

Full-Length 16S rRNA Gene Amplicon Profiling of Soil Bacterial Communities under Rubber–Canna Intercropping and Rubber Monoculture

Cut Riska Arista¹, Nita Noriko¹, Hidayat Yorianta Sasarela¹, Yunus Effendi^{1*}

¹Magister in Natural Resources Management, Faculty of Science and Technology, Universitas Al-Azhar Indonesia, Jl. Sisingamangaraja Kebayoran Baru, West Jakarta, 12110.

Corresponding Author/E-mail: effendiy@uai.ac.id

Abstract- Rubber-based intercropping may modify soil bacterial communities through changes in vegetation cover, root inputs and soil chemistry. This comparative observational study evaluated bacterial-community profiles in rubber–canna (*Hevea brasiliensis*–*Canna indica*) intercropping and rubber monoculture plots at PTPN VIII, Subang, West Java. Three spatial soil subsamples per system were collected from 0–20 cm depth. Community DNA was extracted from 0.25 g soil, near-full-length 16S rRNA genes were amplified with 27F/1492R primers and amplicons were sequenced using Oxford Nanopore GridION. Reads were profiled using a Centrifuge/Krona read-based workflow. Shannon diversity was higher in intercropping than monoculture (5.00 ± 0.16 versus 4.78 ± 0.21), whereas Chao1 richness was lower (1575.8 ± 145.8 versus 1778.8 ± 223.6), exploratory Mann–Whitney tests were not significant. The systems shared 2,221 classified taxonomic units, with 531 units only in intercropping and 375 only in monoculture. *Proteobacteria* relative abundance was higher in intercropping (49%) than monoculture (27%). Soil organic C, total N, available P, Mg and Ca were also descriptively higher in intercropping. These findings indicate contrasting plot-level bacterial and soil-chemical profiles, but independent plot replication and functional validation are needed before causal conclusions are made.

Keywords - *Canna Indica*, *Hevea Brasiliensis*, Intercropping System, Rhizosphere Bacteria.

INTRODUCTION

Rubber (*Hevea brasiliensis*) is an economically important perennial crop in Indonesia. In Subang Regency, West Java, rubber plantations are managed under state, private and smallholder systems. Conversion of structurally complex vegetation to simplified monoculture can reduce soil organic carbon, alter nutrient balance and modify soil-biological communities [1-2]. These concerns have encouraged interest in rubber-based intercropping and agroforestry as strategies for maintaining ground cover, diversifying farm products and supporting ecological functions in plantation soils.

Canna indica was selected as the intercrop because it tolerates partial shade and variable soil-moisture conditions, develops relatively rapid above-ground cover and can grow beneath woody stands [3-4]. Its

canopy and litter may protect the soil surface, while roots and rhizodeposits may alter the quantity and composition of organic substrates entering the rhizosphere. These characteristics provide a biologically plausible basis for expecting differences in bacterial-community structure between rubber–canna and rubber-only soils. However, such mechanisms should be treated as hypotheses unless root exudation, microbial activity, nutrient transformation, or plant performance is measured directly.

Soil bacterial communities participate in organic-matter decomposition, nutrient transformations, plant–microbe interactions, and other biogeochemical processes. Their composition is shaped by vegetation, soil physicochemical properties, management history and spatial heterogeneity [5]. Marker-gene sequencing enables culture-independent characterization of bacterial

communities, but sequencing PCR products of the 16S rRNA gene should be described as 16S rRNA gene amplicon sequencing, not shotgun metagenomic sequencing. Alpha-diversity indices, taxonomic relative abundance, and functional potential also represent different analytical levels and should not be conflated.

The earlier work of Effendi et al. [6] compared rubber soil with and without Canna using Illumina-based 16S rRNA profiling and reported differences mainly at class and family levels. The present study extends that work by examining soil after approximately four years of rubber–canna establishment, using near-full-length 16S rRNA gene amplicons sequenced with Oxford Nanopore technology, presenting species-level taxonomic labels and considering bacterial-community patterns together with selected soil chemical measurements. Because only one plot per planting system was represented and the three samples within each plot were collected approximately 1 m apart, the current data provide plot-level comparative evidence rather than replicated evidence of a general intercropping effect.

