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Effect of Synthetic Fertilizer on Diazotrophic Bacteria in Secondary Forest and Oil Palm Soils in Central Kalimantan

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ABSTRACT

Soil microorganisms, especially diazotrophic bacteria, are vital for ecosystem sustainability, significantly contributing to nitrogen cycling and biodiversity conservation. Understanding the impact of different land-use practices on soil microorganisms, especially synthetic fertilizer addition, is essential for sustainable agriculture. This study compares soil properties, bacterial densities, and responses to fertilization in secondary forest soils and adjacent oil palm plantation soils located in PT Kerry Sawit Indonesia, Central Kalimantan. A greenhouse experiment was conducted using both secondary forest and oil palm plantation soils to evaluate the impacts of different synthetic fertilizers on nitrogen-fixing bacteria and soybean agronomic performance. Total soil bacteria and diazotrophic bacteria, essential for nitrogen fixation, were analyzed through microbiological assays and qPCR focusing on the nifH gene. Our findings show that oil palm plantation soils had higher levels of nitrogen, phosphate, and nitrate, while secondary forest soils had a higher abundance of diazotrophic bacteria. Furthermore, excessive nitrogen fertilization was found to reduce microbial density, negatively impacting plant sustainability, highlighting the importance of customized fertilizer management. The study highlights the intricate connections between land-use practices and microbial populations, providing valuable insights for balancing agricultural productivity with ecological sustainability.

1. Introduction

The rapid expansion of oil palm (*Elaeis guineensis*) plantations in Southeast Asia, particularly in regions like Central Kalimantan, Indonesia, has led to significant land-use changes, often at the expense of ecologically rich forests. Deforestation and oil palm expansion rates in this province are one of the highest in Indonesia (Broich *et al.* 2011; Sumarga and Hein 2016), accounted for 36% of total deforestation in Kalimantan (Gaveau *et al.* 2019). The ecological consequences of this transformation extend far beyond deforestation, encompassing significant alterations to soil properties and microbial communities in the long-term impacts (Pahalvi *et al.* 2021; Tripathi *et al.* 2020).

The transition from forested areas to agricultural land has significantly increased the use of synthetic fertilizers. Even though synthetic fertilizer has been important in boosting agricultural productivity and fulfilling the needs of an expanding global populations, this extensive application has come with consequences for the environment, human well-being, and soil microorganisms, particularly in relation to diazotrophic bacteria (Liao *et al.* 2018; Imran *et al.* 2021). These bacteria convert atmospheric nitrogen (N2) into ammonia (NH3), forms that plants can utilize, thus supporting plant growth in

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nitrogen-limited environments (Udvardi *et al.* 2021). Diazotrophic bacteria are essential to the sustainability of tropical soils, where they contribute to the natural nitrogen inputs that support plant growth and maintain ecological balance (Pankievicz *et al.* 2019).

Despite the critical role that diazotrophic bacteria play in maintaining soil health, there is a surprising lack of research on how synthetic fertilizers impact these microorganisms, particularly in the context of land-use change from forests to oil palm plantations in Indonesia. Zulfarina *et al.* (2017) found that soil bacterial community abundances differed between tropical rainforest and oil palm plantation samples. Fertilization temporarily increased bacterial abundances, even in the oil palm plantation. This result contradicts the idea that the conversion of rainforests to agricultural lands leads to a reduction in bacterial populations. Moreover, nitrogenase activity experienced a marked increase when organic manure and crop residue were applied together but decreased when chemical fertilizers were used alone in paddy fields (Tang *et al.* 2021).

This gap in knowledge is particularly concerning given the stark contrast between secondary forest soils, which are typically rich in organic matter and microbial diversity, and oil palm soils, which are often subjected to fertilization and reduced organic inputs. The differences between these two soil types provide a unique opportunity to investigate how synthetic fertilizers affect diazotrophic bacteria in distinct ecological contexts. To clarify the relationship between land-use practices, synthetic fertilization, and microbial communities, we conducted a greenhouse assay using soils from a secondary forest and an adjacent oil palm plantation in PT Kerry Sawit Indonesia, Central Kalimantan. We utilized both culture-independent and culture-dependent approaches to determine the density of diazotrophic and total bacterial communities in soil samples collected from these sites. Furthermore, we investigated changes in these bacterial communities in response to synthetic fertilizer application and evaluated the agricultural yield of soybean plants grown in these soils.

By comparing these two contrasting soil ecosystems, the research aims to determine the impact of synthetic fertilization on diazotrophic microbial communities and agricultural productivity. The findings from this study will not only contribute to a deeper understanding of the ecological consequences of agricultural intensification but also provide valuable insights into sustainable soil management practices. These practices are essential for balancing the need for agricultural productivity with the conservation of soil health and biodiversity, helping to mitigate the negative impacts of synthetic fertilizers on both microbial diversity and overall soil health.

2. Materials and Methods

2.1. Soil Sampling

High Conservation Value (HCV) forest site (South: S2°46'40.6", East: E112°30'13.5") and oil palm plantations site (South: S2°46'45.4", East: E112°30'22.9") located in PT Kerry Sawit Indonesia, East Kotawaringin Regency, Central Kalimantan is selected for soil sampling. HCV forest site is the area for conservation efforts, aiming to balance environmental preservation with no agricultural use for at least 20 years. Meanwhile, the oil palm plantations are used for commercial agricultural activities, focusing on the production of palm oil. Soil sampling is carried out randomly at six different locations at geographically close site (400-600 m distance) to each other (Zuluaga *et al.* 2020). At each sampling position, we performed soil excavation 1 meter away from the tree, reaching a depth of 20 cm (Zulfarina *et al.* 2017; Kirkman *et al.* 2022; Berkelmann *et al.* 2020). In each sampling point, four sub-samples were taken according to Lima *et al.* 2009 & Yaghoubi *et al.* 2021 resulting in 24 soil cores in total for each site.

