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by m umar harun

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G Baskara[1*], N Gofar[2], U Harun[2], J Widada[3], P A Hardini[1], R Wandri[1] and D Asmono[1]

[1] Sustainability, Research, and Development Division, PT Sampoerna Agro Tbk., Jl. Basuki Rahmat 788, Palembang, Indonesia, 30128.
[2] Faculty of Agriculture, Sriwijaya University, Raya Palembang-Prabumulih Street Km. 32, Indralaya District, Ogan Ilir Regency, Indonesia, 30662.
[3] Department of Agricultural Microbiology, Faculty of Agriculture, Gadjah Mada University, Flora Street, Yogyakarta - 55281, Indonesia

*Corresponding author: gregorius.nugraha@sampoernaagro.com

Abstract. During the drought stress period, oil palms experienced increasing ACC substance synthesis converted to ethylene. It decreases root growth and plant tolerance to drought stress. Ethylene production can be controlled by transforming ACC into α-ketobutyrate and ammonia via ACC deaminase (ACCd). This enzyme is produced by bacteria in the plant rooting system when the plant experiences drought stress. This study aimed to characterize ACCd bacteria diversity from selected oil palm progenies with difference responses to drought stress using ribosomal intergenic spacer analysis (RISA). The method used was by isolating bacteria from oil palm root surface using the Dworkin-Foster media enriched with ACC. Bacteria were then isolated, identified molecularly based on 16S rRNA genes, and profiling their population from selected oil palm progenies. The study results show that nine isolates managed to be isolated and characterized based on their morphology. Molecular identification based on their gene bank and phylogenetic analysis revealed that the ACCd bacteria community were divided into three major groups, i.e., *Firmicutes, Actinobacteria,* and *Proteobacteria.* Genus *Pantoea, Acinetobacter, Pseudomonas, Burkholderia, Kocuria*, and *Bacillus* were identified and could be utilized as bioagents to overcome the drought stress on oil palm crops. Based on the composition of the PCR-RISA fragments, showed that the oil palm rhizosphere of progeny P8 had a higher functional genetic diversity than progeny P1 and P13. Analysis of the similarity pattern of the ACC deaminase producing bacterial community divided 2 large clusters with a similar pattern of up to 69 %.

1. Introduction

Oil palm is one of Indonesia's superior commodities due to its major contribution to the Indonesian economy. The demand for vegetable oil derived from palm oil for the food and fuel sector (biodiesel) has risen in tandem with the global population growth [1,2]. Indonesia currently holds the title for the largest oil palm plantations globally, although they are yet to reach their full productivity potential.

The productivity of oil palm was impacted by various elements such as environmental conditions, genetic traits, and cultivation techniques. Environmental conditions, especially abiotic, such as drought stress, have decreased oil palm plant productivity [1,3,4]. The drought stress inhibits vegetative and generative growth of oil palms. It decreases fresh fruit bunch (FFB) production by 10-40% and crude palm oil (CPO) by 21-65% [5,6].

The biological reaction of oil palm undergoing drought pressure was shown by a rise in the production of 1-aminocyclopropane-1-carboxylic acid (ACC) in plant tissue. ACC oxidase subsequently converts ACC into ethylene. Ethylene is a hormone that plants need to stimulate root growth and help the fruit ripening process. However, concentrations that are too high can inhibit root growth and weaken the plant's resistance to various stress conditions [7,8]. Ethylene production can be controlled by transforming ACC into α-ketobutyrate and ammonia by ACC deaminase produced by ACC deaminase (ACCd) producing bacteria on the plant rooting system or rhizosphere [9–12].

A study analysed potentials of ACC deaminase coding genes, i.e., *Acd*S genes in the IMG (Integrated Microbial Genomes) database and discovered 485 strains included in the *Acidovorax, Bacillus, Brenneria, Bordetella, Burkholderia,Collimonas, Curvibacter,Cupriavidus, Dickeya, Halomonas, Herbaspirillum, Lonsdalea, Methylibium, Phytophthora, Pantoea, Pseudomonas, Polaromonas, Ralstonia, Serratia, Tatumella, Variovorax*, and *Xenophilus* [13]. These ACCd bacteria are genetically able to help the plant to survive during the drought stress or act as a bioagent inoculated to the plant.