This study aimed to compare Shannon diversity and Chao1 richness estimates between the sampled rubber–canna intercropping and rubber monoculture plots, describe shared and system-specific classified taxonomic units, compare taxonomic profiles including *Proteobacteria* and selected high-count bacterial labels and describe co-occurring patterns in selected soil chemical properties. The study did not directly test bacterial function or establish causal relationships among intercropping, microbial communities, soil nutrients and plant performance.

METHODS

Study Site, Design, Soil Collection and Handling

Soil sampling was carried out at PTPN VIII Rubber Plantation, Jalan Raya Kalijati Timur Km 7, Subang District, Subang Regency, West Java (6°34'03.0"S 107°47'00.0"E) in May 2023. Each planting system was represented by one field plot. Six soil samples were collected from two sites under investigation: rubber–canna intercropping and rubber monoculture. Three sampling points were positioned approximately 1 m apart within each plot. Therefore, the three samples per system were spatial subsamples within a plot rather than independent plot-level biological replicates. Statistical comparisons are consequently exploratory and

cannot be generalized as treatment effects across rubber plantations.

At each sampling point, soil was collected from 0–20 cm depth around the plant/root zone using a cleaned shovel. Approximately 1 kg of soil was transferred to a labeled zip-lock bag and placed in a cool box for transport to the laboratory. A 0.25-g portion was used for DNA extraction, while the remaining soil was allocated for chemical analysis.

DNA Extraction and Near-Full-Length 16S rRNA Gene Amplification

Community DNA was extracted from 0.25 g soil using the ZymoBIOMICS DNA Miniprep Kit. Soil was added to a ZR BashingBead Lysis tube containing 0.1- and 0.5-mm beads, mixed with 750 µL lysis solution and vortexed for 20 min. Subsequent filtration, binding, washing and centrifugation steps followed the manufacturer workflow. DNA was eluted in 55 µL DNase-free water. DNA concentration was assessed using NanoDrop and Qubit measurements before sequencing-library preparation.

The bacterial near-full-length 16S rRNA gene was amplified using primers 27F and 1492R in a 25-µL PCR reaction containing 12.5 µL MyTaq Red Mix, 6.5 µL primer mixture containing 10 ng of each primer, 2 µL DNA template and nuclease-free water to the final volume. Thermal cycling consisted of initial denaturation at 95 °C for 1 min; 35 cycles of denaturation at 95 °C for 15 s, annealing at 55 °C for 15 s, and extension at 72 °C for 20 s. PCR products were expected at approximately 1,500 bp. Amplicons were checked by electrophoresis on a 0.8% TBE agarose gel at 70 V for 25 min using a 1-kb DNA ladder and visualized under UV illumination.

Library Preparation, Bioinformatic Analysis and Soil Chemistry

Library preparation was conducted using Oxford Nanopore Technologies kits according to the manufacturer instructions. GridION sequencing was performed using MinKNOW software version 20.06.9. Base calling was performed using Guppy version 4.0.11 in high-accuracy mode. FASTQ read-quality summaries were generated using NanoPlot [7]. Filtered reads were classified using Centrifuge [8] with the Bacteria and Archaea index obtained from the Centrifuge distribution. Downstream inspection and visualization were performed using Pavian and Krona Tools. The final analysis reported in this manuscript was read-based and used

classified taxonomic labels from the Centrifuge/Krona output. No OTU clustering or ASV-denoising workflow was used for the diversity and taxonomic-unit results reported here.

