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Studies on the Short- and Long-Term Effects of Rubber-Canna Agroforestry Through Soil Analysis and a Metagenomic Approach

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Abstract. Agroforestry combines trees and crops for sustainable benefits. We explore rubber and canna integration into agroforestry, emphasizing sustainability, biodiversity, and carbon sequestration. This study assesses *C. indica*'s viability beneath 7-8-year-old rubber plantations, examining its impact on soil,microbial communities, and latex production. The research site in Subang, Indonesia, features, at the beginning, six-year-old rubber trees with variations in sunlight under canopies. Wild *C. indica* rhizomes from Mid Java are planted beneath rubber trees and open ground. No significant difference was found in plant height, rhizome weight, leaf area, number of leaves, r/s ratio, SLA, LWR, and LAR between *C. indica* cultivated beneath rubber trees (RC agroforestry) and on open ground. Although not significant, RC soil had higher N, P, K, and organic C levels than rubber monoculture (RM) soil two years after adopting the rubber-canna agroforestry system. After six years, RC soil had a greater pH, C, N, P, and K, clay and silt content,and Shannon E index than RM soil. Analysis of soil metagenomics showed the phylum Proteobacteria dominates and enhances soil fertility, particularly in RC soils. These results increase latex output at the RC site over the RM location. In conclusion, the Rubber-Canna agroforestry system enhances sustainability, soil fertility, and crop yield, addressing food security and environmental concerns. The primary novelty of this six-year study lies in the integration of *C. indica* into Southeast Asia's rubber agroforestry systems, highlighting its unique characteristics such as low-light survival, which can contribute to food security and soil protection.

Keywords: Agroforestry; Canna indica; Rubber; metagenomic; soil properties

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INTRODUCTION

Agroforestry systems have emerged as sustainable land-use practices that combine the cultivation of trees with agricultural crops, creating sustainable agricultural practices that provide many ecological, economic, and social benefits (Dollinger & Jose, 2018; Wilson & Lovell, 2016). One particular form of agroforestry is incorporating rubber trees (*Hevea brasiliensis*) with various crops and tree species to create a symbiotic and productive environment.

Rubber, derived from the H. *brasiliensis* tree, is a crucial cash crop with substantial global economic importance. Globally, rubber supports the live of more than 40 million people with 90% of production being smallholders (Pinizzotto et al., 2021). Even though the conventional rubber monoculture system is plagued by issues such as soil degradation, loss of biodiversity, and susceptibility to diseases and pests (Drescher et al., 2016; Jiang et al., 2017), several studies have demonstrated that rubber plantations can help mitigate the impact of climate change. Brahma et al. (2018), for instance, highlight the significance of increasing carbon stocks as a sustainable practice. Utilizing rubber woods and natural rubber reduces the demand for synthetic elastomers and, consequently, fossil fuel consumption (Pinizzotto et al., 2021). In addition, it was reported that rubber plantation have lower transpiration rates than oil palm plantations, thereby reducing the risk of water shortage (Merten et al., 2016).

The integration of rubber plantations into agroforestry systems offers various advantages, including ecological, economic, and social benefits. It can increase land productivity by utilizing available space more efficiently and allowing the growth of diverse plant species alongside rubber trees (Lin et al., 2018). Intercropping rubber trees with various crops helps improve soil fertility, reduces soil erosion, and promotes nutrient cycling, resulting in better conservation of soil resources (Chen et al., 2017). Rubber agroforestry systems also provide habitat for various plant and animal species, promoting biodiversity and contributing to ecosystem stability. In addition, these systems aid in carbon sequestration and help mitigate climate change (Schroth et al., 2015). Liu et al. (2018) reported that introducing a leguminous shrub to a rubber plantation improved soil carbon and nitrogen fractions and ameliorated soil environments. This suggests that adopting rubber agroforestry practices can provide benefits without compromising yields and help mitigate the environmental problems associated with rubber monoculture.

In Southeast Asia, several plant species have been used in rubber agroforestry, for example, cacao (*Theobroma cacao*), coffee (*Coffea* spp.), tea (*Camellia sinensis*), durian (*Durio zibethinus*),and banana (*Musa* sp.) (Liu et al., 2016). Traditional medicinal plants such as *Flemingo macrophylla, Alpinia oxyphylla, Amomum longiligulare,* and *Morinda officinalis* have also been reported planted with rubber in an agroforestry setting (Liu et al., 2021). Besides rubber, other commercial plantations have also applied agroforestry systems. Indonesia provides an excellent example of how smallholders' adoption of agroforestry systems has promoted sustainable agriculture (Roshetko et al., 2013) in agroforestry with teakwood (*Tectona grandis*).

Unfortunately, the majority of plant species used as intercropping are not carbohydrate-producing staple dietary alternatives. On the other hand, it is essential to cultivate carbohydrate-producing species such as *C. indica* if we were to establish food resilience and help eliminate world hunger through food diversification. *C. indica* has the potential to be developed as an alternative to rice as a source of flour, in addition to its other uses (medicinal, animal feed, etc.). Its ability to survive in a low-light environment could help minimize forest slash and burning foragriculture, an activity that still occurs (Murdjoko et al., 2022). In addition to its role as a carbohydrate producer, *C. indica* is ideal to be used as an intercropping plant because of its unique characteristics and ecological functions. Its large leaves can serve as a natural weed suppressant, reducing the need for herbicides, while at the same time protecting the soil underneath it from direct rainfall, thereby minimizing splash erosion.

There are few studies that investigate the light development requirements of *C. indica*; however, Sasaerila et al. (2019; 2021) reported that *C. indica* can tolerate low to high light levels. To date, however, little is known about the ability of *C. indica* to survive beneath matured rubber stands ina rubber plantation and both its short- and long-term impact on latex production. This study examined the viability of *C. indica* planted underneath 7-8-year-old rubber trees, its effect on the chemistry, physics, and biology of the soil, and latex yield. We applied a metagenomic approach to study soil biology. Soil microbes are a crucial contributor to essential ecological biogeochemical processes, supporting plant nourishment and soil well-being (Arias et al., 2005; Lelario et al., 2018; Maeder et al., 2002; Sofo et al., 2018). The presence of abundant soil microorganisms leads to higher disease control because it creates stable conditions in the soil (Zhang et al., 2017). In addition, plants can impact the variety of soil microorganisms through the release of root exudates, which induce physical and chemical alterations in the rhizosphere, consequently influencing the diversity of soil microorganisms (Zhou et al., 2022). This study compared the diversity of microbial communities from soil under rubber monoculture (RM) and rubber-canna (RC) agroforest after six years of its implementation. Analysis was done by direct extraction of DNA, characterization of culturable and non-culturable microorganisms, and metagenomics analysis.

