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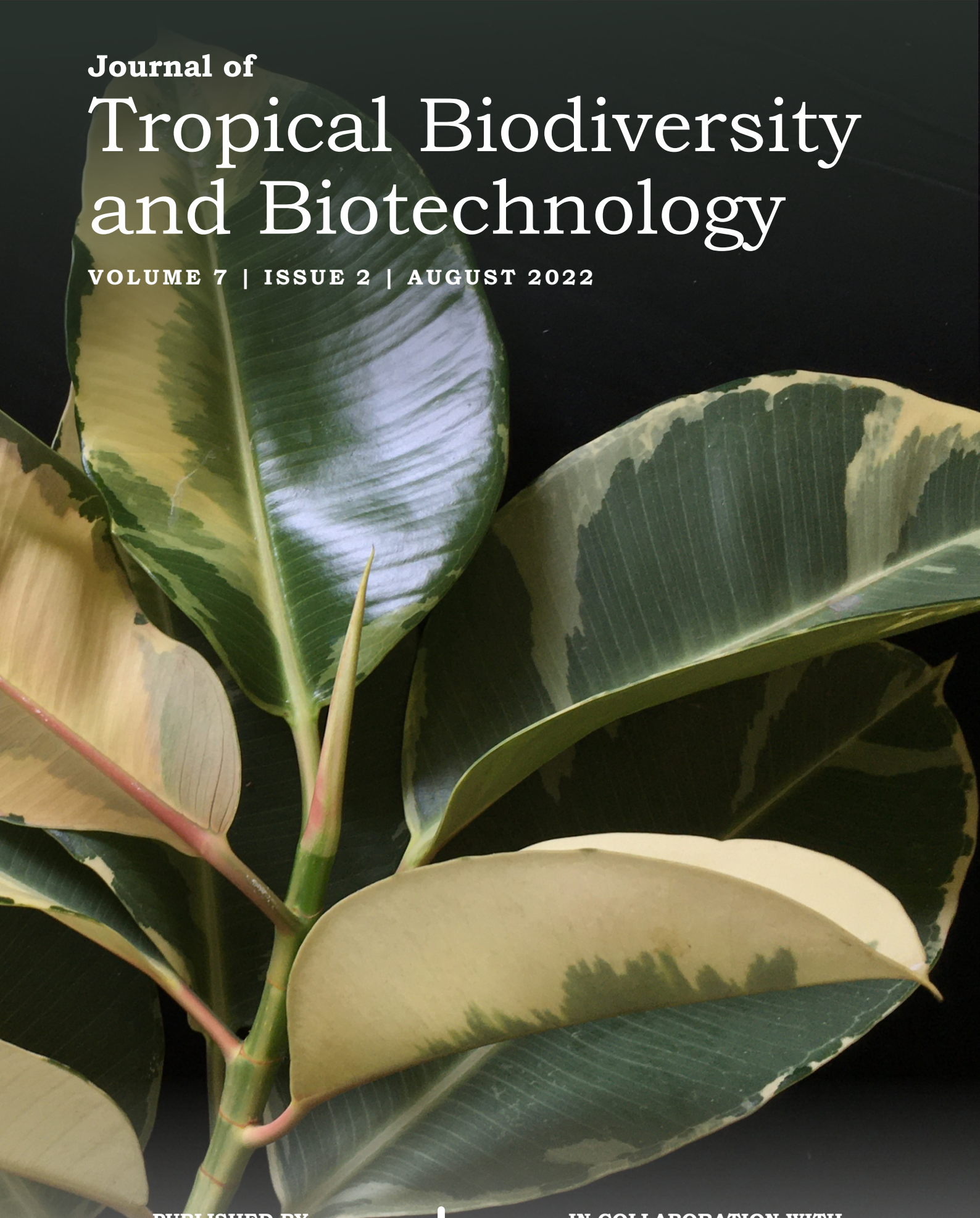