2.2. Greenhouse Experiment

A composite soil sample, formed by combining 24 individual soil cores from each sampling point is thoroughly mixed and 2 kg of the soil from each site were placed in a 40 × 40 polybag (Junier *et al.* 2009; Zuluaga *et al.* 2020). Soybean seeds were immersed in 70% alcohol for 30s and 2.5% sodium hypochlorite solution for 5 minutes for surface sterilization. Then seeds were washed five times with sterile distilled water (de Castro *et al.* 2018). The plants were grown in a randomized complete design with three fertilization treatments: 0% F (no fertilization), 50% F (25 kg N plus 50 kg P plus 50 kg K per hectare), 100% F (50 kg N plus 100 kg P plus 100 kg K per hectare) with eight replications of each treatment. The N, P, and K synthetic fertilizers were applied as urea, superphosphate, and potassium chloride, respectively. Fertilization was done within 5 days after sowing (DAS) and 30 DAS. 15 days after sowing, the seedlings were thinned into one healthy plant per polybag. Soil samples were taken

at 0 days, 30 days, 60 days, and 90 days after seeding. Soil was collected from the area surrounding the main root of the plant (Junier *et al.* 2009). by soil corer of 16 mm diameter. The samples were kept at 4°C until DNA extraction and bacteria isolation.

2.3. Soil DNA Extraction and Conventional Polymerase Chain Reaction (PCR)

Genomic DNA is extracted in triplicate from 250 mg of composite soil samples using DNeasy® Power Soil® Pro Kit (Qiagen, Germany, Catalog No. 47014) according to the manufacturer's protocol. DNA concentrations are quantified with a Nanodrop™ 2,000 (Thermofisher). The composite soil samples are collected and extracted every month from each treatment until harvesting time. Extracted DNA samples were stored at -20°C for further use.

PCR mixture contained 5 µL of GoTaq Green Master Mix (Promega, Madison, Catalog No. M7122), 1 µL of forward primer, 1 µL of reverse primer were mixed in a PCR tube and the volume is adjusted to 10 µL using DNAse-free water to achieve final sample concentration of 1 ng/µL. The amplification nitrogenase gene is conducted using the primer pair IGK3-F (GCIWTHTAYGGIAARGGIGGIATHGGIAA) and DVV-R (ATIGCRAAICCICCRCAIACIACRTC) (Gaby & Buckley 2012). The PCR procedure included an initial step at 95°C for 50 second, followed by 35 cycles at 58°C for 45 seconds, 60 seconds at 72°C, and a final extension at 72°C for 5 minutes. To minimize bias, three technical replicates were prepared and collected for each PCR amplicon. The PCR products were evaluated on a 1% (w/v) agarose gel to ensure efficient DNA amplification.

2.4. Cloning and Sequencing of *nifH* Genes

Specific band sizes were purified with the QIAQuick® Gel Extraction Kit (Qiagen, Germany, Catalog No. 28704) and the QIAquick® PCR Purification Kit (Qiagen, Germany, Catalog No. 28104). The amplicon is then ligated to pGEM®-T-Easy Vector (Promega, Madison, WI, Catalog No. A1360) and transformed into *Escherichia coli* DH5α competent cells. Blue-white screening was conducted using ampicillin (100 mg/ml) and X-gal (40 mg/ml) in Luria agar (LA) plates. White colonies were selected and streaked onto plates containing Luria Agar with ampicillin (LA + ampicillin). Recombinant cells were grown overnights and plasmids were isolated using QIAprep® Spin Miniprep Kit (Qiagen, Germany, Catalog No. 27104). Plasmids isolated from white colonies were subjected to PCR amplification using vector-specific primer sets of M13F and M13R, along with the IGK3/DVV primer pair. The PCR product was checked on a 1% (w/v) agarose gel to confirm positive clones. The presence of the gene was also confirmed by sequencing using the ABI PRISM 3130 Genetic Analyzer (Applied Biosystem, CA, USA). The sequences were then compared to other known nucleotide sequences using BLAST (NCBI, Bethesda, MD).

2.5. Quantitative PCR (qPCR) Standard Curve and Putative N_2-fixing Bacteria

A standard curve is created using the plasmid previously described above. Tenfold dilutions were performed by pipetting 1 µL of plasmid sample into 9 µL of Nuclease Free Water (NFW). This process is repeated until 10^2 dilution is obtained. Each qPCR reaction with a total volume of 10 µL contained 5 µL 2X SensiFastTM HRM Kit (Meridian Bioscience, Catalog No. 32020), 1 µL of each IGK3 and DVV primer, 1 µL of plasmid dilution of 10^2 to 10^9, and nuclease free water was added to each mixture. Two replications of each dilution are prepared. qPCR is carried out on the MyGo Pro real-time PCR instrument (IT-IS International Ltd, Eastleigh, UK) with the following protocol: 95°C for 5 minutes, followed by 45 cycles of 95°C for 20 seconds, 58°C for 15 seconds, and 72°C for 20 seconds. The data were acquired at 72°C for 20 seconds. A melting curve was generated at the end of the reaction to confirm the specificity of the amplicon. A standard curve was generated by a line of regression through all data points. The gene copy number for each dilution was calculated using the equation below as stated by Armstrong 2019.

$$\text{Number of gene copy} = \frac{\text{ng of DNA} * 6.022 \times 10^{23}}{\text{Plasmid length} * 1 \times 10^{9} * 66}$$

Figure 1 Equation to determine nifH gene copy number. Concentration of DNA (ng/µL) multiplied by Avogradro's Number then divided by the weight of each base pair in grams. 1×10^9 represents the conversion from grams into nanograms.