The six soil samples were submitted to PT UNILAB PERDANA for soil chemical analysis. The dataset reports organic C, total N, available P and exchangeable K, Na, Mg and Ca. Values were summarized descriptively as means $\pm$ standard deviations where internally consistent source values were available. Because paired sample-level chemistry values were not available for all parameters, the relationship between bacterial-community profiles and soil chemistry was not evaluated using correlation, constrained ordination, or multivariate modeling. The interpretation therefore treats bacterial and chemical patterns as co-occurring observations rather than evidence of direct mechanistic relationships.

RESULT AND DISCUSSION

Amplification of The Near-Full-Length 16S rRNA Gene

All six samples produced visible amplicon bands near the expected size of approximately 1,500 bp, supporting their use for near-full-length 16S rRNA gene amplicon sequencing (Figure 1). Band intensity varied among samples, indicating that the gel image should be interpreted as a qualitative check rather than a quantitative assessment of library input. The successful amplification confirms that the DNA extracts were adequate for downstream Oxford Nanopore sequencing of near-full-length 16S rRNA gene amplicons.

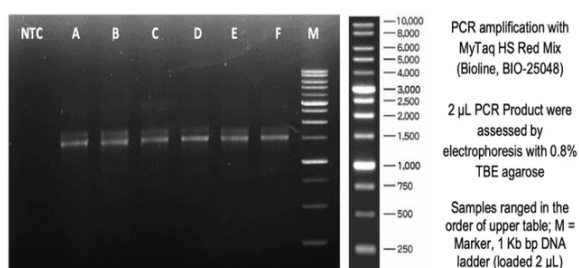


Figure 1. Agarose-gel Visualization of Near-Full-Length 16S rRNA Gene PCR Products. NTC = Non-Template Control; A–F = Soil Samples; M = 1-kb DNA Ladder.

Alpha Diversity and Richness Patterns

Shannon diversity was descriptively higher in the rubber–canna intercropping subsamples, whereas Chao1 richness was descriptively higher in rubber monoculture. The intercropping subsamples showed

Shannon diversity of 5.00 ± 0.16 compared with 4.78 ± 0.21 in monoculture, while Chao1 richness was 1575.8 ± 145.8 in intercropping and 1778.8 ± 223.6 in monoculture (Figure 2). Exploratory exact Mann–Whitney U tests were non-significant for both Shannon and Chao1 ($p = 0.40$), and the lack of independent plot replication precludes treatment-level inference.

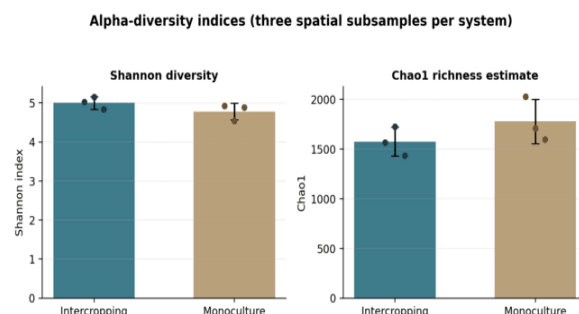


Figure 2. Shannon Diversity and Chao1 Richness Estimates for Three Spatial Subsamples in each Sampled Plot. Error Bars Represent Sample Standard Deviations; Points Show Individual Subsample Values.

The contrasting Shannon and Chao1 patterns show that diversity, richness, and abundance should not be used interchangeably. Shannon diversity integrates richness and evenness, while Chao1 emphasizes rare taxonomic units [9–10]. Thus, the results do not support a simple statement that intercropping increased all aspects of bacterial diversity. Instead, they indicate that the sampled intercropping and monoculture plots differed in alpha-diversity structure, with higher evenness-sensitive diversity in intercropping and higher estimated richness in monoculture.

The non-significant exploratory tests should be interpreted with caution because the three samples within each planting system were spatial subsamples from one plot rather than independent plot-level biological replicates. Microbiome studies are sensitive to sample size, effect size, sequencing depth and diversity metric selection [10]. The alpha-diversity results are therefore best understood as descriptive plot-level observations that generate hypotheses for future replicated studies.