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Short Communications

# The Protective Effect of *Gynura procumbens* Adventitious Root against Lead Acetate Toxicity in Mice

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**ABSTRACT**
Lead induced oxidative stress contributes to increase the productivity of reactive oxygen species (ROS) and to disrupt the antioxidant balance. *Gynura procumbens* adventitious root (GPAR) methanol extract contains abundant phenolic and flavonoids compounds as antioxidants and can be used as traditional medicinal plants. The objective of this study is to evaluate the protective effect of GPAR against lead acetate toxicity in mice to haematological parameter, histological of hepatic cells, and activities of antioxidant enzymes. The data obtained from five groups of treatment: P1 (control), P2 (Pb acetate-100 mg/L), P3 (GPAR-100 mg/L + Pb acetate-100 mg/L), P4 (GPAR-200 mg/L + Pb acetate-100 mg/L), P5 (GPAR-300 mg/L + Pb acetate-100 mg/L). The results indicated that administration of methanol extract of GPAR can prevent the decreasing of haematological parameter, maintain the percentage of normal hepatic cells, activities of superoxide dismutase (SOD), and catalase (CAT) due to lead acetate treatment. The effective dose of GPAR extract was 300 mg/L. This study provides that methanol extract of *G. procumbens* adventitious root exerts protective effects against lead acetate toxicity in mice.



Lead (Pb) is non-biodegradable heavy metal. It can pollute the environment and endanger organisms, especially humans. Lead absorbed into the body through the respiratory tract, digestive tract, and skin; then be stored in tissues or organs, such as bone marrow, muscle, brain, kidney, heart, spleen, and liver. Most of leads accumulate in the liver, which are about 33% because they are related to the function of the liver, such as storage, biotransformation, and detoxification of toxic compounds (Mudipalli 2007; Haouas et al. 2014)

Exposure to high concentrations of lead is inducing oxidative stress because of the formation of reactive oxygen species (ROS) or as free radicals. If the ROS production is excessive and the endogenous antioxidants cannot overcome, it will trigger the occurrence of lipid peroxidation, which impacts changes in cell membrane integrity and eventually results in cell damage. Metwally (2015) reported swelling of the liver cells of mice lead-treated for 2-4

weeks. It is caused by swelling of intracellular organelles, particularly mitochondria and endoplasmic reticulum. Lead also inhibits the activity of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx). High production of ROS leads to the suppression of the antioxidant in the human body. SOD is an oxidoreductase that acts as a superoxide radical detoxifier by converting superoxide into a less reactive form. In humans, the lower the SOD activity, the more various trigger reactions to metabolic diseases. CAT includes hydroperoxidase enzymes that catalyzed the breaking down $H_2O_2$ into $H_2O$ (Ercal et al. 2001; Kumar et al. 2015; Patra et al. 2011).

Several previous studies reported that to compare the control group, treatment by lead was significantly decreasing red blood cell (RBC), hemoglobin concentration (HGB), hematocrit (HCT) (Sugiharto et al. 2019a) and reducing SOD and CAT activity (Carocho & Ferreira 2013). On the contrary, leads were increasing platelet (PLT), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular hemoglobin (MCH) (Sugiharto et al. 2020a), malondialdehyde (MDA) level, and lead concentrations in the liver (Sugiharto et al. 2019b), and were also significantly shifting Bowman's capsule diameters, and glomeruli–Bowman's ratio (Sugiharto et al. 2020b). The other studies showed that administration of 1000 ppm lead acetate for 30 days caused parenchymal degeneration, hydropic and liver cell necrosis in mice (Mauliku & Gustian 2019), while administration of 4% lead acetate for 17 days in mice could cause damage to several parts of the brain (Naqi 2015).