Using of ACCd bacteria to control ethylene synthesis, especially during the vegetative and generative growth period of plants, is an effort to increase the growth and resistance of oil palm to drought stress. Research on the capability and application of ACCd bacteria for oil palm has not been documented. Its utilization for oil palm farming is a hopeful technological option to counter the decline in productivity resulting from water shortages during dry periods. This study aimed to characterize ACCd bacteria diversity from selected oil palm progenies with difference responses to drought stress using ribosomal intergenic spacer analysis (RISA).

2. Materials and Method

2.1. Study Site & Planting Materials

Trial was conducted in Surya Adi Estate, Mesuji, Ogan Komering Ilir, South Sumatera, Indonesia with coordinates of 104.20º N and 106.00º E, and 2.30º to 4.15º S in December 2019. The planted oil palm variety was superior oil palm of the DxP Sriwijaya Semi Clonal aged 13 years. Three selected progenies give the difference response to drought stress and divided into tolerance progeny, moderate progeny, and susceptible progeny. Each progeny consists of two replicates which is use for isolation ACC deaminase producing bacteria and ribosomal intergenic spacer analysis.

2.2. Sample Collection

The method used in sample collection was the composite sampling method. Soil and root sample collections were carried out at a 2 m distance from the trunk base of 30 x 30 cm with 0-20 depths surrounding the selected oil palms. In total, there were 18 sampling points, where 500-gram samples were collected from each point. Soils were composited and put into plastic containers and carried out to the laboratory using an icebox.

2.3. Isolation & Morphological Characterization of ACCd Bacteria

The ACCd bacterial obtained from positive test tube from MPN then isolated and carried out by macroscopic and microscopic morphological characterization. Macroscopic morphological

characterization included observation of colony colour, colony shape, edge shape, and elevation [14], while the microscopic morphological characterization included Gram staining.

2.4. 16S rRNA PCR Amplification

Isolates were grown on Luria Bertani (LB) medium for 24 hours at 30oC. Extraction of bacterial genomic DNA was carried out using the Quick-DNA Bacterial Miniprep Kit. The extracted DNA was measured for concentration and purity using BioDrop. PCR amplification in the 16S rRNA region used primers 27F and 1492R. The PCR reaction volume conduced was 25 µl consisting of 12.5µL GoTaq Green Master Mix 2X (Promega, USA); 0.6 µL primer 27F dan 1492R (10 pmol concentration); 9.3 µl nuclease-free water, and 2 µl bacterial genome DNA (20 µg µL-1) as the template. The conditions of PCR Bio-RAD C1000 Thermal Cycler used were pre-denaturation (95^{o}C for 3 minutes), denaturation (95^{o}C for 30 seconds), annealing (55^{o}C for 15 second), extension (72^{o}C for 20 seconds), and final extension (72^{o}C for 5 minutes) for 35 cycles. The amplified DNA was sequenced and then aligned with GenBank data using the BLAST-N program on the NCBI website. The construction of the evolutionary relationship or phylogenetic tree was conducted utilizing the MEGA 6.0 software program.

2.5. DNA Extraction for RISA Amplification

Positive test tube from MPN collected based on selected progenies for DNA genome extraction. The Quick-DNA Bacterial Miniprep Kit was used to extract the bacterial genomic DNA in accordance with the manufacturer's instructions. PCR-RISA amplification with RISA primer 926F (5'> AAAACTYAAAKGAATTGACGG<3'), and primer 23SR (5'>GGGTTBCCCCATTCRG<3'). The final volume of the PCR mix used was 25 µl. The non-coding region between the 16S and 23S genes of ACC deaminase-producing bacteria was amplified using primers 926F and 23 SR. Prior to amplification, DNA was first diluted to a concentration of 2.5 ng/ml. The reaction for PCR contained a mixture of 2 µl DNA (2.5 ng/ml), 12.5 µl MasterMix PCR GoTaq Green 2 µl 926F primer, 2 µl 23 SR primer, and 6.5 µl nuclease-free water into a PCR microtube. Amplification was carried out using a PCR machine with an initial denaturation of 95°C for 5 minutes, then followed by 35 cycles consisting of denaturation, primer attachment, and polymerization each at a temperature of 95°C for 15 seconds, a temperature of 50°C for 15 seconds, and a temperature of 72°C for 1 minute [15]. The final polymerization was extended at 72°C for 7 minutes.