Shared and System-Specific Classified Taxonomic Units

The two planting systems shared 2,221 classified taxonomic units, while 531 units were detected only in the intercropping samples and 375 only in the monoculture samples (Figure 3). The larger number of system-specific units in the intercropping profile suggests that the presence of *Canna indica* may be

associated with additional bacterial labels in the sampled plot. This interpretation is ecologically plausible because intercrops can alter vegetation cover, litter inputs, root-derived substrates, and soil microhabitats that influence bacterial community assembly in the rhizosphere [5], [11].

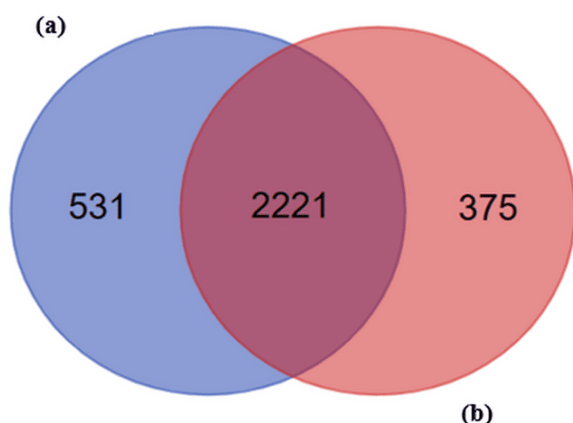


Figure 3. Shared and System-Specific Classified Taxonomic Units Reported For Rubber–Canna Intercropping and Rubber Monoculture. Left circle = Intercropping; Right Circle = Monoculture.

These system-specific values must remain conservative because the final analysis was read-based and did not include a documented OTU or ASV workflow. The term classified taxonomic units is therefore more appropriate than OTUs or ASVs. Unique-label counts in amplicon datasets can be influenced by sequencing depth, read-quality filtering, classification thresholds, database completeness and low-frequency reads. For stronger inference, future analyses should report per-sample sequencing depth, quality-score distributions, length filtering, classification thresholds, database version, normalization strategy and final analytical unit [12–13].

Taxonomic Composition and Selected Bacterial Labels

One of the clearest taxonomic differences was the higher relative abundance of *Proteobacteria* in the rubber–canna intercropping profile. Krona visualization indicated that *Proteobacteria* accounted for approximately 49% of classified reads in intercropping compared with 27% in monoculture

(Figure 4). This result suggests that the bacterial community in the sampled intercropping plot was compositionally distinct from that in monoculture.

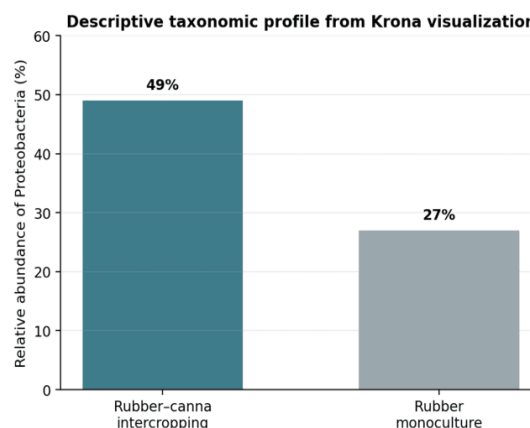


Figure 4. Descriptive Relative Abundance of *Proteobacteria* in The Aggregated Krona Profiles

Proteobacteria are frequently reported as dominant or responsive members of plant-associated and rhizosphere bacterial communities, including taxa that respond to labile carbon sources, root exudates, and nutrient-rich rhizosphere environments [5]. Intercropping systems can modify belowground microbial communities by changing plant diversity, root architecture, organic inputs, nutrient availability, and rhizosphere interactions [14–15]. The higher *Proteobacteria* fraction in the rubber–canna intercropping profile is therefore consistent with the hypothesis that *Canna indica* contributed to a different rhizosphere environment beneath the rubber stand (Figure 5).