To mitigate the impact caused by lead exposure, the exogenous antioxidants can be given because of their function to stop or to break the chain reaction of free radicals in the human body. The administration of endogenous antioxidants is expected to reduce cell or tissue damage caused by free radicals. *Gynura* is one of the plants that contain secondary metabolites which has potential antioxidants. Puangpronpitag et al. (2010) and Teoh et al. (2013) reported that rats treated with *Gynura* methanol extract did not show significant toxicity symptoms and were safe to use as natural medicinal ingredients. *G procumbens* is rich in phenolic and flavonoid compounds, which are also known to contribute to antioxidant properties. The results of adventitious roots culture of *G. procumbens* influenced by phenylalanine and tyrosine could increase in flavonoid compounds (Manuhara et al. 2019). Based on the High-Performance Liquid Chromatography (HPLC) assay, we found that methanol extract of the adventitious root of *G. procumbens* contains abundant of flavonoids compounds, such as myricetin, kaempferol, quercetin, and catechins (Sugiharto et al. 2021). Krishnan et al. (2015) reported that the root extract of *G. procumbens* had higher antioxidant potential than leaves and stems extract.

The present study was aimed to investigate the protective effect of *G. procumbens* adventitious root extract against lead acetate toxicity in mice. RBC, HGB, AST levels, ALT levels, histological analysis of hepatic cells, and activities of antioxidant enzymes (SOD and CAT) were also determined. There

are limited reports regarding the application of *G. procumbens* adventitious root extract produced from tissue culture for treatment in animal as well as to be used for traditional medicinal plants.

*G. procumbens* adventitious root in a laboratory-scale bioreactor was harvested after 28 days of culture (Faizah et al. 2018; Manuhara et al. 2019). Briefly, 25 g of dry root powder of *G. procumbens* were immersed in 250 mL of methanol (1:10) and macerated in a shaker for 24 h and supernatant filtered through filter paper. This procedure was treated three times using methanol as solvent and the filtrates were concentrated using an evaporator.

The use of animal subjects has been approved by Faculty of Veterinary Ethics Committee, Airlangga University (certificate no. 2.KE. 151.07.2019). Total of 25 (8-10 weeks old) male mice (*Mus musculus*, strains Balb/C) obtained from Faculty of Pharmacy, Airlangga University. Mice were randomly gathered into five treatment groups:

P1: 0.25 mL of distilled water (control)
P2: 0.25 mL of Pb acetate 100 mg/L
P3: 0.25 mL of GPAR 100 mg/L and 0.25 mL of Pb acetate 100 mg/L
P4: 0.25 mL of GPAR 200 mg/L and 0.25 mL of Pb acetate 100 mg/L
P5: 0.25 mL of GPAR 300 mg/L and 0.25 mL of Pb acetate 100 mg/L

*G. procumbens* treatment was given every morning around 09:00 to 10:00 a.m., and lead treatment was given 2 h after that. Preparation of 100 mg/L Pb stock solution was carried out by dissolving 10 mg of lead acetate into 100 mL of distilled water. Meanwhile, the preparation of a 300 mg/L stock solution of methanolic extract GPAR was carried out by dissolving of 3 mg the GPAR extract into 10 mL of distilled water. The same method was also used to make 100 and 200 mg/L GPAR solutions. Treatment by oral administration for 30 days using *cannula*. Blood samples were taken using the cardiac puncture technique. The blood samples were mixed and incubated (3000 rpm) for 10 min at 10 °C to harvest serum for measurement of hematological parameters and activities of antioxidant enzyme (SOD and CAT) analysis. In addition, the preserved liver analyzed using hematoxylin eosin (HE) staining that embedded into paraffin for histological analysis. The histological hepatic cell observed by light microscopic (Olympus CWHK) with graticulae (magnification 400x). Histological observations of the liver were carried out in four (4) fields of view, namely superior, median right side, median left side, and inferior. The observed cells were normal cells (cells with oval shape, solid round nucleus in the middle); swollen cells (cells have clear and wide cavities, irregular cytoplasm); and necrotic cells (cells are small, nucleus not very clear and eosinophilic). The total number of cells in one field of view is assumed to be 100% (Sugiharto et al. 2019b).

