertilizer	

DNA Extraction from Soil

Culture Dependent DNA Extraction. Bacteria from soil samples were cultured on NA medium and DNAs were extracted using CTAB Method (Istiqomah, 2015).

Culture Independent DNA Extraction. DNA was extracted directly from samples by following protocols from Soil DNA extraction kit (Zymo-BIOMICSTM DNA Mini Kit).

DNA amplification and visualization. The DNA was amplified on the basis of PCR using T100 Thermal Cycler Bo-Rad. A master-mix of 10 μl reaction was prepared with PCR components with the total volume 25 μl (master-mix 9 μl; ddH₂O or RNase free water 8 μl; primer forward and reverse each 3 μl; and 2 μl genomic DNA). Bacterial 16S rRNA were amplified using RISA primers ITSF (5'-GTC GTA ACA AGG TAG CCG TA-3') and ITSReub (5'-GCC AAG GCA TCC ACC-3') (Cardinale *et al*., 2004). Amplification of isolated DNA was performed under the following condition: 94°C for 3 minutes; then 30 cycles of 94°C for 45 seconds, 55°C for 1 minute, 72°C for 2 minutes; 72°C for 7 minutes and

hold at 4°C. DNA fragment was separated using 4% polyacrylamide gel under TBE 1X solution buffer by Promega pH 7.4 by electrophoresis machine for 90 minutes on 75 V. Gel was stained by 100 ml of 0.001% Ethidium Bromide (EtBr) solution for 15 minutes. The stained gel was rinsed with distillated water for distaining, illuminated on UV transillu-minator and photo-graphed by gel documentation unit for measuring the bands of amplified DNA fragments.

Data Analysis

Community composition based on relative band intensity and position was analyzed by analysis of diversity using numerical taxonomy system (NTSYS) 2.10 program and zoned by unweighted pair groups with arithmetical averages (UPGMA) method.

RESULTS AND DISCUSSION

Community Structure of Rhizobacteria

Culture Dependent

Culture-dependent studies indicated that bacterial communities were representasted by bacterial divisions

that are cosmopolitan in the environment, whereas others appear restricted to certain habitats (Schlegel & Jannasch, 1992). Hugenholtz *et al.* (1998) reported that several bacterial divisions sequence representatives have been identified in a wide range of habitats, suggesting cosmopolitan characteristics or ever present distribution of the corresponding organisms in the environment and, potentially, their broad metabolic capabilities. Some microbial species have a cosmopolitan distribution and occur in many environmental conditions if particular habitat requirements are met. The distribution of microbes does not depend on contingencies occurring over evolutionary or ecological time scales but only on the properties of the habitat (Fenchel, 2005).

This method showed that each treatment had more than 15 bands of DNA fragments before (Figure 1) and after treatment (Figure 2). Mostly bands amplified between 250–750 bp. The differences between before and after biological control agent application were at the bands between 250–750 bp. Fingerprinting of bacterial community by electrophoretic separation of amplified IGS sequences between the rrs and rrl genes (RISA) allows quick characterization of a community within various environmental conditions.

Using this approach, changes in bacterial community structure in paddy soil were observed. Before and after biological control agent treatment showed that soil samples contained β-subdivision of Proteobacteria, γ-subdivision of Proteobacteria, high GC gram positive bacteria, low GC gram positive bacteria, Chlamydiae, Cyanobacteria, Spirochetes, and Cytophagales (Ranjard *et al.*, 2000). The similarity between each treatments showed by unweight pair group method with arithmetic average (UPGMA) analysis (Figure 3).

Figure 3 showed that the fourteen samples of paddy soil appeared to fall into two zones (before and after biological control agent application) and based on 0.70 coefficient of similarity also clustered into seven zones. Additionally, we observed that paddy soil samples with biological control agent application clustered together and separated from samples without biological control agent application. The liquid formulation treatments except (L1-A and L1-B, L2-A and L2-B) showed different bacterial community based on phylogenetic analysis, also for other soil samples for each plot (L3-A different with L3-B, G-A different with G-B, WP-A different with WP-B, C1-A different with C1-B, and even C2-A different with C2-B). Between L1, L2, L3, C2 samples before and after BCA application had 50% similarities of bacterial community, while WP, G, C1 samples had 56%.