A standard curve was generated by a line of regression through the means of each dilution obtained. To quantify *nifH* copy number from the soil samples, DNA concentrations of all samples obtained every month were adjusted by dilution to 50 ng µL−1 and quality (260:280 ratio) was confirmed at ~1.8. qPCR

was used to quantify nitrogen-fixing bacterial gene (*nifH*) using the primer mentioned above with the same PCR conditions. The gene copy number in soil samples is quantified by applying the established regression curves for gene copy number.

2.6. Plant Harvesting and Data Collection

Harvesting is carried out when the soybean crop has reached its physiological maturity. The soybean pods were measured and recorded. The plants were then carefully uprooted from the entire polybag to gather data on fresh root weight and dry root weight. The uprooting process involved exposing the entire root system to prevent loss. Adhering soil was carefully removed by gently washing the roots with tap water. Data of pod weight, number of pods, number of leaves, and plant height are also measured (Ntambo *et al.* 2017; Li *et al.* 2021). For statistical analysis, the data underwent an Analysis of Variance (ANOVA) to assess the impact of different fertilizer treatments using R (R Core Team).

2.7. Counts of Total Culturable Bacteria on Soil Extract and N-Free Malate Medium

Estimation of total culturable bacteria is conducted using soil extract medium (Nguyen *et al.* 2018) and nitrogen-free malate medium with bromothymol blue (BTB) indicator (Baldani *et al.* 2014) with the direct plating method involving a series of dilutions (Zakavi *et al.* 2022) 1 g of soil samples was diluted with 9 ml of physiological saline (0.85% w/v) then vortexed for 1 minute. The suspension was subsequently serially diluted (10^{-2} to 10^{-6}). Then, 100 µL of this mixture was spread onto the surface of agar media using a sterile spreader. The petri dishes were then incubated upside down in the dark at 30°C for 6-7 days. Initial screening of potential nitrogen-fixing organisms is carried out by observing the formation of a blue region when these isolates were grown on nitrogen-deficient malate media containing bromothymol blue (BTB) as an indicator. A color change to blue was considered a positive indicator indicating the presence of potential nitrogen-fixing microorganisms (Gothwal *et al.* 2008). Colony Forming Units (CFU) are counted during each observation for all samples.

2.8. Statistical Analysis

The culture-dependent (dilutions and plating) as well as -independent (DNA extractions, PCR, and qPCR) procedures were performed in triplicates. The data was analyzed using a one-way ANOVA, and comparisons were conducted for each pair using Tukey HSD test in R (R Core Team). For some datasets, t-tests were also performed. All values are given as means ± standard error of the mean (SEM). Differences were considered significant when the P-value was ≤0.05. Principal Component Analysis (PCA) was also conducted to reduce the dimensionality of the dataset and identify the main components contributing to variance. The PCA was performed using the "prcomp" function in R, and the resulting principal components were visualized using the ggplot2 package, which provided a clear graphical representation of the data structure and the relationships between variables.

3. Results

3.1. Soil Properties and Nutrient Contents of Secondary Forest and Oil Palm Plantation

In our study, we investigated the initial differences in soil properties and nutrient levels between secondary forest soils and adjacent oil palm plantation soils (Table 1). The pH of secondary forest soil was neutral at 6.8, whereas oil palm plantation soil was slightly lower with a pH of 6.3. Nutrient analysis revealed that oil palm soils had significantly higher levels of nutrients. Specifically, the total nitrogen content was 22% higher, phosphate levels were 89% higher, and nitrate levels were 83% higher compared to secondary forest soils.

Additionally, secondary forest soils had an ammonium content of 319.16 mg/kg, which was higher than the 285.7 mg/kg found in oil palm soils. Despite these differences in nutrient content, soil temperature did not significantly differ between the two land-uses (Table 1). Overall, our findings indicate that oil palm plantation soils have higher levels of nutrients, especially nitrogen, phosphate, and nitrate, and they

Table 1. Initial soil properties and nutrient contents of different land-use types

Soil parameters	HCV	OP	p-value
pH	6.8±0.11^{a}	6.3±0.14^{b}	0.02
Soil temperature	29±0.26^{a}	29±0.14^{a}	0.12
Nitrogen content (%)	0.18±0.003^{b}	0.23±0.005^{a}	0.01
Phosphate (mg kg^{-1})	37.7±0.13^{b}	361.7±0.66^{a}	0.00
Nitrate (mg kg^{-1})	6.51±0.09^{b}	39.3±0.22^{a}	0.00
Ammonium (mg kg^{-1})	319.16±6.58^{a}	285.7±5.37^{a}	0.05
Texture	Clay	Clay	

Mean ± SE values with different letters (a-b) represent significant differences when compared with each other (t-test, P<0.05). All data, except for texture, is included in this analysis

also tend to have a more acidic pH compared to soils in secondary forests.