$$\% \text{ normal cell} = \frac{\text{normal cell}}{\text{total number cell}} \text{ x } 100\% \quad \text{.....} \quad (1)$$

All data were expressed as means ± standard deviations. The statistical analyses ($p < 0.05$) for the RBC. HGB, AST level, SOD activity, percentage of swollen and hepatic necrosis cells were subjected by SPSS 25.0. ANOVA

and Duncan's test were applied. The Kruskal-Wallis and Mann-Whitney tests are used to determine ALT level and percentage of normal hepatic cells because the data are not normally distributed based on the Kolmogorof-Smirnov test ($p < 0.05$).

This study showed that lead could induce toxicity due to an increase in ROS and lipid peroxidation. Lead toxicity can cause changes in several parameters such as haematological parameter, enzyme activities of SOD and CAT in blood serum, as well as histological hepatic cells (Table 1, Figure 1, Figure 2, and Figure 3). Most of the lead accumulate in the liver, generating changes in cell membrane integrity and inhibition of endogenous antioxidant enzymes activity, cell or organ damage, and metabolic disorders (Assi et al. 2016; Mudipalli 2007).

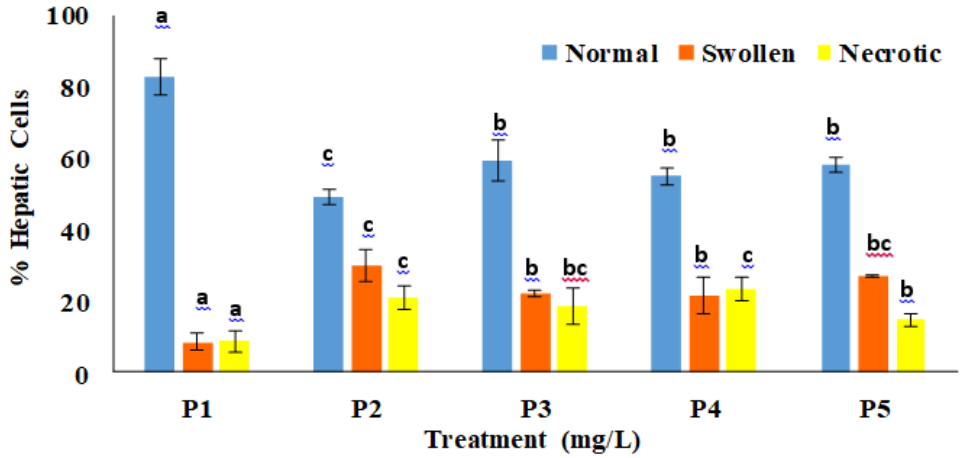


**Figure 1.** Effect of *G. procumbens* adventitious root against lead acetate on histological hepatic cells. P1=control, P2=Pb acetate-100 mg/L, P3=GPAR-100 mg/L + Pb acetate-100 mg/L, P4=GPAR-200 mg/L + Pb acetate-100 mg/L, P5=GPAR-300 mg/L + Pb acetate-100 mg/L. One-way ANOVA and Duncan's test performed statistical analysis for the percentage of swollen and hepatic necrosis cells. The Kruskal-Wallis and Mann-Whitney tests were conducted for the normal cells ($p < 0.05$).