The purpose of this research is to enhance our understanding of the potential benefits and challenges associated with integrating *C.indica* into rubber agroforestry systems, with a focus on ecological and metagenomic aspects. The implications extend to addressing key challenges in agriculture, such as food security and environmental sustainability.

METHODS

Experimental Site and Plant preparation

This research was conducted in the rubber plantation of PTPN 8, blocks 9 and 10, Soklat-Pasirkareumbi, Subang, West Java, Indonesia (6º

34’ 3” S, 107^{o} 47’ 0” E). The age of the rubber trees that were used at the beginning of this experiment were six years old. Both block 9 and block 10 had not been fertilized for the past seven years before this experiment and continued as such until this experiment was terminated in 2023. Ratios of light intensity and the temperature between open ground and beneath rubber trees were measured four times during this experiment: January and June 2018, July and November 2019. Rainfall, temperature, and humidity data were taken from Indonesia’s Agency for Meteorological, Climatology and Geophysics (BMKG) Subang. Light measurements were done using a Lux Meter (LX-1010B), and the temperature was measured using a digital hygrometer (Sanfix TH-308).

***C. indica* plant materials**

Rhizomes of wild *C. indica* of white cultivar were used in this experiment, obtained from Gombong, Kebumen, Mid Java, Indonesia. Samples of this rhizome were sent to the National Research and Innovation Agency (BRIN) for identification and confirmed that they were *C. indica*. Before planting, the rhizomes were washed with tap water and cut into small pieces (± 5 cm, ± 50 g), each with a growing nodal point. The rhizomes were directly planted in the experimental plots that had been previously prepared to a depth of approximately 10 cm below the soil surface.

Twenty days prior to planting, the experimental plots beneath the rubber trees were randomly selected, cleared of grasses, and tilled. Fourteen days before planting rhizomes, manure was added and thoroughly mixed with the soil. During the duration of this experiment, no additional fertilizer or pesticide was used.

Experiment 1: Effects of implementing rubber-canna agroforestry on latex yield

This study was conducted between July 2017 and August 2023, applying a completely randomized block design with two treatments and three replicates. The treatments consisted of: 1. Rubber Monoculture (RM) plots containing only rubber trees, and 2. Rubber-Canna (RC) agroforestry plots in which C. *indica* was planted as an intercropping plant between rubber trees (Figure 1A, 1B). The average distance between rubber trees is 1.5 meters. *C. indica* plants were planted 1 m apart from other *C. indica* plants and 1 m from rubber trees. Each treatment was administered in a 25 x 25 m plot, with three plots per treatment in Block 9 for the RM and Block 10 for the RC agroforestry treatment. To maintain the independence of each treatment, the distance between the RM and RC sites was maintained to be more than 100 m away. There were a total of 321 rubber trees in each treatment. Employees of PTPN 8 tapped (harvested) the latex regularly in accordance with the company's harvest schedule. Latex production data for all treatment plots were collected from the company.

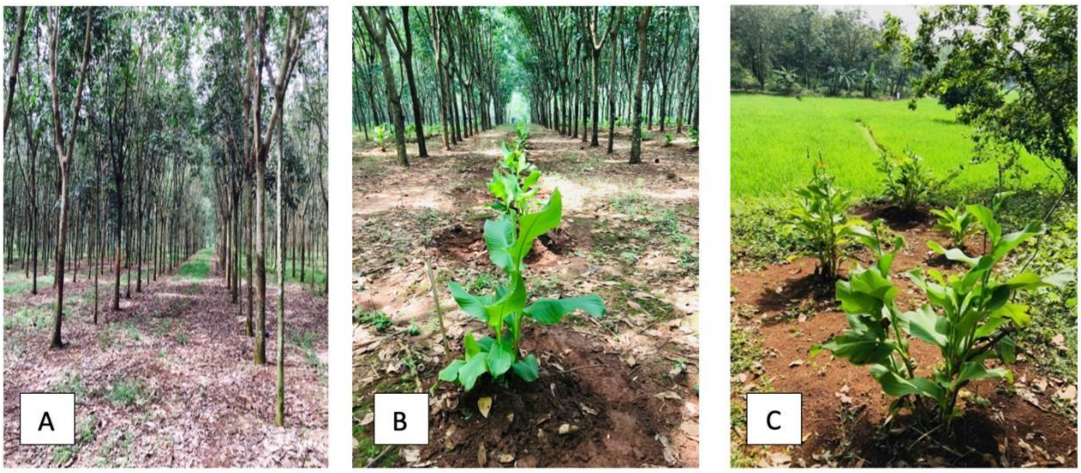

Figure 1. Field experimental layout A. Rubber monoculture (RM); B. Rubber-Canna agroforestry(RC); C. Canna monoculture (CM).

Experiment 2: Effect of rubber leaf shade on the growth of intercropped *C. indica*

An experiment with two treatments was conducted to determine whether *C. indica* can grow under a light-reduced environment caused by the rubber leaves. Two 5 x 10 m sites were chosen at random, one for the Canna monoculture site and the other for the Rubber-Canna (RC) agroforest site. The RC plot consisted of *C. indica* planted as an intercropping plant between rows of rubber trees, whereas the CM plot consisted only of *C. indica* planted on open ground, with no light restriction by rubber leaves (Figure 1B, 1C). In preparation for planting, grasses were eliminated, and the soil was tilled. Each treatment plot was planted with 20 rhizomes of *C. indica*. To maintain the independence of each treatment, the distance between the RC and CM sites was more than 100 meters. This experiment was carried out twice, once in 2018 and repeated in 2019, at the same sites using identical planting protocols as used for the previous experiment done in 2018.

Experiment 2 was running from February to September 2018. After sampling and measurement were done, experimental plots were left alone for four months, after which Experiment 2 was repeated, also from February to September 2019, using the previous hole to plant *C. indica*.