3.2. Density of Bacterial Communities Derived from Secondary Forest and Oil Palm Plantation

To compare the total bacterial count in soil from secondary forest and oil palm plantation, soil extract medium (SE) was used for culturing bacteria. The densities of total bacteria in SE for both secondary forest (HCV) and oil palm plantation (OP) soils were similar, with counts of 3.37×10^7 CFU g^{-1} soil and 2.79×10^7 CFU g^{-1} soil, respectively (Figure 1). To measure the density of diazotrophic bacteria, which are favored by N-free malate medium (NFM), this medium was employed. The mean density of diazotrophic bacteria in HCV soil was 2.19×10^7 CFU g^{-1} soil, slightly higher than the 1.58×10^7 CFU g^{-1} soil in OP soil, although this difference was not statistically significant. Additionally, the abundance of *nifH* genes, indicative of nitrogen-fixing bacteria, was assessed using qPCR. HCV soils exhibited a significantly higher copy number of *nifH* genes (1.58×10^6 copy number g^{-1} soil) compared to OP soils (1.02×10^6 copy number g^{-1} soil).

3.3. Effect of Fertilizer Application on Bacterial Density of Secondary Forest and Oil Palm Plantation Soils

To assess how synthetic fertilizer affects the density of total bacterial and diazotrophic bacteria in soils of secondary forests and oil palm plantations, we measured bacterial growth in soil treated with different fertilizer concentrations. Subsequently, the soil samples were plated on soil extract and N-free malate medium each month. In general, the total bacterial and diazotrophic bacteria counts decreased after treatment in both secondary forest and oil palm plantation soils (Figure 2).

In the secondary forest soil, there were significant differences in total bacterial count at one and two months, but not at three months (Figure 2A). In the first month after fertilizer addition, the number of bacteria decreased in the treated soils (F50% and F100%) but slightly increased in the untreated soils. By the second month, the F50% fertilizer treatment resulted in the highest bacterial count compared to the F0% and F100% treatments. For the oil palm plantation soil, significant differences were observed only in the third month (Figure 2B). The F50% treatment resulted in the highest bacterial count of 1.36×10^7 CFU g^{-1} soil, followed by the F0% treatment with 1.22×10^7 CFU g^{-1} soil. The F100% treatment showed the lowest bacterial count at 9.36×10^6 CFU g^{-1} soil.

For total bacteria observed in nitrogen-free malate medium, significant differences were observed at two and three months (Figure 2C) in secondary forest soil. In the second month, the no fertilizer treatment resulted in the highest bacterial count of 1×10^7 CFU g^{-1} soil, followed by the F50% treatment with 7×10^6 CFU g^{-1} soil, while the F100% treatment showed the lowest count at 2.86×10^6 CFU g^{-1} soil. By the third

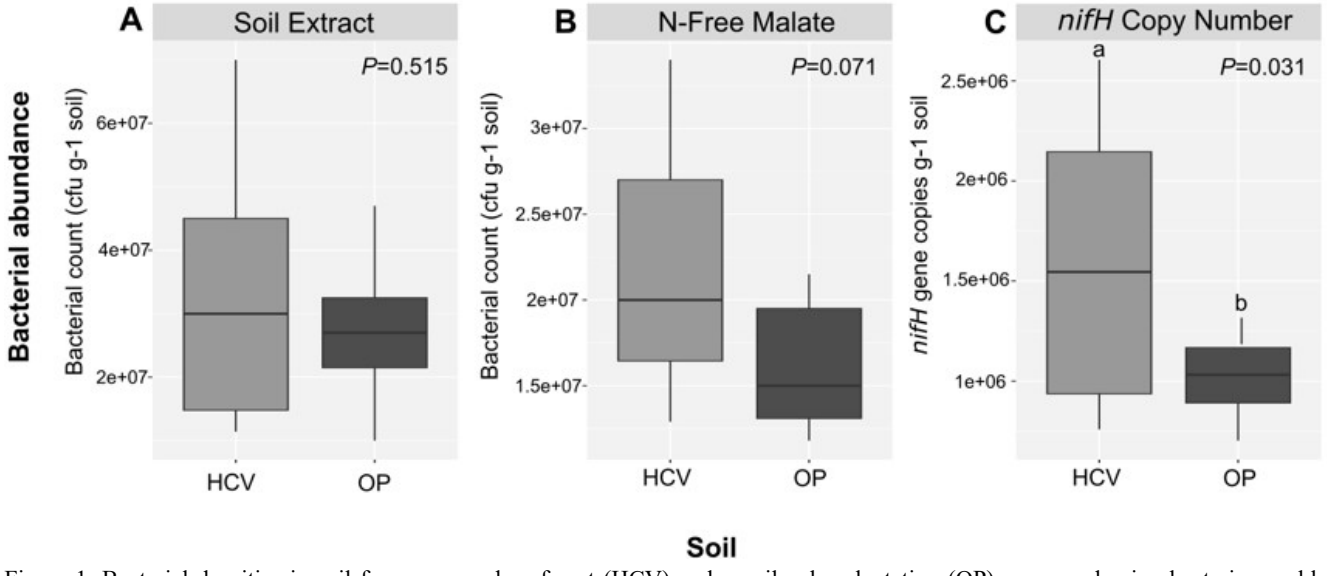

Figure 1. Bacterial densities in soil from a secondary forest (HCV) and an oil palm plantation (OP), measured using bacteria capable of growing in soil extract medium (A) and N-free malate (B), and quantified as nifH copy number using qPCR (C). Different lowercase letters denote statistical differences ($P<0.05$, t-test)