2.6. Analysis of Diversity & Phylogeny of ACC Deaminase Producing Bacteria

The analysis of genetic diversity of electrophoresis results was measured based on the similarity coefficient. All bands of DNA fragments from polymerization visualized on the gel were counted as binary data with the provisions, a value of 1 if there were bands of DNA fragments and a value of 0 if there were no bands in the same row. CorelDRAW© 2018 software was used in the digitization process. NTSYSpc 2.0 (Numerical Taxonomy and Multivariate Analysis Systems) was used to perform kinship analysis [16,17]. This program can be used to see the relationship between several samples by looking at whether or not a physical parameter/factor appears in each sample.

3. Results and Discussion

3.1. Screening of ACCd Bacteria

ACCd bacteria isolates were characterized by colony and cell morphologies by observing the shape, edge, elevation, and color, and were subjected to Gram staining. Positive Gram bacteria demonstrated purple color while negative Gram bacteria demonstrated red color at the end of the staining process [18].

In Table 1, bacterial isolates show various colony morphologies, namely circular, irregular, and punctiform. Colony edges are smooth, irregular, and lobate. Colony elevation includes convex, flat, raised, and umbonate. Observation of the shape, edge, elevation, and color of microbial colonies' growth is determined by the genetic makeup of the organism and environmental factors around it.

Table 1. Characteristics of ACCd Bacterial Isolates on Modified DF Medium

Isolate	Colony Morphology				Cell Morphology	
	Shape	Edge	Elevation	Colour	Cell Shape	Gram *
P6	*circular*	*smooth*	*raised*	yellow	short rod	-
P8	*irregular*	*lobate*	*umbonate*	cloudy white	short rod	-
P11	*irregular*	*irregular*	*flat*	beige	rod	-
P14	*punctiform*	*smooth*	*convex*	green	short rod	-
P15	*punctiform*	*smooth*	*flat*	beige	cocci	+
P16	*irregular*	*irregular*	*flat*	cloudy white	rod	+
P17	*punctiform*	*smooth*	*umbonate*	white	rod	+
P25	*irregular*	*lobate*	*umbonate*	chocolate	short rod	-
P27	*irregular*	*irregular*	*umbonate*	yellow	short rod	-

Information: * + Gram positive bacteria; - Gram negative bacteria.

The genetic expression of organisms is contingent upon environmental factors, including the availability of nutrients, temperature, and humidity [14,19]. Gram staining is used to determine the cells' shape, and the properties of the Gram ACCd bacteria obtained. Gram staining can be seen in Fig 2; three isolates were Gram-positive, while six other isolates were Gram-negative.

Previous studies have shown that demonstrated that bacteria capable of producing ACC deaminase can assist plants in enduring and adapting to drought stress conditions [20,21]. Hence, further investigation was required to determine the overall quantity of bacteria and the quantity of bacteria producing ACC deaminase in oil palm offspring undergoing drought stress.

3.2. Identification of ACCd Bacteria 16S rRNA Genes

The DNA visualization results of PCR products demonstrate that all ACCd bacterial isolates had bands with a ± 1500 bp size. The success of 16S rRNA gene amplification was characterized by forming a single band parallel to the size of 1500 bp. Nucleotide base sequence of the 16S rRNA gene is most often used for classification or as a molecular marker to determine bacterial species at this time [22,23].

Table 2. Results of BLAST-N Sequence Gen 16S rRNA ACCd Bacteria

Isolate Code	**BLAST results**	**Accession Number**	***Query Coverage***	**Similarity**
P6	*Acinetobacter nosocomialis* strain *RUH 2376*	NR_117931.1	99%	98.38%
P8	*Burkholderia arboris* strain *R-24201*	NR_042634.1	99%	98.76%
P11	*Burkholderia cepacia* strain *NBRC 14074*	NR_113645.1	97%	97.92%
P14	*Burkholderia metallica* strain *R-16017*	NR_042636.1	97%	97.83%
P15	*Bacillus subtilis* strain *BCRC 10255*	NR_116017.1	98%	99.83%
P16	*Kocuria palustris* strain *TAGA27*	NR_026451.1	98%	99.42%
P17	*Bacillus tequilensis* strain *10b*	NR_104919.1	99%	99.17%
P25	*Pantoea dispersa* strain *DSM 30073*	NR_116797.1	100%	98.60%
P27	*Pseudomonas knackmussii B13*	NR_121733.1	100%	98.23%

Identification of ACCd bacterial isolates was carried out by analysing the nucleotide base sequences of the conservative DNA short strands as bacterial species markers. The conservative 16S rRNA gene had ±

500 bp hypervariable region at the end of the sequence, which is different for each type. This area was used as a marker for the type of bacteria obtained by comparing the nucleotide sequence with the nucleotide sequence in the gene bank database.