Eight months after planting, five randomly selected *C. indica* were harvested from each treatment plot for measurements. Measured parameters include plant height, rhizome dry weight, leaf area, number of leaves, specific leave area (SLA), leaf weight ratio (LWR), and leaf area ratio (LAR). Subsequently, samples were transported to the laboratory for oven drying. The plant components were oven-dried at a temperature of 80^{o}C for 48 h or until a constant weight was achieved, indicating no further changes in weights.

Soil physics and chemical analysis

To examine its physical, chemical, and biological properties, soil samples were collected in three replicates at a depth of 10 cm after removing the litter horizon at each site. Each soil sample, which contained a mixture of five subsamples and weighed around 2 kg per replicate, was taken from each experimental plot in a diagonal pattern, four samples from each corner and one sample from the center. Soil from each sample point was mixed thoroughly, placed in a plastic bag, and brought back to the university lab for further analysis. In the laboratory, the root, stones, and litter debris were carefully removed from soils, and each soil samples were kept at a constant temperature of 25±2^{o}C. Each sample was divided into two parts: one part was used to determine the physical properties of soil, while the other was used to determine chemical properties, enzyme activity, and metagenomics analysis.

Soil chemical analysis was conducted at the commercial laboratory of Unilab, Kebayoran Lama, Jakarta Selatan, Indonesia, by using established methods: total N (Kjeldahl method), available P (Bray II and molybdenum-blue method), exchangeable K (1 N NH4OAc, pH 7 and flame photometry method), soil pH (pH meter). To investigate soil microbial activities, two methods were applied: 1. Assessment of hydrolysis of fluorescein diacetate [3′, 6′- diacetyl fluorescein (FDA)] as described in Patle et al. (2018), was done in Universitas Al Azhar Indonesia laboratory, and 2) Metagenomic analysis was done using two steps, which are DNA extraction and nanoporesequencing and followed with bioinformatics analysis.

For Bulk density measurement, five soil cores were randomly collected using a can cylinder (7.5 cm in diameter and 7.5 cm in height, and transported to the laboratory for measurement. The bulk density (BD) and soil moisture content (SMC) were calculated using the following formulas:

$$BD(gcm^{-3}) = \frac{WSCD\ (g) - WECC(g)}{200\ (volume\ of\ cylinder)(cm^3)}$$

$$SWC\ (\%) = \frac{WSAT\ (g) - WSCD(g)}{WSCD\ (g) - WESC\ (g)} x100\%$$

Note: WSCD is the weight of the cylinder with fresh soil;WESC is the weight of an empty can cylinder; WSAT weight of the saturated sample

DNA extraction

Six soil samples consisted of 3 samples from RC agroforest sites and 3 samples from RM sites were used for DNA extraction. Each soil sample contains 3 composites taken from 3 different soil sampling points. Each soil composites then homogenized, and 5 grams were collected for DNA extraction. Total soil DNA was extracted using Quick-DNA Fecal/Soil Microbe Miniprep Kit (Zymo Research) according to the protocols of the manufacturer. First, DNA was extracted after bacterial cell lysis via mechanical homogenization and purified using column-based methods. Removal of inhibitors and continued with collecting the DNA with buffer elution. DNA concentration was determined using both NanoDrop spectrophotometers and Qubit fluorometers. The quality of DNA was visualized using 1% agarose electrophoresis.

Nanopore sequencing and bioinformatics analysis

Library preparations were prepared using the 16S barcoding kit from Oxford Nanopore Technology according to the manual of the manufacturer. A total of 6 libraries were mixed in equal concentration and used further used for sequencing using GridION (Oxford Nanopore Technologies). GridION sequencing was operated by MinKNOW software version 20.06.9. Base calling was performed using Guppy version 4.0.11 with high accuracy mode (Wick et al., 2019). The quality of FASTQ files was visualized using NanoPlot (De Coster et al., 2018). Only reads with a length above 1000 base and has quality score >8.0 were used for further analysis. Filtered reads were classified using a Centrifuge classifier (Kim et al., 2016). The bacteria and Archaea index was downloaded from the centrifuge website (https://ccb.jhu.edu/software/centrifuge).
Downstream analysis and visualizations were performed using Pavian (https://github.com/ fbreitwieser/pavian) and Krona Tools (https:// github.com/marbl/Krona). Statistical analysis was performed using Microsoft Excel.

Statistical Analysis

To assess the impact of treatments, analysis of variance (ANOVA) was performed using the R-studio 2022.12.0+353 statistical package. One-factor ANOVA was deployed to compare treatment effects. The least significant difference (LSD at 0.05 level of probability) test was applied to assess the differences between means.

RESULTS AND DISCUSSION

Experimental Conditions

Under the 6 to 7-year-old rubber trees, the quantity of sunlight received varied between 10 and 50 percent less than on open ground. During the dry months, when there was less than 100 mm of precipitation and 0 rainy days, rubber trees lost their leaves, producing a similar light condition to that of the open ground (Figure 2). The data for temperatures exhibited a similar pattern. The ambient temperatures beneath the rubber trees were approximately 2 to 6 ºC cooler than those on the open ground. The least difference in temperature was also observed during dry months when the majority of rubber trees had shed their leaves. On days with clear, bright sunshine, the warmest temperature and most intense solar radiation occurred between 12:30 and 13:30. Rubber plants have been observed to discard leaves in response to dry conditions (e.g., Giambelluca et al.,2016; Kobayashi et al., 2014).

Figure 2. Rubber trees lose their foliage, stimulating the light and temperature conditions of an openfield.