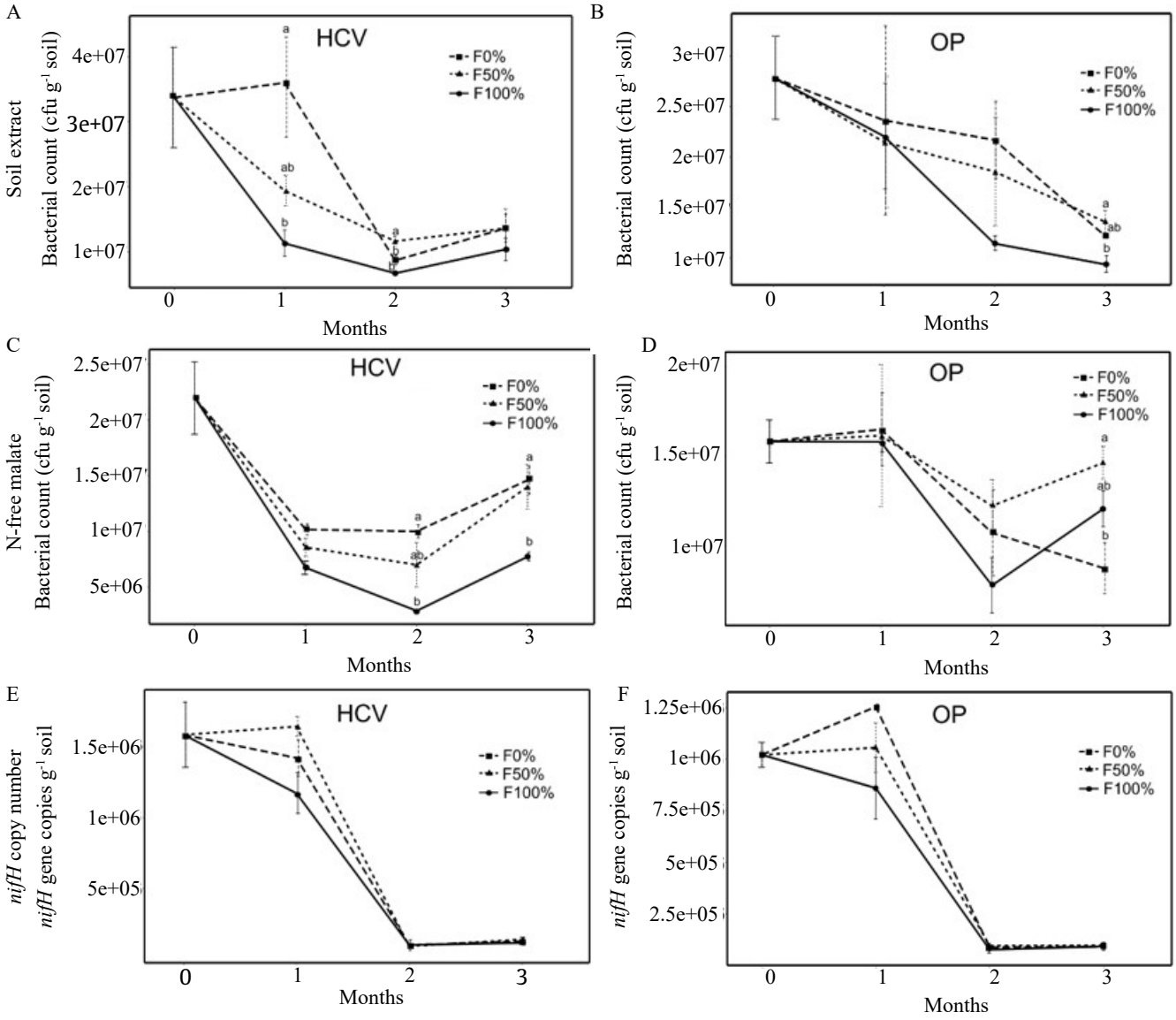

Figure 2. Changes in total colony forming unit (CFU) counts of bacteria grown in soil extract medium (A, B) and N-free malate medium (C, D), as well as quantification of nifH gene copy number using qPCR (E, F) in secondary forest (HCV) and oil palm plantation (OP) soils. The soils were treated with different fertilizer concentrations: no fertilizer (F0%), 50% fertilizer (F50%), and 100% fertilizer (F100%). Different letters indicate statistically significant differences between treatments (ANOVA followed by Tukey's HSD test; $P<0.05$)

month, there were no significant differences between the F0% and F50% treatments. In oil palm plantation soil, significant differences in diazotrophic bacteria were only observed in the third month (Figure 2D). The F50% treatment resulted in the highest bacterial count of 1.46×10^7 CFU g^{-1} soil, followed by the F100% treatment with 1.21×10^7 CFU g^{-1} soil, and the F0% treatment showed the lowest count at 8.77×10^6 CFU g^{-1} soil.