Based on identification results in Table 2, nine ACCd bacteria isolates in oil palm rhizosphere during the drought stress period were identified as the *Acinetobacter nosocomialis* strain RUH 2376, *Burkholderia arboris* strain R-24201, *Burkholderia cepacia* strain NBRC 14074, *Burkholderia metallica* strain R-16017, *Bacillus subtilis* strain BCRC 10255, *Kocuria palustris* strain TAGA27, *Bacillus tequilensis* strain 10b, *Pantoea dispersa* strain DSM 30073, and *Pseudomonas knackmussii* B13. The query coverage value is the percentage of the length of the nucleotides that are in line with the database. The higher the value, the higher the match between the bacterial sequence and the database sequence. The similarity value of 99% indicates that the isolates are considered the same species. Meanwhile, the similarity of ≥ 97% can be stated that the isolates compared were in the same genes, and the species homology was between 89-93%. Phylogenetic analysis was employed as the further analysis observe the branches produced by ACCd bacteria isolates through position observation placed between other species or comparing species.

The evolutionary tree in Figure 1 displays two significant categories of ACCd bacteria in the oil palm's rhizosphere, i.e., Gram-negative and Gram-positive ACCd bacterial isolates. Gram-negative ACCd bacterial isolates included the *Pantoea, Acinetobacter, Pseudomonas*, and *Burkholderia* genes, while Gram-positive ACCd bacterial isolates included the *Kocuria* and *Bacillus* genes. The bootstrap value indicates how well the model dataset was used in the reconstruction of the phylogenetic tree. Bootstrap value is used to estimate the phylogenetic tree's trust level, where a higher bootstrap value means a higher phylogenetic tree topology trust [16,24,25].

Pantoea dispersa ICGV-2 isolated from peanut plants had an ACC deaminase activity of 0.90 ± 0.06 (nmolμg/protein hr-1) [26]. 50% of *Acinetobacter nosocomialis* from sugarcane roots produced high amounts of ACC deaminase from the *Acinetobacter* gene, which was isolated [27]. The *Pseudomonas* gene isolated from oil palm plants in this study was *Pseudomonas knackmussii* B13. This species has not been widely reported in terms of drought stress; however, could induce plant resistance to salinity stress and produce the IAA hormone, which was reported to synergize with ACC deaminase in overcoming drought stress [28]. *Burkholderia arboris* strain R-24201, *Burkholderia cepacia* strain NBRC 14074, and *Burkholderia metallica* are the *Burkholderia* genes found in oil palm plants as a phosphate solvent and a phytohormone producer [29]. *Burkholderia* was confirmed to have the acdS gene successfully isolated from the roots of pineapples, gripped by herbicide accumulation, waterlogging, and the attack of *Phytophthora* spp [30].

P16 isolates have similarities with *Kocuria palustris* TAGA27, which is included in the *Actinobacteria* filum. *Kocuria palustris* JRT2, another species that may be closely related, was detected to use ACC as a nitrogen source and produced IAA in drought conditions [31]. Another possible species is Kocuria rhizophila, which has the capacity to endure NaCl concentrations exceeding 10% and displayed the capability to enhance plant growth by dissolving phosphate and producing IAA [32].

The isolated γ-*Proteobacteria* group was P17 isolates, the *Bacillus tequilensis* bacteria, and P15 isolates, the *Bacillus subtilis* bacteria. *Bacillus tequilensis* S2-H16 [33] and *Bacillus tequilensis* J12 [34] could survive well in osmotic stress induced with 25-30% PEG in the growth medium. Moreover, elevated ACC deaminase activity was also accompanied by the production of exopolysaccharide compounds, plant hormones such as jasmonic acid and salicylic acid, and inhibition of abscisic acid synthesis, which has similar effects to ethylene [35]. Another species, i.e., *Bacillus subtilis* could induce tomato resistance to drought stress [36], increased plant physiological adaptation to drought [37], formulated exopolysaccharide substances [38], and increased plant biomasses [39].