Table 1. Light intensity and Temperature conditions during Rubber-Canna Agroforestry experiment

Jan-18	Light Intensity ± SD (Lux)			Temperature (ºC)		
Time	Open ground	UnderRubber	% sunlight reduction	Open ground	UnderRubber	Temp.diff.
10.00-11:15	84795±155	43757±106	52	28±1	24±0	-4
12:00-12:15	98751±465	59867±74	61	30±1	24±0	-6
14:00-14:15	60643±100	35677±248	59	30±1	24±1	-6
16:00-16:15	21194±391	10578±158	50	28±1	24±0	-4
Jun-18						
10.00-11:15	84363±524	54836±340	65	27±0	27±0	0
12:00-12:15	99015±408	81610±141	82	30±0	29±0	-1
14:00-14:15	60566±872	48673±244	80	30±0	29±0	-1
16:00-16:15	20654±1025	12290±306	60	30±0	28±0	-2
Jul-19						
10.00-11:15	86579±310	55506±319	64	29±0	29±0	0
12:00-12:15	99618±134	90673±120	91	31±0	30±0	-1
14:00-14:15	60425±124	54673±244	90	31±0	30±0	-1
16:00-16:15	21869±	17233±211	79	29±0	29±0	0
Nov-19						
10.00-11:15	84851±156	54892±82	65	27±0	24±0	-3
12:00-12:15	99499±218	63569±386	64	31±0	25±0	-6
14:00-14:15	61771±209	36562±420	59	31±0	25±0	-6
16:00-16:15	20740±157	11856±	57	26±0	24±0	-2

The effect of rubber shade on C. *indica* growth

No statistically significant differences were observed in plant height (F = 2.34, p = 0.15), rhizomedry weight (F = 2.23, p = 0.15), number of leaves (F = 1.16, p = 0.37), leaf area (F = 1.58, p = 0.24), specific leaf area (F = 1.20, p = 0.27), leaf weight ratio (F = 0.40, p = 0.84), and root/shoot ratio (F = 0.32, p = 0.58) between C. *indica* plants grown under rubber trees (Rubber-Canna, RC) agroforestry system in both years of the experiments, 2018 and 2019 (Table 2). The growth patterns seen in this experiment were similar to those seen in the previous study, which showed that *C. indica* can adapt to different light situations. For example, a greenhouse study showed that *C. indica* can grow best when exposed to 25% natural light (Sasaerila et al., 2019; 2021).

Table 2. Mean values for growth parameters of C. *indica* grown beneath six- to seven-year-old rubber trees (rubber-canna agroforestry, RC) versus canna monoculture (CM). Experiments were conducted twice, the first in 2018 and the second in 2019

Year Growth parameters	2018		2019	
	Canna Monoculture	Rubber-Canna Agroforestry	Canna Monoculture	Rubber-Canna Agroforestry
Plant height (cm)	60.5±9.5	104.5±28.2	113.6±7.3	107.3±12.2
Rhizome dry weight (g)	86.2±20.65	74.0±29.2	86.6±12.9	50.92±4.6
Root dry weight (g)	6.5±1.55	4.7±1.23	3.37±0.5	5.95±1.1
Leaf area (cm^2)	318.8±41.1	344.2±48.7	1914.8±627.6	1576.2±518.7
Number of leaves	26.6±3.9	19.6±2.1	48.2±8.6	45.2±7.3
Root to Shoot ratio	1.018±0.094	1.589±0.65	0.73±0.06	0.540±0.09
SLA	11.66±1.64	19.32±5.85	54.41±24.2	32.99±11.1
LAR	2.25±0.60	2.56±0.32	12.67±4.7	8.13±3.1
LWR	0.18±0.04	0.16±0.04	0.27±0.002	0.26±0.001

Mean ± SE with different letters within rows is significantly different, LSD (p<0.5). The absence of a letter indicates the lack of a statistically significant difference between the two treatments.

The ratio of plant leaf area to leaf dry weight (SLA), the amount of leaf area produced per unit of biomass (LAR), and the number of photosynthetic organs of the plant (LWR), can be used to measure plant adaptation to light. In low-light environments, the SLA, LAR, and LWR values tend to increase (Neufeldt et al., 2000; Sasaerila et al., 2021; Wyka et al., 2012). In this study, these three values did not differ significantly between C. *indica* grown in RC agroforestry systems and those grown in CM systems (open areas), indicating that C. *indica* could grow well in the shade of mature rubber trees. The slight difference between the results of this experiment and those of the greenhouse experiments may be attributable to the shading effect of rubber trees at the age of 6-7 years. Data show that underneath the rubber trees of this age in this experiment only reduced light by only 30–50% relative to natural light. On the other hand, in greenhouse experiments, the light growing condition was consistently maintained at 75% lower than natural light.

Short-term (two-year) effects of *C. indica* planting on soil characteristics

Soil moisture content (SMC), soil pH and bulk density (BD). The SMC significantly differed between treatments ($F=8.91$; $p< 0.004$, Table 3). Both RM and RC soils have an SMC that is 1.4 times greater than CM soil, the *C. indica*-planted open-groundareas. This result is consistent with the fact that the CM site was formerly a grassland with no other vegetation. This area has a higher sand content than RM and RC soils, most likely due to splash erosion that leads to the erosion of clay and sediment (Table 1). Both RM and RC soils contain a higher proportion of clay and sediment. The higher SMC in RM and RC soils can be attributed to the larger proportion of clay and silt and lower sand (Zhu et al., 2023).

There was no significant difference between CM, RM, and RC soils in terms of pH and bulk density ($F = 0.23$; $p = 0.80$). However, the highest BD was observed in the RC agroforestry soil, which may indicate the effect of leaf shading that produced multilayered canopies that shield the soil beneath them from direct raindrop impact (Liu et al., 2016; Zhu et al., 2023).

Table 3. Properties of soils from rubber monoculture (RM), canna monoculture (CM), and rubber-canna (RC) after two years of agroforestry implementation at the rubber plantation of PTPN VIII, blocks 9 and 10, Soklat-Pasirkareumbi, Subang, West Java, Indonesia. BD: bulk density; SWC: soil water content

Soil parameters	Light Environment		
	Canna Monoculture	Rubber Monoculture	Rubber-Canna Agroforestry
pH	5.03	4.76	4.40
C organic (%)	1.63	1.53	1.82
N total (%)	0.15	0.38	0.45
P2O5 (mg/100g)	na	82.13	77.71
K2O (mg/100g)	211.67	33.74	150.13
Soil Texture (%):			
Sand	19.67	14.86	14.38
Loam	28	36	34.67
Clay	53	49.15	50.96
Loam + Clay	81	85.15	85.63
BD ($g.cm^{-3}$)	1.74±0.09	1.75±0.04	1.80±0.06
SWC (%)	19.17±0.67^{b}	26±2.34^{a}	25±2.36^{a}
Enzyme activity (mg FDA g^{-1}soil dw. h^{-1})	3.37±0.25^{a}	2.22±0.30^{b}	3.67±0.19^{a}

Mean ± SE with different letters within rows are significantly different, LSD ($p<0.5$). Soil chemistry data for 2018 were done with n=1.