Culture Independent

The culture-independent approach was used to examine changes in rhizosphere after application of biological control agents. Noticeable differences were shown at bands with sizes greater than 1000 bp. Soil samples taken after biological control agent treatments showed more bands within size greater than 1000 bp compared than before treatment. Based on Ranjard *et al.* (2000), could be predicted that the paddy soil after treatment contained more α-subdivision of Proteobacteria, even though the number of bands from this method were less than results from culture dependent method, and it affected the phylogenic analysis. This method showed that each treatment had about 8–18 bands of DNA fragments (Figure 4 and Figure 5). Similarity between each treatments are showed using UPGMA analysis (Figure 6).
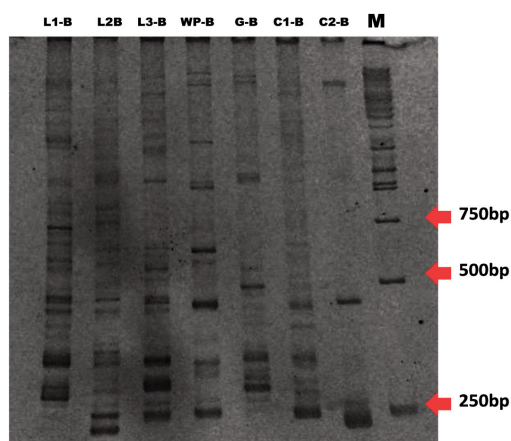

Figure 1. Soil bacterial community structure revealed by PCR-RISA analysis in soil samples before application of biological control agents using culture dependent method of DNA extraction; Lanes: M: Marker 1Kbp DNA ladder, L1-B: Liquid 1 before application of BCAs; L2-B: Liquid 2 before application of BCAs; L3-B: Liquid 3 before application of biological control agents (BCAs), WP-B: Wettable Powder before application of BCAs, G-B: Granule before application of BCAs, C1-B: Control before application of BCAs 1, C2-B: Control 2 before application of BCAs
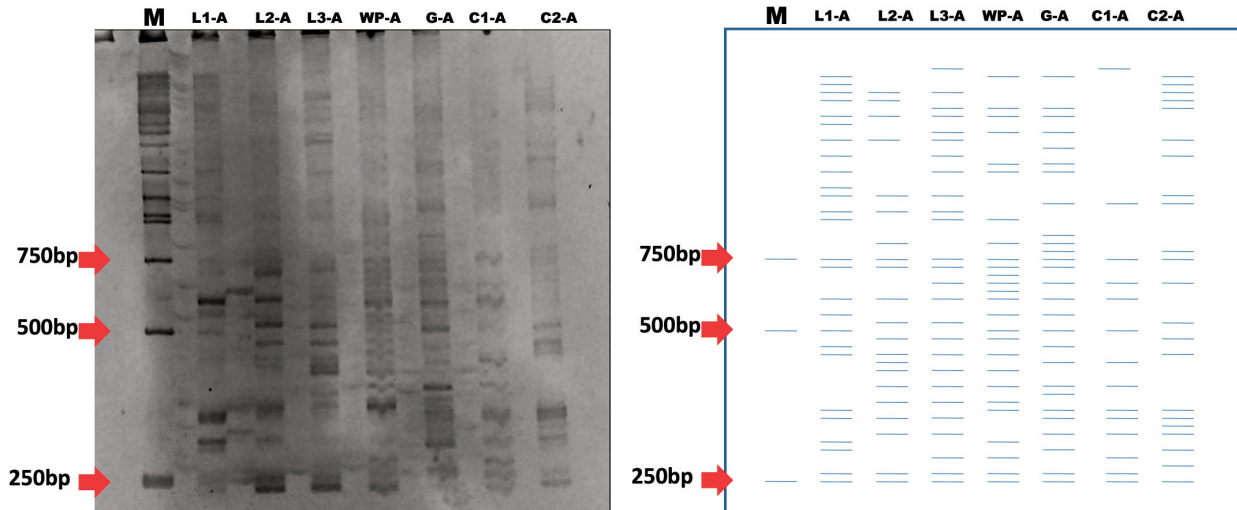
Figure 2. Soil bacterial community structure revealed by PCR-RISA analysis in soil samples after application of biological control agents (BCAs) using culture dependent method of DNA extraction; Lanes: M: Marker 1Kbp DNA ladder L1-A: Liquid 1 after application of BCAs; L2-A: Liquid 2 after application of BCAs; L3-A: Liquid 3 after application of BCAs , WP-A: Wettable Powder after application of BCAs, G-A: Granule after application of BCAs, C1-A: Control after application of BCAs 1, C2-A: Control 2 after application of BCAs
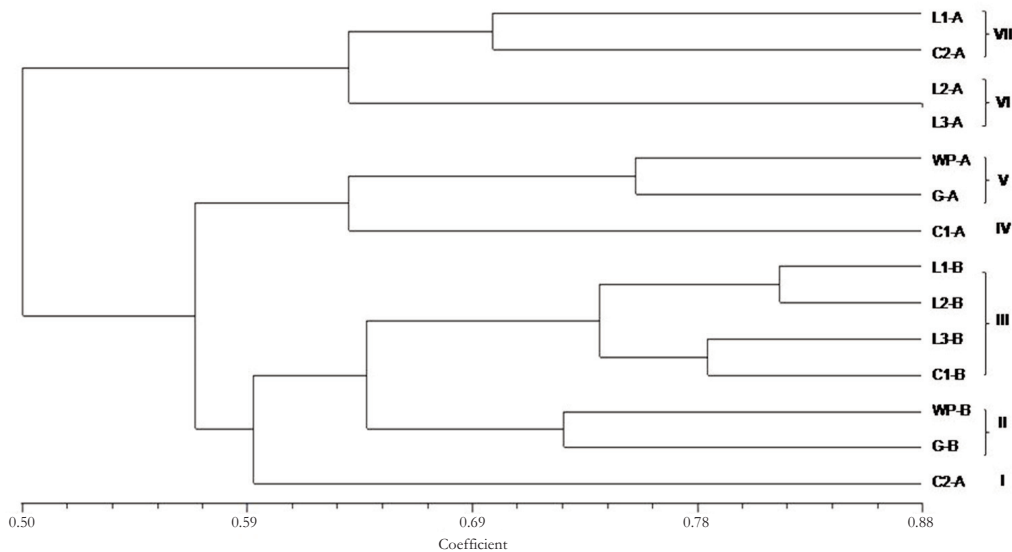