To assess *nifH* gene copy numbers in secondary forest and oil palm plantation soils, qPCR was performed. No significant differences were observed between treatments across all months (Figure 2E and F). However, unlike the traditional plating method, there was a slight increase in the abundance of the *nifH* gene after one month of treatment. Specifically, there were 1.64×10^6 copies g^{-1} in the F50% treatment for secondary forest soils and 1.25×10^6 copies g^{-1} in the F0% treatment for oil palm plantation soils. Similar with this observation, correlating diazotrophic bacteria and total *nifH* gene abundances showed a weak but significant linear increase ($R = 0.12$, $p = 0.004$) (data not shown).

3.4. Effect of Fertilizer Application on Soybean Growth in Secondary Forest and Oil Palm Plantation Soils

The impact of different fertilizer doses on soybean growth in secondary forest (HCV) and oil palm plantation (OP) soils is summarized in Table 2. The results show significant differences in agronomic characteristics such as leaf number, plant height, root dry weight, stem dry weight, number of pods, pod weight, and seed weight among the various treatments. Overall, soybeans grown in oil palm plantation soils generally exhibited higher values in the tested parameters compared to those in secondary forest soils.

In secondary forest soils, fertilizer application generally enhanced growth compared to the non-fertilized treatments. Soybeans grown with F50% fertilizer had an average leaf number of 4.8±1.65, which was higher than those in the F0% (2.6±0.49) and F100% (2.8±0.37) treatments. Plant height was consistent across treatments, with F50% resulting in 25.1±2.05 cm, similar with F0% (25.0±1.43 cm) and slightly lower than F100% (27.9±1.79 cm). Root and stem dry weights were higher in the F50% treatment (0.3±0.05 g and 0.7±0.16 g, respectively) compared to the F0% treatment (0.2±0.02 g and 0.4±0.04 g) but were comparable to the F100% treatment. The number of pods and pod weight were higher in the F50% and F100% treatments compared to F0%, with the F100% treatment yielding the highest seed weight (3.4±0.17 g).

In oil palm plantation soils, the non-fertilized treatment exhibited better results in some traits compared to the fertilized treatments. The F0% treatment resulted in the highest number of leaves (14.3±0.91) and comparable plant height (54.7±3.61 cm) to the F50% treatment (52.3±3.69 cm). The F100% treatment led to the tallest plants (66.6±2.79 cm). Root and stem dry weights were highest in the F100% treatment (4.8±0.75 g and 5.9±0.96 g, respectively), followed by F0% and F50%. The number of pods was also highest in the F100% treatment (25±1.75), although not significantly different from F0% (24.7±1.73) and F50% (20.3±2.56). Pod weight and seed weight were highest in the F0% treatment (39.7±3.98 g and 3.7±0.19 g, respectively), suggesting that moderate fertilizer application could be more advantageous for certain yield components.

3.5. Clustering of Land Type Uses with Different Fertilizer Treatments Based on Microbiology, Molecular and Phenotypic Variation

To reduce the dimensionality of this multivariate data and assess the relationship between various land types and fertilizer treatments based on biological factors, Principal Component Analysis (PCA) was conducted. The first two principal components account for almost 81% of the variation (Figure 3). The proximity between all agronomy parameters (NL, PH, NS, SDW, RDW, NP, PW, SW) indicates a positive correlation among them. Furthermore, the similarity between the total bacterial count in soil extract and the N-free malate and *nifH* gene copy numbers suggests a positive correlation among them.

The representation of different types of land and fertilizer treatments defined by the two principal components is also shown in the PCA graph. Using different colors to represent secondary forest soils with different fertilizer treatments (in blue) or the oil palm plantation group (in green), the plot demonstrates PCA's ability to appropriately differentiate both groups. Noteworthy, secondary forest soils with F100% are somewhat separated into groups below, far from F0% and 50%.

Based on the PCA, we observed that PC1, which explained 71% of the variance, was responsible for distinguishing between secondary forest and oil palm plantation soils. This was based on agronomic

Table 2. Mean values and variation of agronomic traits in soybean grown in soils from secondary forest and oil palm plantation with different fertilizer doses

Soil	Treatment	Leaf number	Plant height (cm)	Root dry weight (g)	Stem dry weight (g)	Number of pods	Seeds weight (g)
HCV	F0%	2.6±0.49^{a}	25.0±1.43^{a}	0.2±0.02^{a}	0.2±0.02^{a}	1.8±0.58^{a}	1.5±0.18^{b}
	F50%	4.8±1.65^{a}	25.1±2.05^{a}	0.3±0.05^{a}	0.3±0.05^{a}	2.9±0.62^{a}	2.9±0.19^{a}
	F100%	2.8±0.37^{a}	27.9±1.79^{a}	0.3±0.05^{a}	0.3±0.05^{a}	2.6±0.48^{a}	3.4±0.17^{a}
p-value		0.273	0.445	0.331	0.331	0.428	0.000
OP	F0%	14.3±0.91^{a}	54.7±3.61^{b}	3.6±0.76^{ab}	5.3±0.78^{a}	24.7±1.73^{a}	3.7±0.19^{a}
	F50%	9.2±1.28^{b}	52.3±3.69^{b}	2.5±0.39^{b}	4.7±0.87^{a}	20.3±2.56^{a}	3.6±0.13^{a}
	F100%	13.4±1.63^{ab}	66.6±2.79^{a}	4.8±0.75^{a}	5.9±0.96^{a}	25±1.75^{a}	3.5±0.15^{a}
p-value		0.02	0.01	0.05	0.608	0.212	0.486

Mean ± SE (One-Way test ANOVA followed by Tukey HSD multiple comparison post-hoc test)