Organic C, N, P, and K content

After two years of implementation of the RC agroforestry system, the data showed that the RC soil had 1.1, 5, 1.1, and 3.4 times greater organic C, N, P, and K content, respectively, than the soil in the RM system (Table 3). This result is likely due to the higher clay content and lower sand content of the soil in the RC agroforestry system compared to the RM system. It seems that the combination of rubber and *C. indica* in an agroforestry system has beneficial effects on the physicochemical properties of the soil. Compared to RM and CM, RC agroforestry soil had higher levels of C, N, P, BD, and SWC at each sampling time. These findings are likely attributable to the increased root exudates and metabolic rates of *C. indica* (Wang et al., 2021).

Although they were not statistically significant, the pH and N content of the CM soil was higher than that of the RM soil (Table 3). This result demonstrates the potential of C. *indica* as a soil remedial plant with the ability to enhance soil nutrients, most likely as a result of a greater numberof soil microorganisms in areas where C. *indica* was cultivated. This result is in accordance with previous reports on C. *indica* as a soil-remediating plant (Gunarathna et al., 2016). It seems that *C. indica* is capable of enhancing the soil's capacity to retain water and enhancing soil nutrition byincreasing organic C, N, P, and K. In turn, this causes the soil to discharge organic and inorganic nutrients that support the development and diversity of soil microorganisms, resulting in an increase in the soil's concentration of these elements. Improved soil properties have been reportedin rubber cacao and rubber-*F. macropylla* agroforestry (Chen et al., 2019). Our results on the enzyme hydrolysis activity of soil microorganisms and soil metagenomics support this explanation.

Effect of C. *indica* on soil enzyme activity (Soil microorganism hydrolysis activity)

The hydrolysis activity of microorganisms was significantly higher ($p<0.05$) in CM and RC soil than in RM soil (Table 3). This may be due to the increased availability of organic matter to primary soil decomposers, which has been observed to increase FDA activity in RC agroforestry and CM sites. C. *indica* will lose its old leaves and replace them with new ones. The decomposition of these senescence leaves may have contributed to high levels of organic C and N that were utilized by the primary soil decomposer, resulting in high levels of FDA activity (Rodrigues et al.,2015; Miguel et al., 2020). This result is supported by our metagenomic analysis.

Long-term effects of Rubber-Canna (RC) agroforestry on soil properties and latex yield

This study shows that planting *C. indica* as an intercropping plant between rubber trees has a positive effect on latex yield over time. Prior to the implementation of the RC agroforestry systemin 2017, the yield of latex in RC sites was only 81% of that of RM sites (Figure 3). In 2022, or six years after the implementation of the RC agroforestry system, the latex production was 1.03 times that of RM sites, which was a significant improvement over the initial year (Figure 3).

This outcome is consistent with soil data from 2023 showing enhanced soil physics, chemistry, and biology due to the presence of *C. indica* (Figure 4), which demonstrates that all parameters are higher in RC agroforestry soil after six years of deployment. In Thailand, where latex yields from 34 monocultural and 47 agroforest rubbers were evaluated, a similar outcome was noted (Warren-Thomas et al., 2020). It should be noted that the rubber plantation blocks used in this study have not received fertilizer in 7 years, making them perfect study land in the sense that the findings are truly attributable to the effects of the given treatment. During this experiment, it was observed that national rubber production, including that of PTPN 8 (the location of this research), decreased. The Indonesian Ministry of Agriculture stated that a prolonged drought season afflicted Indonesia during 2018–2019, resulting in a decrease in rubber production in various Indonesian provinces (Directorate General of Estate Crops, 2019).

Effect of long-term Rubber-Canna agroforestry system on soil biology

Soil as a medium for plants to grow is a complex and dynamic environment, where biologically most of the activity in the soil is influenced by the abundance and diversity of soil microbiomes (Zhou et al., 2011). Soil microbe is an important component of soil microbiomes. Based on Nanopore sequencing, a total of 587981 reads have been obtained from 6 samples, with average read number 110371.3 for RC and 85603 for RM (Table 4). Only reads with a length >1000 base are used for further analysis. On average, 1923 ± 67 species were identified from 6 samples. The RC samples have higher species numbers on average 2008 ± 99 species in comparison to RM samples (1837.33 ± 40 species).

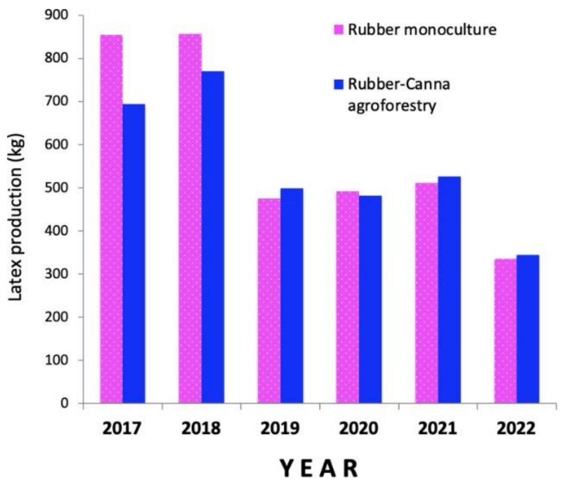

Figure 3. Comparison of annual latex production between Rubber Monoculture (RM) and Rubber-Canna (RC) agroforestry experimental plots at the rubber plantation of PTPN VIII, blocks 9 and 10, Soklat- Pasirkareumbi, Subang, West Java, Indonesia. The size of each plot is 25 x 25 m, with three plots per treatment.

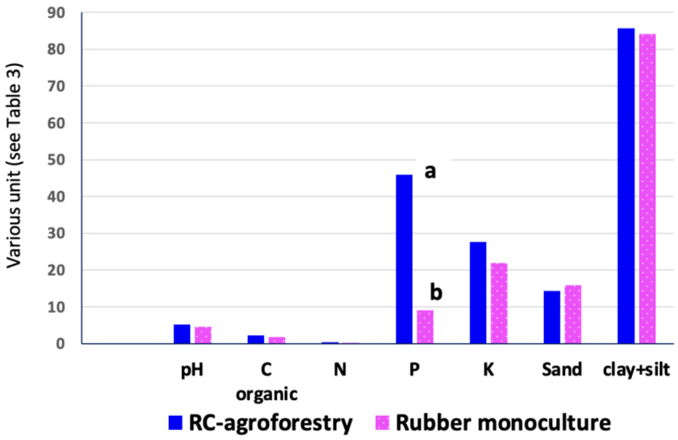

Figure 4. Effect of six years application of Rubber-Canna (RC) agroforest soil chemistry. Bars with different letters are significantly different, LSD ($p<0.5$).