Figure 3. Un-weighted pair group method with arithmetic mean dendogram of paddy soil before and after treatment of biological control agents (BCAs) using culture dependent method of DNA Extraction; L1-B: Liquid 1 before application of BCAs; L2-B: Liquid 2 before application of BCAs; L3-B: Liquid 3 before application of BCAs, WP-B: Wettable Powder before application of BCAs, G-B: Granule before application of BCAs, C1-B: Control before application of BCAs 1, C2-B: Control 2 after application of BCAs. L1-A: Liquid 1 after application of BCAs; L2-A: Liquid 2 after application of BCAs; L3-A: Liquid 3 after application of BCAs, WP-A: Wettable Powder after application of BCAs, G-A: Granule after application of BCAs, C1-A: Control after application of BCAs 1, C2-A: Control 2 after application of BCAs

There were few differences in soil bacteria communities from different soil treatments using DNA extraction kit. Based on 0.90 coefficient similarity, soil samples from paddy field were clustered into seven (7) zones and the samples were not separated between before and after application of biological control agent unlike the culture dependent method. It implies that only slight differences at bacterial communities for each treatment based on two (2) zones. At zone I, there was L3 area which was treated with Liquid 3 formulation, which had about 10% difference with L3 area before treated
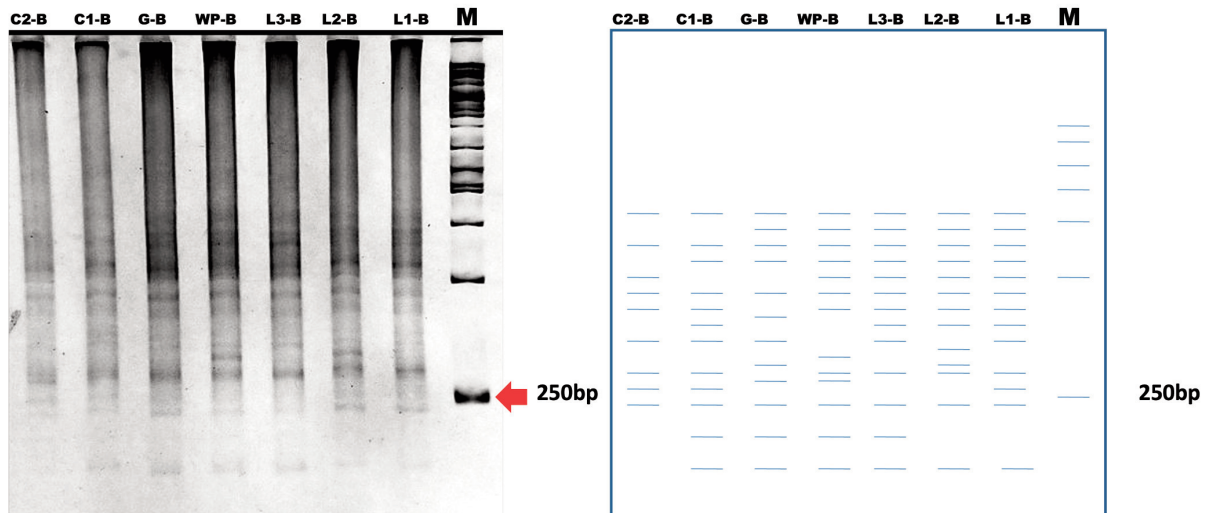