This *Proteobacteria* result remains a relative-abundance pattern. Amplicon sequencing data are compositional, and an increase in the relative abundance of one group may reflect a decrease in others rather than a true increase in absolute abundance [13]. A higher *Proteobacteria* percentage therefore does not by itself prove higher bacterial biomass, improved soil fertility, or increased nutrient-cycling activity. Such claims would require absolute quantification, normalized abundance tables, functional-gene assays, enzyme measurements, or metagenomic/metatranscriptomic evidence.

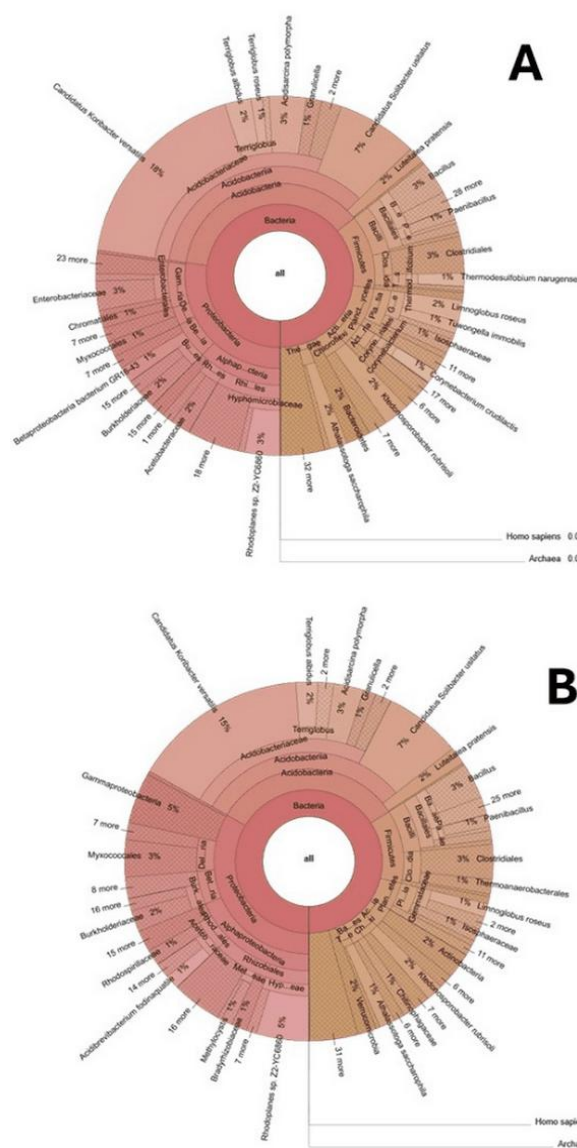


Figure 5. Representative Krona visualizations of Taxonomic Profiles for Rubber–Canna Intercropping (A) and Rubber Monoculture (B)

Several high-count bacterial labels were more frequently reported in the intercropping profile, including *Pseudolabrys* sp. FHR47, *Azospirillum* sp. CFH 70021, *Phenylobacterium* sp. HYN0004, *Phenylobacterium zucineum* and *Bradyrhizobium zhanjiangense*. In contrast, *Ktedonosporobacter rubrisoli* and *Corynebacterium crudilactis* were higher in the monoculture profile. Seven of the ten high-count labels belonged to *Proteobacteria*, supporting the broader observation that this phylum contributed strongly to the intercropping profile (Figure 6).