To assess the bacterial diversity within soil samples, we conducted an alpha diversity analysis utilizing the Shannon and Chao1 indices. The results revealed that the microbial diversity in the RC sample was greater than that in the RM, as indicated by the Shannon index values of 5.00 ± 0.16 for RC and 4.78 ± 0.21 for RM. This suggests a more varied species presence in the RC sample. Conversely, the Chao1 index pointed to a higher species richness in the RM sample, with values of 1567 ± 145.9 for RC and 1778.7 ± 224.2 for RM, implying a lower abundance in RC. We hypothesize that this reduced abundance in RC may be attributed to the dominance of certain microbial groups within the sample. This dominance could also explain why the microbial community in RM appears more evenly distributed, contributing to its higher Chao1 index value (Wu et al., 2018).

Krona analysis at the phylum level revealed distinct patterns in the relative abundance of microbial groups between the two samples. Specifically, the phylum Proteobacteria was the most prevalent in the RC samples, constituting an average of 49% of the microbial population, as opposed to a significantly lower presence of 27% in the RM samples (Figure 5). Although Proteobacteria is a prominent component of the bacterial community in the RC samples, the RM samples displayed a more balanced distribution, with Proteobacteria and Acidobacteria appearing in comparable proportions. However, it is important to note that both phyla are less abundant in the RM samples when compared to the RC samples (Figure 6).

Table 4. The quality of FASTQ files was assessed by NanoPlot

	Soil samples					
Sample ID	RC1	RC2	RC3	CM1	CM2	CM3
Reads number	88171	151329	91614	85013	104898	66916
Mean read length	1543.3	1477.7	1542.3	1541.8	1533	1540.9
Mean read quality	12.5	12.3	12.5	12.4	12.4	12.4
Read length N50	1567	1556	1567	1567	153	1568
Number of species	1920	2206	1899	1881	1876	1756

Numerous bacterial species within the Proteobacteria phylum are known to play a significant role in soil functionality, including the maintenance of soil health and the cycling of nutrients, and they are commonly found in agricultural soils, as documented by Borneman et al. (1996), Smit et al. (2001), and Valinsky et al. (2002). An increase in the relative abundance of Proteobacteria members, as demonstrated by Solanki et al. (2020), has been linked to enhanced microbial diversity in the rhizosphere, a phenomenon often associated with elevated levels of soil organic carbon and total nitrogen content. These findings are consistent with our soil chemistry results (Figure 4) which show that the RC soil contains higher amounts of nitrogen and organic carbon compared to RM soil.

To elucidate the relationship between the abundance, dominance, and functional implications of the Proteobacteria phylum in the RC and RM ecosystems, we conducted a detailed analysis focusing on the soil microbial species with the highest abundance. Within the top ten species identified in both ecosystems, seven were predominantly found in the RC system (Figure 7). Notably, seven of these species belong to the Proteobacteria phylum, specifically *Labilithrix luteola*, *Pseudolabrys* sp., *Blastochloris viridis*, *Azospirillum* sp., *Phenylobacterium* sp., *P. zucineum*, and *B. zhanjiangense*. The remaining three species include *K. rubrisoli* from the Chloroflexi phylum, *Edaphobacter* sp. from Acidobacteria, and *C. crudilactis* from Actinobacteria, as depicted in Figure 7. These findings suggest a significant presence of Proteobacteria in the RC system, which may influence its soil functions and ecosystem dynamics.

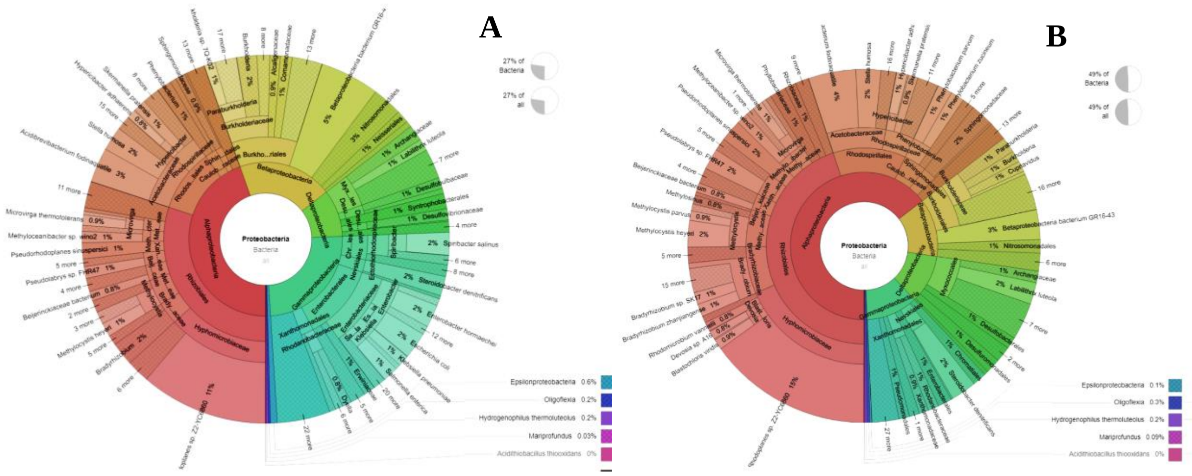

Figure. 5. Representative Krona visualization of phylum abundance. A. Krona of Proteobacteria in Rubber-Canna (RC) agroforestry sample. Phyla Proteobacteria is a dominant phylum in the RC samples. B. Phyla Proteobacteria has an average of 27% in the Rubber Monoculture (RM) samples.