Figure 4. Soil bacterial community structures revealed by PCR-RISA analysis in soil samples before application of biological control agents (BCAs) using culture independent method of DNA extraction; Lanes: M: Marker 1Kbp DNA ladder, L1-B: Liquid 1 before application of BCAs; L2-B: Liquid 2 before application of BCAs; L3-B: Liquid 3 before application of BCAs , WP-B: Wettable Powder before application of BCAs, G-B: Granule before application of BCAs, C1-B: Control before application of BCAs 1, C2-B: Control 2 before application of BCAs
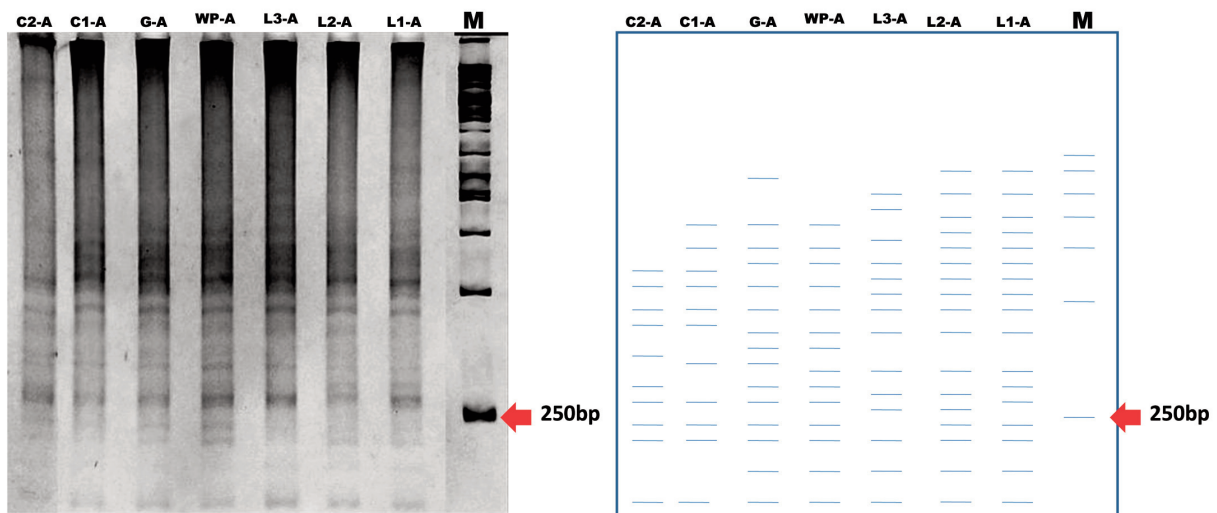

Figure 5. Soil bacterial community structures revealed by PCR-RISA analysis in soil samples after application of biological control agents (BCAs) using culture independent method of DNA extraction; Lanes: M: Marker 1Kbp DNA ladder, L1-A: Liquid 1 after application of BCAs; L2-A: Liquid 2 after application of BCAs; L3-A: Liquid 3 after application of BCAs, WP-A: Wettable Powder after application of BCAs, G-A: Granule after application of BCAs, C1-A: Control after application of BCAs 1, C2-A: Control 2 after application of BCAs

with the same treatment. Control 1 and Control 2 area had similar bacterial communities. Different bacterial communities were spotted from WP area which was treated with Wettable Powder formulation and granule formulations, it also appeared at Control 1 and Control 2 area (Figure 6). L1, and L2 samples before and after treatment had 80% similarities of bacterial community, L3 samples had more than 90% similarities, while WP, G, C1, C2 samples

about 71% before and after treatment for each samples.