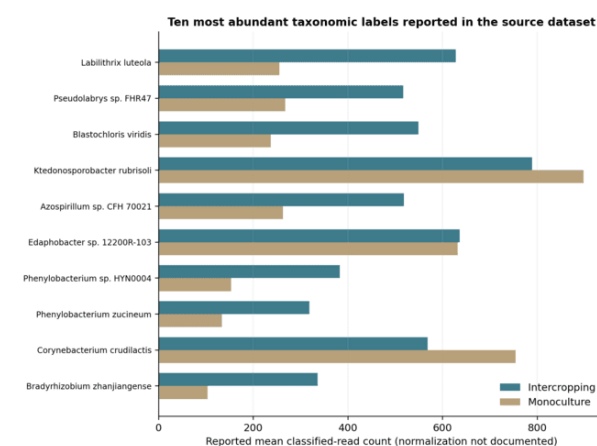


Figure 6. Reported Mean Classified-Read Counts for Ten Taxonomic Labels. Normalization was not Documented in The Source Dataset; Values are Interpreted Descriptively

The ecological interpretation of selected bacterial labels must remain hypothesis-generating. *Azospirillum* species are widely discussed as plant-growth-promoting bacteria, with potential mechanisms including biological nitrogen fixation, phytohormone production, stress tolerance and rhizosphere colonization [16-17]. Members of *Bradyrhizobium* are strongly linked to nitrogen cycling and plant-associated soil functions, although functional capacity can vary across strains and environments [18]. Phosphate-solubilizing bacteria and phosphatase-associated microbial processes can influence plant phosphorus acquisition, but these functions require direct biochemical or molecular confirmation [19].

Near-full-length 16S rRNA gene amplicon sequencing can improve taxonomic resolution compared with short-read 16S approaches, but it does not demonstrate gene expression, metabolic activity, nitrogen fixation, phosphate solubilization, phytohormone production, or plant-growth promotion [12]. Thus, the manuscript does not claim that these bacteria directly improved soil fertility or plant performance. The intercropping plot instead showed higher reported counts of bacterial labels whose related taxa have been associated in the literature with plant-associated or nutrient-cycling functions.

Soil Chemical Properties and Co-Occurring Microbial Patterns

The soil chemical data showed higher reported values of organic C, total N, available P, Mg and Ca in the intercropping plot, whereas K was higher in the monoculture plot and Na values were relatively similar. These patterns are biologically compatible with the expectation that intercropping may influence soil properties through plant residue inputs, root activity, nutrient uptake patterns and rhizosphere microbial processes. Agroforestry and diversified cropping systems can modify soil organic matter, nutrient cycling, aggregation and microbial habitats by changing plant inputs and belowground resource flow [11], [20].

The higher organic C and total N reported in the intercropping plot may reflect greater organic inputs from *Canna indica* litter, root turnover, or rhizodeposition. The higher available P, Mg, and Ca may also indicate differences in nutrient availability or initial soil conditions. These co-occurring soil chemical patterns provide useful ecological context for interpreting the bacterial community results, particularly the higher *Proteobacteria* relative

abundance and the higher reported counts of several plant-associated bacterial labels in the intercropping profile.

The current dataset cannot establish causal relationships among planting system, bacterial taxa, and soil chemical properties. The study did not include paired replicate-level soil chemistry for robust correlation analysis, constrained ordination, or multivariate modeling. The soil chemistry results should therefore be described as co-occurring descriptive patterns rather than mechanistic evidence that specific bacterial groups altered nutrient availability. Initial plot heterogeneity, fertilization history, soil texture, topography, drainage, plant age and local management practices may also have contributed to the observed differences.

Study Limitations and Implications for Rubber-Based Intercropping Systems

The most important limitation of this study is the absence of independent plot-level replication. Each planting system was represented by one field plot, and the three samples per system were spatial subsamples collected approximately 1 m apart. This design enables preliminary plot-level comparison but does not support generalized inference about the effect of rubber–canna intercropping across rubber plantations. In microbiome research, true biological replication, adequate sample size and clear effect-size reporting are essential for robust inference [10].

A second limitation is incomplete sequencing metadata. Per-sample read counts, read-length distributions, quality-score summaries, length-filtering criteria, chimera-handling procedures, database version and classification thresholds should be reported in future work. These details are particularly important for Oxford Nanopore full-length 16S rRNA gene amplicon sequencing because full-length reads can improve taxonomic resolution but also require careful handling of read length, error rate and taxonomic assignment [12]. A third limitation is the lack of direct functional validation. Although several bacterial labels were related to genera with potential plant-associated or nutrient-cycling functions, 16S rRNA gene amplicon data cannot demonstrate microbial activity.