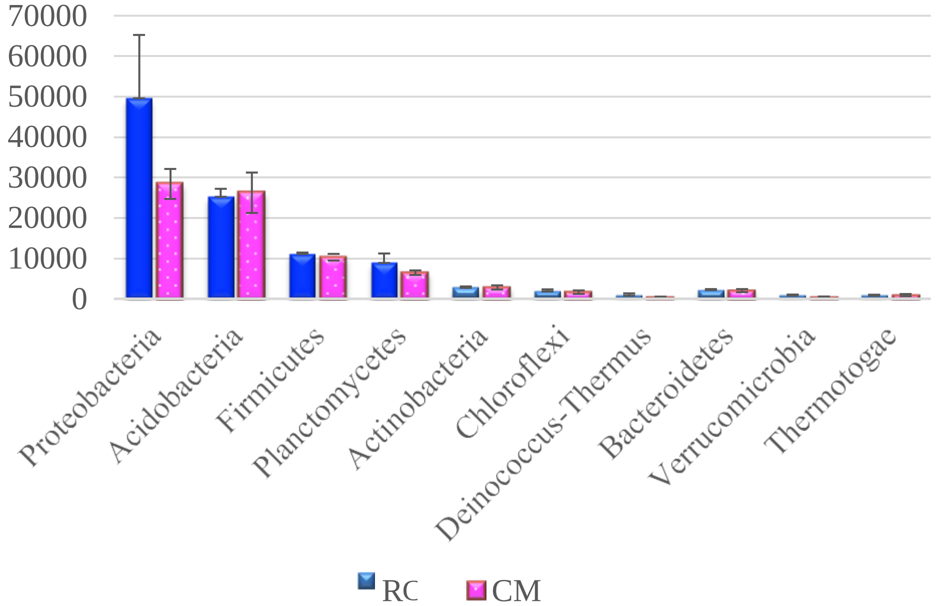

Figure 6. Relative abundance of top 10 phylum in this study. RC: Rubber-Canna agroforestry; CM: Canna Monoculture system.

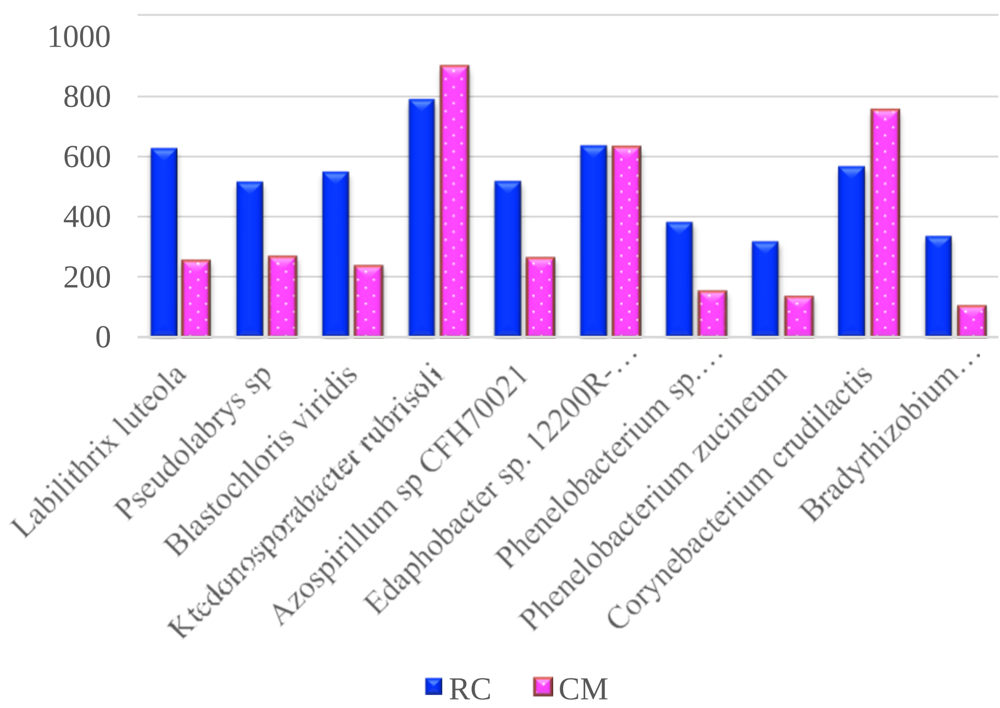

Figure 7. The abundance of the top ten species in the Rubber-Canna (RC) agroforestry and Rubber Monoculture (RM) systems. Seven out of these ten species belong to the phylum Proteobacteria.