This study used two different DNA extraction methods to identify soil bacteria community structures. Culture dependent method was done by using CTAB, meanwhile culture independent method was done by using commercial DNA extraction kit. After cell lysis, the commercial kit used column filtration during DNA purification, which had low
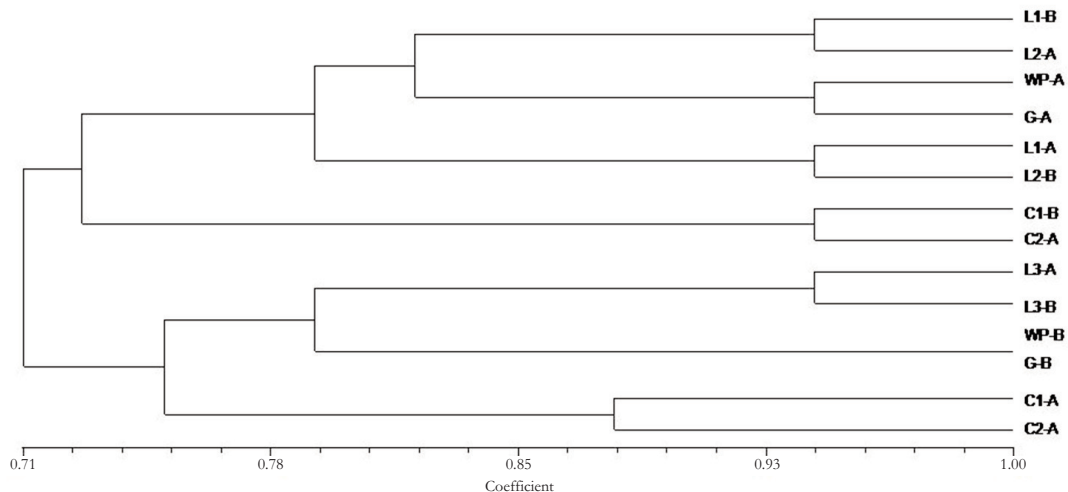
Figure 6. Un-weighted pair group method with arithmetic mean dendogram of paddy soil before and after treatment of biological control agents (BCAs) using culture independent method of DNA Extraction. L1-B: Liquid 1 before application of BCAs; L2-B: Liquid 2 before application of BCAs; L3-B: Liquid 3 before application of BCAs, WP-B: Wettable Powder before application of BCAs, G-B: Granule before application of BCAs, C1-B: Control before application of BCAs 1, C2-B: Control 2 after application of BCAs. L1-A: Liquid 1 after application of BCAs; L2-A: Liquid 2 after application of BCAs; L3-A: Liquid 3 after application of BCAs , WP-A: Wettable Powder after application of BCAs, G-A: Granule after application of BCAs, C1-A: Control after application of BCAs 1, C2-A: Control 2 after application of BCAs

efficiency with respect to DNA purity and yield for our samples. Because DNA is a negatively charged molecule, we suggest that the filtration column method may not effectively work to separate DNA from contaminants derived from paddy soil due to characteristics that are shared between DNA and these contaminants.

Results obtained from PCR-RISA analysis of the paddy soil microbial community revealed a clear influence of the DNA extraction method in the detected microbial diversity and community composition. The CTAB method yielded DNA with an enriched profile compared to soil DNA obtained using commercial kits, which yielded a lower number of weaker bands with lower intensities. The culture independent method was used to confirm the bacterial community directly from soil without culturing on synthetic media. This method should amplify more DNA fragments rather than culture dependent method because only less than 1% bacteria can be cultured. But, in this study this culture independent method showed less DNA fragments rather than culture dependent method. Other processes are still needed to get optimal result, for example by using purification kit to get highest quality of DNA samples. The results obtained for the DNA isolated with the

commercial kit may be related to the lower amount of DNA recovered after extraction, which may have interfered with the PCR-RISA profiles. The DNA extracted with the commercial kit demonstrated that bacterial communities in the Control 2 and another samples are more similar to each other. The similar patterns were observed when commercial kit was used to extract DNA (Leite *et al.*, 2014). Even though the culture independent method showed less bands than culture dependent method, it showed higher similarity of bacterial community structure. It means there was no impact on bacterial community after application of biological control agent because the soil already contained many microorganisms including bacteria.