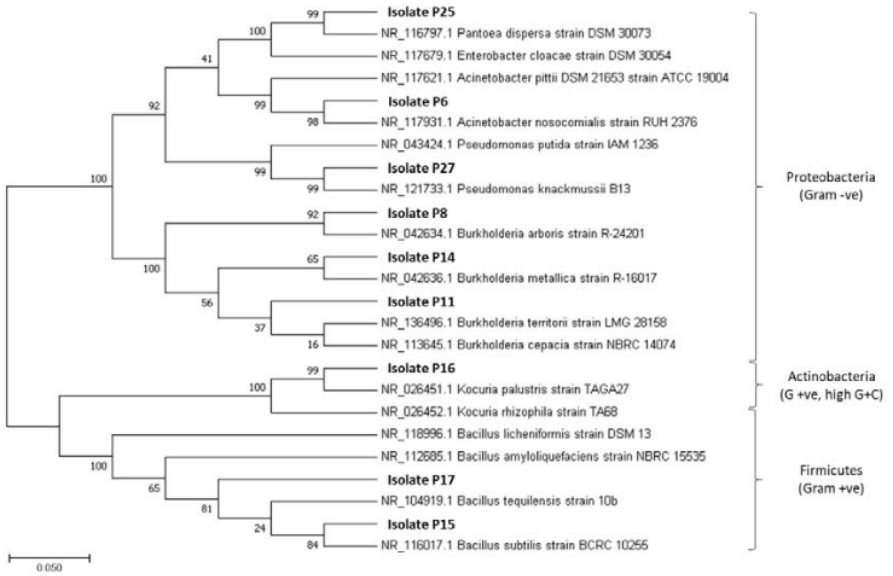

Figure 1. Phylogenetic Tree of ACCd Bacteria Isolates Based on the Neighbor-Joining Method with a bootstrap value of 1000 replications. The evolutionary distance was calculated using the Maximum Composite Likelihood method.

3.3. Community Structure of ACC Deaminase Producing Bacteria Based on PCR-RISA

The results of polymorphic analysis of the IGS sections have sizes from 200 bp to 3.0 kb. The results of amplification using PCR-RISA obtained DNA banding pattern as a result of gel electrophoresis visualization as shown in Figure 2. The specific pattern of DNA bands is the DNA blueprint for each type of microbe. It also describes the community profile with each specific DNA band indicating the microbial population in the rhizosphere of oil palm plants. These results indicate that the rhizosphere of tolerant plants in all replicate has the highest diversity of bacterial communities. Tolerant progenies (P8) in samples showed more diverse communities than moderate progenies (P1) and susceptible progenies (P13). Diversity of ACC deaminase producing bacteria in P1 and P13 was not significant. It was suspected that the two progenies have high similarity of phenology response to drought stress and have a close genetic characteristic. In addition, the response to normal conditions of the two progenies needs further investigation.

The variations observed in the DNA fragment profiles within the rhizosphere of different oil palm progenies result from disparities in the quantity and intensity of bands, as well as the structure of the prevailing pattern. Similar numbers and sizes of fragments indicate the dominance of a specific population within the community. However, it is important to note that the fragment pattern derived from RISA analysis cannot provide precise information about the taxonomic composition of the community due to the presence of overlapping size classes among related populations. The results of polymorphic analysis of the section that separates the *rrs* and *rrl* IGS (intergenic spacer) genes have size variations from 200 bp to 3000 bp.

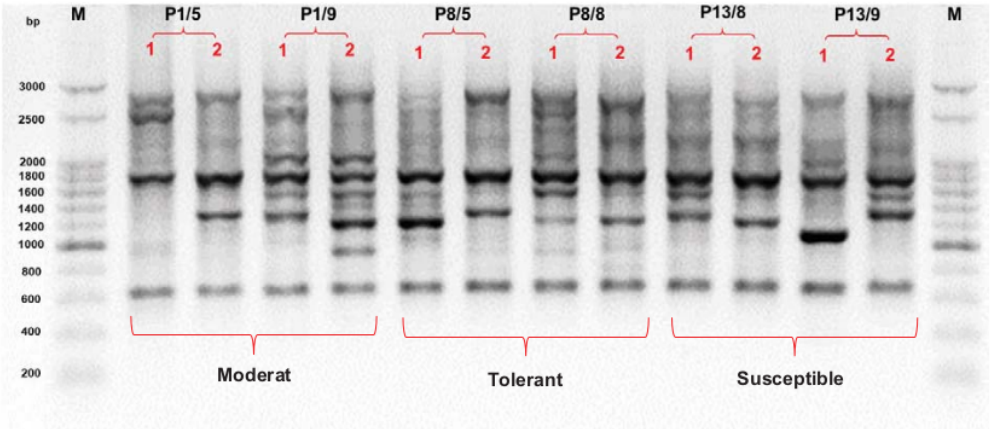

Figure 2. Visualization results of intergenic spacer region amplicon (16s rRNA – 23s rRNA) using Ribosomal Intergenic Spacer Analysis (RISA). Concentration of fine agarose gel 3%, 50 volts for 3 hours at a temperature of 15-20°C. P1/5 and P1/8 were moderate progenies, P8/5 and P8/8 were tolerant progenies, while P13/8 and P12/9 were susceptible progenies with 2 replicates.