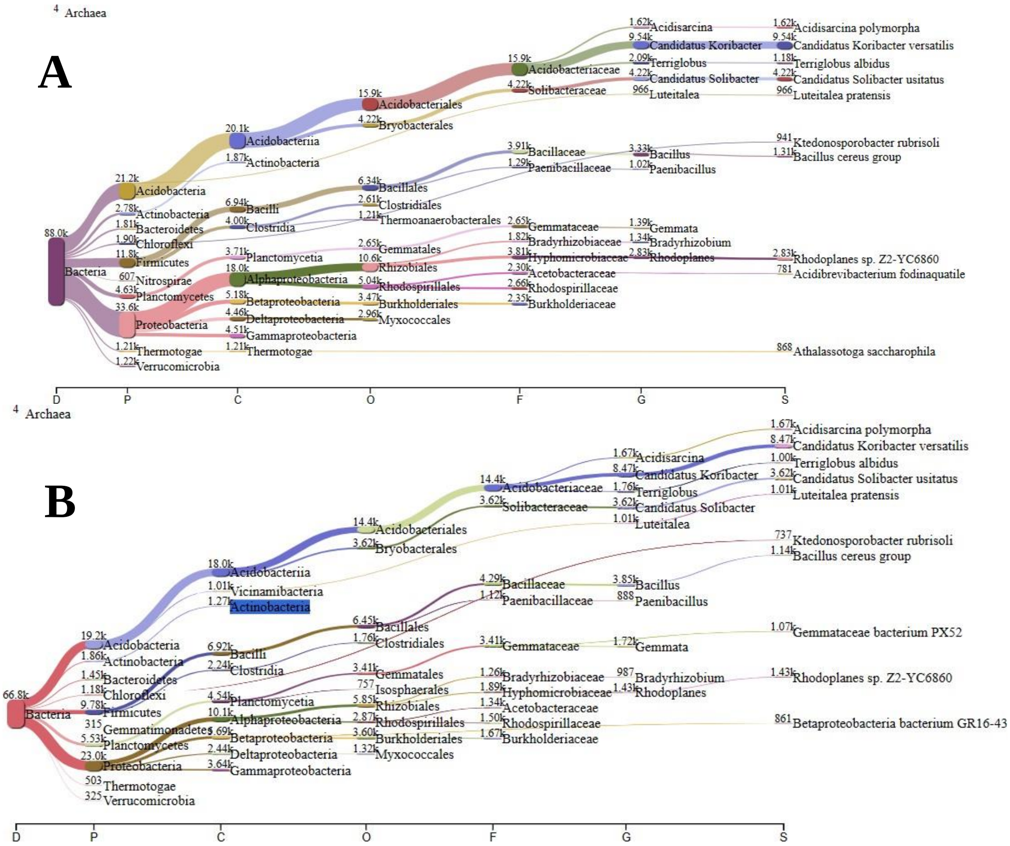

Figure 8. illustrates the relative proportions of all taxa based on the number of amplicon sequence variants (ASVs). **A**. Sankey Diagram for the Rubber-Canna (RC) sample; **B**. Sankey Diagram for Rubber Monoculture (RM) sample. Sankey diagrams are instrumental in visualizing the microbial composition of samples, where the width of the arrows correlates with the quantity of ASVs, thereby visually representing the hierarchical changes over time between different nodes in the ecosystem.

The presence of bacterial communities in soil is complex, influencing fertility in various ways. While some bacteria act as pathogens and can negatively impact soil health, many bacterial species, particularly within the Proteobacteria phylum, are known to enhance soil quality significantly. Akob et al. (2022) and Billah et al. (2019) have documented the functional benefits of certain Proteobacteria species on soil conditions. Dang et al. (2020) further explored this topic by studying the effects of intercropping systems on rhizosphere microbial composition and structure in legume-planted lands. Their research confirmed an increase and dominance of the Proteobacteria phylum in intercropping system soils, which is associated with an elevation in nitrogen content both in the soil and legume plants, in comparison to monoculture fields. Our findings, as presented in Figure 8, parallel these observations, suggesting that the higher abundance of the Proteobacteria phylum contributes to improved soil fertility. This is evidenced by the superior chemical properties of the RC soils over those of the RM soils.

In addition to its presence as an indicator of good nutritional conditions in a field, several functional species of Phyla Proteobacteria are reported to play a role in improving nutritional conditions in the soil (Zhang et al., 2022). From the analysis of the abundance and diversity of the top 10 species, it is known that 7 of them come from the phyla Proteobacteria (Figure 7, 8). Some of these bacteria have a high abundance in intercropping systems and are reported to be related to biogeochemical processes in improving soil quality by creating nutrient-rich soil as an ideal condition for the development and growth of plants and rhizosphere bacterial communities (Akobet al., 2022; Billah et al., 2019; Wu et al., 2018). For example, *Pseudolabrys* sp., which has the second highest abundance (Figure 7), with 517 ± 237 and 267 ± 86 in RC and RM, respectively, is reported to play a role in secreting naphthol-ASBI-phosphohydrolase, an enzyme needed to dissolve P so that it can be used by plants (Shen et al., 2021). *Azospirillum* sp. plays a role as a vibroid cell with a single polar flagellum and is reported to be very important as a plant growth promoter and a provider of N in the soil (Lin et al., 2016). *Bradyrhizobium zhanjiangense* is also known to play a role as a nitrogen fixer and denitrifier involved in the N cycle. In addition, it has the acetylene hydratase (AH) enzyme, which is sensitive to the presence of N, so it can catalyze the reduction of dinitrogen into ammonia (Zhou et al., 2022). The acetylene enzyme is quickly absorbed by the soil to improve soil health and enhance plant growth by providing biologically available N (Akob et al., 2022; Zhang et al., 2022). The higher abundance of *Azospirillum* sp. and *Bradyrhizobium zhanjiangense* in the RC soil systems than in RM soil systems (Figure 7), in this study is suggested to contribute to the high content of nitrogen in the soils. This study demonstrates that an intercropping system combining Rubber and Canna significantly enhances the diversity of the soil bacterial community compared to a Rubber monoculture. This is critical, as the presence of keystone species or bacterial groups, such as Proteobacteria, may be augmented through the effects of intercropping, thereby elevating microbial soil activity. Consequently, this approach may serve as a novel strategy to promote soil fertility and sustainability without adversely affecting plant productivity.

This paper introduces a novel comprehensive approach to analyzing both the immediate and extended effects of Rubber-Canna agroforestry, utilizing soil analysis and a pioneering metagenomic method. The research investigates a relatively new concept—the integration of rubber and canna within agroforestry—and explores the ensuing benefits and challenges. It provides insightful revelations about the interactions between these plant species and their collective impact on soil health and microbial communities. These insights are instrumental in understanding the complex dynamics of agroforestry systems, which encompass both the symbiotic relationships and the intricate soil ecosystem shaped by these plant interactions.

Additionally, the research's distinctive focus on metagenomic techniques to study soil microbial communities offers an advanced perspective on the genetic composition and biodiversity within the agroforestry environment. This approach allows for an in-depth analysis of environmental genetic material, leading to a richer understanding of microbial diversity and its functional potential. The emphasis on sustainability, biodiversity, and carbon sequestration further positions the study at the forefront of sustainable land use and agroecology discourse. Moreover, by evaluating the practical applications of its findings for sustainable agriculture in Southeast Asia, the paper provides actionable insights for farmers, land managers, and policymakers. This ensures that the study's implications extend beyond theoretical research, influencing real-world sustainable agroforestry practices.

CONCLUSION

The study's findings have addressed several research objectives. Firstly, the research has successfully demonstrated that *C. indica* can thrive under rubber trees, showing similar growth parameters and plant characteristics as those grown in open areas. Furthermore, the study has uncovered significant variations in soil properties, microbial diversity, and soil fertility when comparing the Rubber-Canna agroforestry system with traditional Rubber Monoculture systems. Additionally, evidence from the research points to an increase in latex production at the Rubber-Canna agroforestry sites, suggesting that the agroforestry system may have positive long-term effects on latex output. Lastly, the findings endorse the view that the Rubber-Canna agroforestry system bolsters sustainability and improves soil fertility and crop yields, which contributes towards food security and mitigates environmental concerns. In summary, the research has provided solid empirical evidence supporting the viability of *C. indica* in an agroforestry setting, its beneficial impact on soil and microbial communities, its positive influence on long-term latex production, and its role in promoting sustainability and food security.

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