These results demonstrated that the choice, evaluation and standardization of DNA extraction methods are critical, further highlighting the importance of DNA extraction steps in microbial community and ecology studies (Leite *et al.*, 2014). Soil bacterial communities and the soil processes mediated by bacteria are critical for ecosystem functioning and productivity in agriculture lands. There is a need to integrate soil bacterial community into our understanding of ecosystem interactions at a scale relevant to the whole-plant, between-plants,

and landscape levels. However, successfully accomplishing this has been extremely difficult. The microscopic nature and the immense diversity of soil bacteria have so far precluded accurate of comprehensive surveys of soil bacterial species, and heterogeneity within the soil matrix has made it difficult to obtain meaningful ecological information from single-sample investigations.

In bacterial communities, different organisms will perform the same processes and probably be found in the same niches (Zhao *et al.*, 2012). Factors that affect microbial diversity can be classified into two groups, i.e., abiotic and biotic factors. Abiotic factors include both physical and chemical factors such as water availability, salinity, oxic/anoxic conditions, temperature, pH, pressure, chemical pollution, heavy metals, pesticides, antibiotic, etc. (Bååth, 1998). In general, all environmental variations different affect and to different degrees, resulting in shifts in the diversity profiles. Biotic factors include plasmids, phages, transposons that are types of accessory DNA that influence the genetic properties and in most cases, the phenotypes of their host and thus have a great influence on the microbial diversity (Zhao *et al.*, 2012). In addition, protozoans are also reported to influence microbial diversity (Clarholm, 1994).

Since only minority of bacterial communities are culturable, only a limited fraction has been fully characterized and named. Prokaryotic organisms are difficult to classify, and the validity of the classifications have often been questioned. The morphological characteristics such as cell shape, cell wall, movement, flagella, Gram staining etc. may not be adequate to establish a detailed taxonomic classification of microbes. Advances in molecular and chemical ecology have provided promising alternatives in estimating microbial diversity without having to isolate the organisms (Giovannoni *et al.*, 1990).

Bacterial abundance and community composition were affected by soil depth. In the present study, the amount of organic matter within soils of this region at different depths could affect soil bacterial communities. Total extractable DNA and cultured heterotrophy abundance decreased with soil depth, suggesting a decrease in soil biomass with depth. Bolton *et al.* (1993) showed where total microbial

biomass (including bacteria, fungi, and other microscopic eukaryotes), as measured by biomass C, N, ATP, and enzyme activities were found to decrease with soil depth. After PCR-RISA, care should be taken with the interpretation of the RISA-ITS amplicon profiles, as these are measurements of bacterial diversity rather than an absolute means to identify specific species (Flight *et al.*, 2015). PCR-RISA can deduce highly reproducible bacterial community profiles. Limitations of RISA include requirement or large quantity of DNA, relatively longer time requirement, and low resolution (Fisher & Triplett, 1999). Although the original protocols of RISA are simple and fast in comparison with other gel-based DNA fingerprinting methods, the detection step of DNA fingerprinting are cumbersome due to the use of a silver staining. While the sensitivity of these detection procedures is sufficiently high within gel-based fingerprinting methods, they are time consuming and require high level of technical skill and experience to achieve high reproducibility.

Soil microbial communities act as a biotic factor involved in the supressing soil pathogens (Garbeva *et al.*, 2004). Garbeva *et al.* (2006) reported that the diversity of soil microbial communities is one of the keys to the functioning of soil supressing ability, also a positive correlation between suppression of *Rhizoctonia solani* AG3 and microbial diversity was observed. The diversity of soil microbes in an area provides less opportunity for pathogenic microbes such as *Rhizoctonia solani*, the common pathogen of rice, to affect plant growth. Fungicides are mostly used for managing plant pathogens. High doses of fungicides greatly disturb the structure of the microbial communities within soil and usually lead the diminution of the soil microbial population and the decrease enzymes activities (Roman *et al.*, 2021).

CONCLUSION

There were differences of soil bacterial community structure from paddy fields before and after application of biological control agents. Based on culture dependent method, bacterial community showed approximately 50–56% similarities and 70–90% similarities based on culture independent method. Study of soil microbial diversity using alternative sensitive molecular methods and DNA sequencing

of species belonging to these communities are still required for further information.

ACKNOWLEDGEMENTS

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