Figure 2 demonstrates that there are two groups of bacteria that produce ACC deaminase and are prevalent in the rhizosphere of susceptible, moderate, and tolerant palm oil progenies. The ACC deaminase-producing bacterial community with sizes of 600 bp and 1800 bp are bacteria belonging to the α-proteobacteria and β-proteobacteria groups [42]. Fragments with a size above 1800 bp in the P1 and P8 progenies were seen to be very diverse, both in terms of size and thickness of their DNA bands. While in the P13 progenies, fragments with large sizes above 1800 bp only a small part that can be detected and the population is very small. Furthermore, each DNA band that appears will be further analysed to determine the close relationship between communities of ACC deaminase-producing bacteria in each selected progeny.

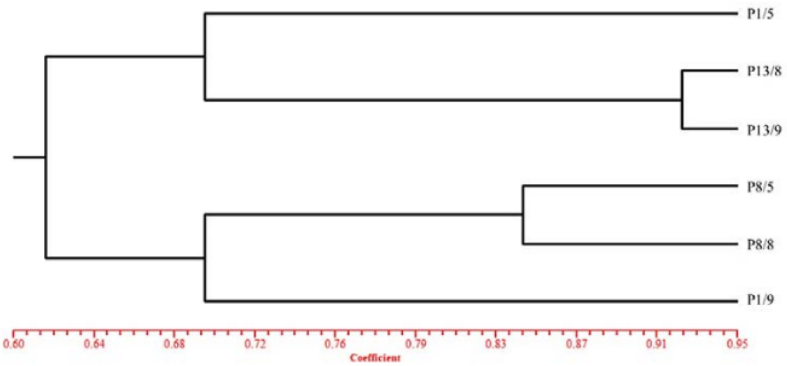

Figure 3. Dendrogram-UPGMA based on the pattern of DNA fragments from PCR-RISA

The findings of the cluster analysis in Figure 3, which assess the resemblance among all communities of ACC deaminase-producing bacteria in chosen plants, are categorized into 2 primary clusters based on the placement of the DNA bands from RISA. Analysis of the pattern of similarity between the community of ACC deaminase-producing bacteria with binary data on samples P13/8 and P13/9 isolated from susceptible oil palm plants had a similarity of ± 92%. Nevertheless, the group of bacteria in oil palm plants that produce ACC deaminase, specifically P8/5 and P8/8, showed a similarity of 84% in their community composition, which remained identical to the cluster found in moderately tolerant palm oil P1/9. Meanwhile, the community pattern of ACC deaminase-producing bacteria sample P1/5 in moderate oil palm was separated from the tolerant oil palm cluster and had a closer relationship to the susceptible oil palm cluster. In general, the tolerant and susceptible progeny clusters had a community similarity of 69%. Primers that attach between the 16S and 23S rRNA genes, bacteria that live dominantly in a sample or environment show a certain profile in the RISA method [42,43]. RISA reveals a specific community profile, at least one organism in the community is indicated by one DNA band [44,45].

4. Conclusion

Oil palm plants are vulnerable to the adverse effects of drought stress, which can significantly diminish their productivity. To address these challenges, there is a growing need for microbial-based biological strategies. In the investigation of ACCd bacteria within oil palm roots during periods of drought stress, *Pantoea, Acinetobacter, Pseudomonas, Burkholderia, Kocuria*, and *Bacillus* groups were successfully isolated based on their ability to utilize ACC as a nitrogen source. ACC serves as a precursor to ethylene, which can be converted into ammonia and α-ketobutyrate by ACCd bacteria. These bacteria exhibit potential in enhancing plant resilience to drought stress and stimulating plant growth under unfavorable environmental conditions. Nevertheless, additional investigation was necessary to comprehensively evaluate the efficacy of ACCd bacteria in alleviating water scarcity pressure for oil palm crops, both in the nursery and in the field.

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