The results suggest that incorporating *Canna indica* into rubber plantations may be associated with shifts in bacterial-community composition, including higher Shannon diversity, a higher *Proteobacteria*

fraction and higher reported counts of selected plant- or nutrient-associated bacterial labels. These patterns are ecologically relevant because they suggest that the intercropping plot differed from the monoculture plot not only in vegetation structure but also in belowground microbial profiles. Similar principles have been reported in intercropping and agroforestry systems, where plant diversification can influence soil microbial communities and nutrient dynamics [14-15], [20].

Practical recommendations should not yet be made solely from the present data. Before rubber–canna intercropping can be recommended as a microbial or soil-fertility intervention, future studies should include multiple independent intercropping and monoculture plots, standardized soil sampling, full sequencing-quality metadata, normalized or absolute abundance data, complete soil analytical methods, and direct functional validation.

CONCLUSION

The sampled rubber–canna intercropping and rubber monoculture plots showed contrasting bacterial-community patterns. Shannon diversity was higher in intercropping, whereas Chao1 richness was higher in monoculture. *Proteobacteria* represented a larger proportion of the intercropping taxonomic profile, and several plant- or nutrient-associated bacterial labels had higher reported mean counts in intercropping. Selected soil chemical values also differed descriptively between plots. These findings indicate associations among planting system, bacterial-community structure and soil chemistry at the sampled site, but they do not demonstrate causal effects or direct bacterial function. Independent plot replication, complete sequencing and soil-method metadata, normalized taxonomic analyses and functional validation are required before broader conclusions can be made.

ACKNOWLEDGMENT

The authors thank PTPN VIII for access to the sampling site, LPIPM UAI for partial financial support, and Kemendikisaintek for the research grant awarded to Y.E.

REFERENCE

- [1] Ali, M. I. Ghani, Y. Li, H. Ding, H. Meng, and Z. Cheng, “HiSeq-based molecular characterization of soil microbial community diversity, structure, and predictive functional profiling in continuous cucumber soil affected by diverse cropping systems,” *Int. J. Mol. Sci.*, vol. 20, no. 11, p. 2619, 2019, doi: 10.3390/ijms20112619.
- [2] A. Liu, M. Y. Liang, J. W. Tang, Y. Q. Jin, Z. B. Guo, and K. H. M. Siddique, “Challenges of the establishment of rubber-based agroforestry systems: decreases in the diversity and abundance of ground arthropods,” *J. Environ. Manage.*, vol. 292, p. 112747, 2021, doi: 10.1016/j.jenvman.2021.112747.
- [3] H. Y. Sasaerila, S. Sakinah, N. Noriko, and R. S. Wijihastuti, “Effects of light environments on leaf traits and phenotypic plasticity of *Canna indica*,” *Biosaintifika*, vol. 13, no. 2, pp. 169–177, 2021, doi: 10.15294/biosaintifika.v13i2.30175.
- [4] P. N. Karungamye, “Potential of *Canna indica* in constructed wetlands for wastewater treatment: a review,” *Conservation*, vol. 2, no. 3, pp. 499–513, 2022, doi: 10.3390/conservation2030034.
- [5] N. Ling, T. Wang, and Y. Kuzyakov, “Rhizosphere bacteriome structure and functions,” *Nat. Commun.*, vol. 13, p. 836, 2022, doi: 10.1038/s41467-022-28448-9.
- [6] Y. Effendi, N. Aini, A. Pambudi, and H. Y. Sasaerila, “Metagenomics analysis of soil microbial communities in plant agroforestry system rubber tree (*Hevea brasiliensis*)–Ganyong (*Canna sp.*),” *IOP Conf. Ser. Earth Environ. Sci.*, vol. 468, p. 012045, 2020, doi: 10.1088/1755-1315/468/1/012045.
- [7] W. De Coster, S. D’Hert, D. T. Schultz, M. Cruts, and C. Van Broeckhoven, “NanoPack: visualizing and processing long-read sequencing data,” *Bioinformatics*, vol. 34, no. 15, pp. 2666–2669, 2018, doi: 10.1093/bioinformatics/bty149.
- [8] Kim, L. Song, F. P. Breitwieser, and S. L. Salzberg, “Centrifuge: rapid and sensitive classification of metagenomic sequences,” *Genome Res.*, vol. 26, no. 12, pp. 1721–1729, 2016, doi: 10.1101/gr.210641.116.
- [9] J. Gauthier and N. Derome, “Evenness-richness scatter plots: a visual and insightful representation of Shannon entropy measurements for ecological community