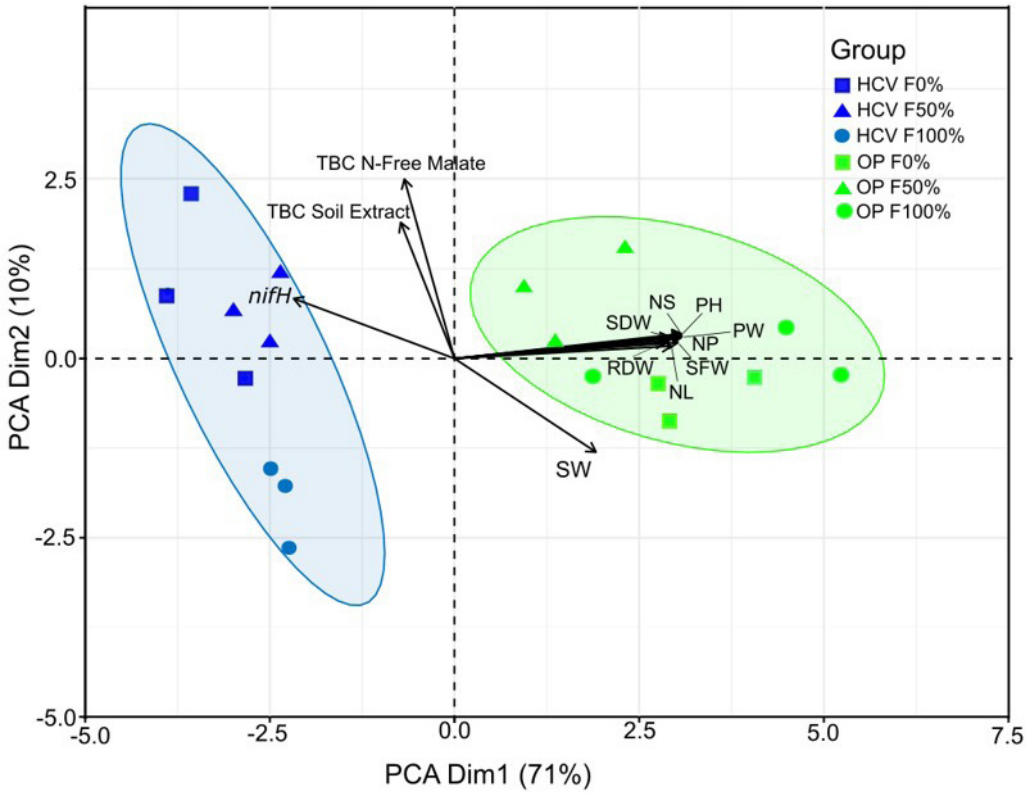

Figure 3. Principal component analysis (PCA) results from variables tested characterizing the effect of different fertilizers in two types of land uses. Codes for parameters: TBC, total bacterial count; *nifH, nifH* gene copy numbers; NL, number of leaves; PH, plant height; RDW, root dry weight; SDW, shoot dry weight; NP, number of pods; PW, pod weights; SW, seed weight

traits such as the number of pods, plant height, pod weight, and the number of seeds. The second principal component mainly separated samples based on the total bacterial count in nitrogen-free malate and soil extract, specifically distinguishing HCV F100% from the other samples. Notably, some trends were clearly exhibited: secondary forest soils were typically associated with *nifH* gene copy numbers, while oil palm soils were linked with higher agronomic traits.

4. Discussion

Understanding the impact of different land-use practices on soil microorganisms is crucial for developing sustainable agricultural systems. Secondary forests and oil palm plantations represent two contrasting land-use types, each with distinct management practices and ecological implications. As oil palm cultivation expands, it is essential to assess how these practices, especially the use of synthetic fertilizers, alter nitrogen fixation and associated microbial populations. Our study revealed several significant differences in initial soil properties, bacterial densities, and the impact of fertilizer treatments between secondary forest and oil palm plantation soils. By understanding these effects, we can develop strategies to enhance microbial activity and promote sustainable agricultural practices.

4.1. Contrasting Soil Dynamics and Diazotrophic Bacteria in Secondary Forests and Oil Palm Plantations

Our initial soil test data suggests that oil palm plantation soils exhibited higher levels of total nitrogen, phosphate, and nitrate compared to secondary forest soils, while also showing lower ammonium content. The significantly higher levels of nitrogen, phosphate, and nitrate in oil palm plantation soils can be attributed to the intensive fertilization practices typically employed in such plantations. The lower ammonium content in oil palm soils, despite higher overall nitrogen levels, suggests a different nitrogen transformation dynamic compared to secondary forest soils. Secondary forests, characterized by a higher soil quality index, accumulate organic matter through leaf litter and plant decomposition, enriching the soil and serving as a nitrogen source through microbial decomposition processes (Pereira *et al.* 2018; Ramadhan *et al.* 2022).

Furthermore, while the total bacterial population remains relatively stable across different land uses, the presence of diazotrophic bacteria is notably higher in secondary forest soils based on the *nifH* gene copy numbers. Diazotrophic bacteria can fix atmospheric nitrogen, producing ammonium usable by the plant (Pankievicz *et al.* 2021). Therefore, the combination of organic matter accumulation and microbial activity in secondary forests could promote a nutrient-rich soil environment conducive to plant growth and ecosystem health. This result adds to the body of literature suggesting that secondary forests can sustain microbial functions, which contribute to soil fertility through natural nitrogen fixation processes.