**Table 1.** Effect of *G. procumbens* adventitious root against lead acetate on hematological parameters.

| Data | P1 | P2 | P3 | P4 | P5 |
|---|---|---|---|---|---|
| RBC ($10^6$/mm$^3$) | $9.93 \pm 0.72^{c}$ | $5.96 \pm 0.93^{a}$ | $8.08 \pm 0.36^{b}$ | $8.25 \pm 1.36^{b}$ | $8.12 \pm 0.58^{b}$ |
| HGB (g/dL) | $15.57 \pm 0.85^{c}$ | $8.63 \pm 1.67^{a}$ | $12.25 \pm 0.66^{b}$ | $12.73 \pm 2.53^{b}$ | $12.33 \pm 2.03^{b}$ |
| AST (U/L) | $119.75 \pm 23.96^{a}$ | $230.00 \pm 32.32^{c}$ | $199.25 \pm 3.30^{bc}$ | $202.25 \pm 49.20^{bc}$ | $167.75 \pm 34.73^{ab}$ |
| ALT (U/L) | $50.75 \pm 10.11^{a}$ | $149.00 \pm 47.95^{b}$ | $71.50 \pm 8.89^{bc}$ | $68.75 \pm 18.54^{ac}$ | $78.25 \pm 8.77^{bc}$ |

RBC=red blood cells, HGB=hemoglobin concentrations, AST=aspartate aminotransferase, ALT=alanine aminotransferase. P1=control, P2=Pb acetate-100 mg/L, P3=GPAR-100 mg/L + Pb acetate-100 mg/L, P4=GPAR-200 mg/L + Pb acetate-100 mg/L, P5=GPAR-300 mg/L + Pb acetate-100 mg/L. Statistical analysis one-way ANOVA and Duncan's test performed to RBC, HGB and AST levels. The Kruskal-Wallis and Mann-Whitney tests were conducted for the ALT level. Both analysis were significant at $p < 0.05$.

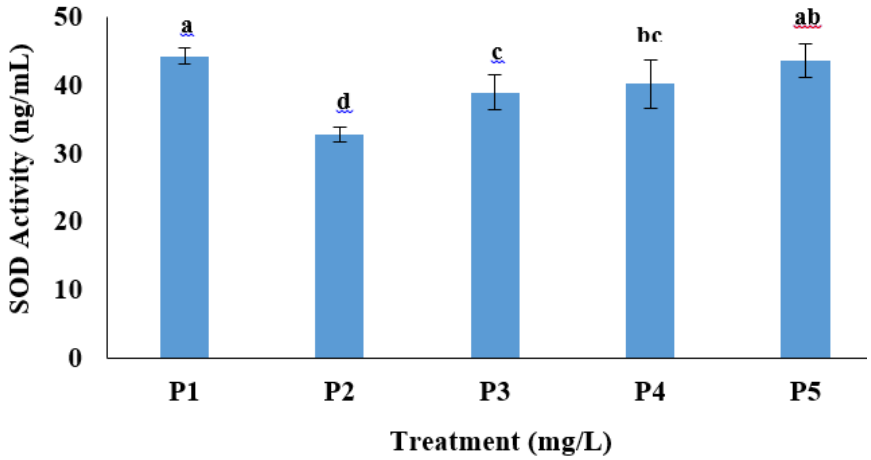


**Figure 2.** Effect of *G. procumbens* adventitious root against lead acetate on SOD activity. P1=control, P2=Pb acetate-100 mg/L, P3=GPAR-100 mg/L + Pb acetate-100 mg/L, P4=GPAR-200 mg/L + Pb acetate-100 mg/L, P5=GPAR-300 mg/L + Pb acetate-100 mg/L. One-way ANOVA and Duncan's test performed statistical analyses ($p < 0.05$).