- analysis,” *mSphere*, vol. 6, no. 2, e01019-20, 2021, doi: 10.1128/mSphere.01019-20.
- [10] J. G. Kers and E. Saccenti, “The power of microbiome studies: some considerations on which alpha and beta metrics to use and how to report results,” *Front. Microbiol.*, vol. 12, p. 796025, 2022, doi: 10.3389/fmicb.2021.796025.
- [11] M. Hartmann and J. Six, “Soil structure and microbiome functions in agroecosystems,” *Nat. Rev. Earth Environ.*, vol. 4, pp. 4–18, 2023, doi: 10.1038/s43017-022-00366-w.
- [12] K. D. Curry et al., “Emu: species-level microbial community profiling of full-length 16S rRNA Oxford Nanopore sequencing data,” *Nat. Methods*, vol. 19, pp. 845–853, 2022, doi: 10.1038/s41592-022-01520-4.
- [13] H. Lin and S. D. Peddada, “Analysis of microbial compositions: a review of normalization and differential abundance analysis,” *NPJ Biofilms Microbiomes*, vol. 6, p. 60, 2020, doi: 10.1038/s41522-020-00160-w.
- [14] K. Dang, X. Gong, G. Zhao, H. Wang, A. Ivanistau, and B. Feng, “Intercropping alters the soil microbial diversity and community to facilitate nitrogen assimilation: a potential mechanism for increasing proso millet grain yield,” *Front. Microbiol.*, vol. 11, p. 601054, 2020, doi: 10.3389/fmicb.2020.601054.
- [15] X. Tang et al., “Cassava/peanut intercropping improves soil quality via rhizospheric microbes and increased available nitrogen contents,” *BMC Biotechnol.*, vol. 20, p. 13, 2020, doi: 10.1186/s12896-020-00606-1.
- [16] F. Cassán et al., “Everything you must know about *Azospirillum* and its impact on agriculture and beyond,” *Biol. Fertil. Soils*, vol. 56, pp. 461–479, 2020, doi: 10.1007/s00374-020-01463-y.
- [17] M. Numan et al., “*Azospirillum* spp. from plant growth-promoting bacteria to their use in bioremediation,” *Microorganisms*, vol. 10, no. 5, p. 1057, 2022, doi: 10.3390/microorganisms10051057.
- [18] M. Akob et al., “Acetylenotrophic and diazotrophic *Bradyrhizobium* sp. strain I71 from TCE-contaminated soils,” *Appl. Environ. Microbiol.*, vol. 88, no. 22, e01219-22, 2022, doi: 10.1128/aem.01219-22.
- [19] M. Billah, M. Khan, A. Bano, T. U. Hassan, A. Munir, and A. R. Gurmani, “Phosphorus and phosphate-solubilizing bacteria: keys for sustainable agriculture,” *Geomicrobiol. J.*, vol. 36, no. 10, pp. 904–916, 2019, doi: 10.1080/01490451.2019.1654043.
- [20] S. Fahad et al., “Agroforestry systems for soil health improvement and maintenance,” *Sustainability*, vol. 14, no. 22, p. 14877, 2022, doi: 10.3390/su142214877.