4.2. Microbial Density Response to Nitrogen Fertilization in Secondary Forest and Oil Palm Plantation Soils

Generally, the addition of fertilizer to secondary forest soil and oil palm soils exhibited similar trends in bacterial density (both total bacteria in soil extract and those in nitrogen-free malate medium) and in *nifH* gene copy number over the three-month observation period. All treatments showed a higher number of bacteria before planting soybeans compared to afterward. It is well-known that plants form microbial communities through root exudates (Pantigoso *et al.* 2022; Santoyo 2022). These exudates act as the main means of communication with their biological environment, promoting nutrient uptake, resource competition, species-specific signaling, microorganism attraction, and various other interactions (Rizaludin *et al.* 2021). By creating a diverse, carbon-rich environment, plants support unique microbial communities in their rhizosphere, which offer numerous fitness benefits to the plant host, influencing their composition and enhancing beneficial traits (Trivedi *et al.* 2020). Since we used soybeans in all treatments, this may explain the similar trend of bacterial density in both secondary forest and oil palm plantation soils. It is also important to note that this experiment was conducted over a period of only three months, which may be insufficient to observe clear trends. Further long-term experiments are needed to better understand the impact of fertilization on secondary forest and plantation soils.

Notable differences in total bacteria grown in soil extract and nitrogen-free malate medium were primarily observed three months post-treatment. In both secondary forest and oil palm plantation soils, both F0% and F50% treatments resulted in the highest total bacteria counts in both mediums compared to F100%. This underscores the impact of nitrogen fertilization levels on microbial populations. The use of chemical fertilizers, particularly nitrogen addition, has been found to decrease bacterial alpha-diversity, which can affect the number of culturable bacteria in the soil (Francioli *et al.* 2016). Excessive nitrogen fertilization has been shown to decrease microbial diversity in both bulk and rhizosphere soils (Wang *et al.* 2018). Similarly, Gu *et al.* (2021) found reduced ACE, Chao1, and observed species under high nitrogen fertilization (N563) compared to N375 and N0 treatments. They suggested that this reduction may be due to the selection pressure exerted by high concentrations of ammonium nitrate and alterations in soil pH on microbial communities. These findings highlight the importance of tailored fertilizer management practices based on specific soil conditions to preserve microbial diversity and ecosystem health.

The assessment of *nifH* gene copy numbers via qPCR in secondary forest and oil palm plantation soils showed no significant differences between fertilizer treatments. A weak correlation was observed between total bacteria and *nifH* gene abundances, suggesting differences in the sensitivity of the methods used. Both methods have limitations and are influenced by various factors, such as soil type, microbial community composition, and environmental conditions. The semi-solid N-free malate medium (NFM), which promotes microaerophilic conditions and lacks nitrogen sources, favored diazotroph growth (Döbereiner *et al.* 1976). However, repeated subculturing often isolates only a limited number of strains, failing to represent the original community's diversity (Mirza and Rodrigues 2012). Similarly, relying solely on *nifH* DNA analysis may underestimate the activity and diversity of nitrogen-fixing microbes due to the dormancy among environmental microbes with the *nifH* gene (Yang *et al.* 2009). Additionally, diazotrophic community composition can vary according to soil type, soil management, plant presence/absence, plant species, and plant varieties (Bouffaud *et al.* 2016). Further research is needed to explore optimal methods for understanding the population dynamics of diazotrophic bacteria.

4.3. PCA Reveals the Impact of Land Type on *nifH* Gene Abundance and Plant Agronomic Performance

The PCA graph visually represents the differentiation of different land types and fertilizer treatments. Clear trends emerged, with secondary forest soils typically associated with *nifH* gene copy numbers, while oil palm soils were linked with higher agronomic traits. This correlation is supported by agronomic data showing that soybeans grown in oil palm plantation soils performed better than those in secondary forest soils. Interestingly, this finding suggests a possible association between *nifH* gene abundance and agronomic parameters such as yield, which, to our knowledge, has not been previously documented. One explanation for these results is that oil palm soils already have readily available nitrogen content, such as nitrate, from fertilization practices, which supports better plant growth and results in higher yields. Caton *et al.* (2018) previously demonstrated that higher nitrate content in soils is negatively correlated with nitrogen-fixing activity. This could explain why oil palm plantation soils, despite having lower *nifH* gene copy numbers, support higher yields due to the abundant available nitrogen, reducing the need for biological nitrogen fixation.

Our study highlights the significant impact of different land-use practices and fertilization treatments on soil microbial communities and agronomic performance. The comparison between secondary forest soils and oil palm plantation soils revealed distinct differences in initial soil properties and bacterial densities. Importantly, this study revealed that excessive nitrogen fertilization can decrease microbial diversity, as supported by previous research. This underscores the necessity of tailored fertilizer management practices to preserve microbial diversity and promote sustainable agricultural systems. The positive correlation observed between agronomic traits and oil palm plantation soils, contrasted with the higher *nifH* gene copy numbers in secondary forest soils, underscores the complex interactions between soil management practices and microbial populations. The weak correlation between total bacterial counts and *nifH* gene abundances, along with the short three-month duration of this study, underscores the need for further research and longer-term experiments to fully comprehend fertilization impacts. Overall, our findings contribute to the growing body of literature on the ecological impacts of land-use practices and fertilization, providing valuable insights for developing sustainable agricultural strategies that balance productivity with environmental health.

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