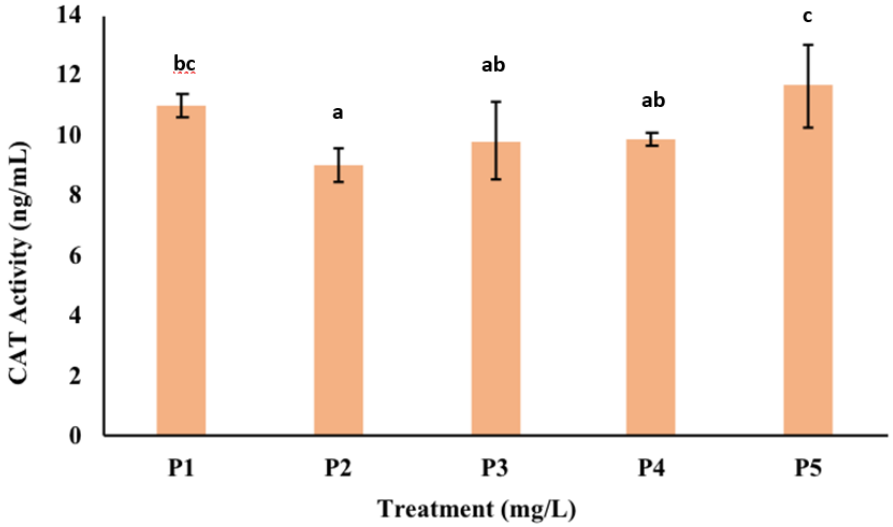


**Figure 3.** Effect of *G. procumbens* adventitious root against lead acetate on CAT activity. P1=control, P2=Pb acetate-100 mg/L, P3=GPAR-100 mg/L + Pb acetate-100 mg/L, P4=GPAR-200 mg/L + Pb acetate-100 mg/L, P5=GPAR-300 mg/L + Pb acetate-100 mg/L. One-way ANOVA and Duncan's test performed statistical analyses ($p < 0.05$).

Compared to the control group, the administration of lead acetate decreased RBC, HGB, percentage of normal hepatic cells, and activity of SOD and CAT. Whereas, lead treatment increased AST levels, ALT levels, the percentage of swollen and necrotic of hepatic cells significantly. Lead toxicity could induce oxidative stress and caused lipid peroxidation in cell membrane. It can be increased cells damage, especially in liver and kidney. Similar to present study, Haouas et al. (2014) reported administration of lead acetate 2 g/L for 35 days increased hypertrophy of hepatic cells and accompanied by an increase in AST and ALT levels. Administration of lead acetate 20 mg/kg BW for 3 weeks increased necrotic of hepatic cells, MDA levels, AST, and ALT levels as indicators of damage in liver cells (Yuniarti et al. 2021). Met-

wally (2015) reported swollen hepatic cells and intracellular organelles for 2-4 weeks due to lead administration. Lead exposures were significant to reduce SOD and CAT activity (Carocho & Ferreira 2013; Sugiharto et al. 2019a). Exposure of 150 mg/kg BW lead acetate for mice showed decreasing of SOD activity and significantly increased $H_2O_2$ formation (Andjelkovic et al. 2019).

Lead is a lipophilic compound and turns into $Pb^{2+}$ in the body. Lead is extremely poisonous and devastating liver cells, induced lipid peroxidation and associated with increase of ROS. When the production of ROS is excessive, it is directly suppressed the body's antioxidative system. Lipid peroxidation due to lead administration could disrupt membrane integrity in the cell, increase cell membrane permeability and the distribution of the ions, it causing extracellular fluid easily through to the cell. As a result, the cells become swollen and then undergo necrosis in hepatic cells (Sipos et al. 2003). Meanwhile, lead bound to the SH group inducing deficiency of the enzyme glucose 6-phosphate dehydrogenase (G6PD) in erythrocytes, and inhibition of the enzymes coproporphyrinogen, δ-aminolevulinic acid dehydratase (δ-ALAD), and ferrochelatase in the bone marrow. It leads to the decreasing of the life span of erythrocytes, increasing the fragility of the erythrocyte membrane, decreasing the number of erythrocytes, and impairing hemoglobin synthesis. In addition, high levels of ROS are an inhibitor of antioxidative enzyme activities such as SOD and CAT. The function of the SOD is to convert two superoxide radical anions ($O_2^-$) into hydrogen peroxide molecules ($H_2O_2$) and oxygen molecules ($O_2$). Moreover, CAT as an antioxidative enzymes produced by peroxisomes, decomposed $H_2O_2$ molecules into $H_2O$ (Sharma & Singh 2014; Sugiharto et al. 2019a; Sugiharto et al. 2020a; Wani et al. 2015).

*G. procumbens* adventitious root extract can be an antioxidant because it contains flavonoid and phenolic compounds (Krishnan et al. 2015; Pramita et al. 2018). Flavonoid and phenolic compounds showed inhibitory effects on ROS and radical scavenging activity by hydrogen atom transfer (HAT) or single electron transfer (SET) that producing more stable free radical. Chelating properties of OH group is probably responsible for the inhibitory effect of flavonoids and protection from oxidative stress; it has been implicated in several of degenerative diseases and exhibited anticancer activities. Wang et al. (2013) and Chaichana et al. (2019) reported that in cancer cell line assay, *Gynura* ethanolic extract (40 – 400 μg/mL) was able to inhibit human cells such as osteosarcoma cell line (U2-OS), gastric carcinoma cell line (KATO-III, HTB-103), human liver hepatocarcinoma cell line (HepG2, HB-8065), human colon adenocarcinoma cell line (Caco-2, CCL-227), and human breast cancer cell line (MCF-7). Furthermore, the myricetin and quercetin compounds in the methanol extract of the adventitious root of *G. procumbens* have exerted potent inhibition to lipid peroxidation and free radical scavenging activity, and it is thought to increase the of the antioxidative enzyme activity such as SOD and CAT by inactivating superoxide (Akan & Garip 2013; Tan

et al. 2016; Terao 2009; Yuniarti et al. 2021). The results of our study indicated that the decreasing of RBC, HGB, percentage of normal hepatic cells, and activity of SOD and CAT could prevent by administration of *G. procumbens* adventitious root extract. *G. procumbens* also significantly prevent increasing of AST and ALT levels, and the percentage of swollen and necrotic hepatic cells in mice exposed to lead. The effective dose of *G. procumbens* extract in this study was 300 mg/L, because there was an increasing in the activity of SOD and CAT enzymes as well as a decreasing in the number of necrotic hepatic cells and AST levels compare to that of *G. procumbens* at the doses of 100 and 200 mg/L. This study provided the scientific potential of *G. procumbens* to be used for traditional medicinal plants.

## AUTHORS CONTRIBUTION

S.S designed the research, wrote the manuscript, and supervised all the process, D.W. collected and analysed data especially for histological analysis, U.I designed the research and supervised the data, A.H.M and A.B.M. analysed the data, Y.S.W.M. prepared *G. procumbens* adventitious root extracts and supervised all the process.

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## CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Short Communications

# Microbial Degradation of Lignocellulose in Empty Fruit Bunch at Various Incubation Time

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**ABSTRACT**
Isolation of cellulose in empty fruit bunch (EFB) is hindered by lignin content. EFB was pretreated with *Pleurotus ostreatus* which has the ability to degrade lignin, in various incubation times (0th, 15th,30th, 45th, and 60th). The result showed lignin and cellulose decreased from 29.3% to 20.3% and from 40.2% to 26.2%, respectively. The optimum degradation time was on 30th day in which lignin and cellulose contents decreased from 29.3% to 20.3% and from 41% to 39.7% respectively. The cellulose to lignin (C/L) ratio increased from 1.40 to 1.99. These data revealed that *P. ostreatus* have a high potential for EFB delignification.



Indonesia is one of the largest palm oil-producing countries in the world with a total production of 40,5 million tons in 2018. Palm oil processing produces waste including empty fruit bunches (EFB). One ton of fresh fruit bunches processing produces 220-230 kg of EFB which is equal to 8.92-9.33 million tons/year (Asari et al. 2015). However, the utilization of EFB is still limited such as for fertilizer, piled up in the ground or just burned.

EFB contents cellulose, lignin, and hemicellulose by 37.3-46.5%, 27.632.5%, and 25.4-33.8%, respectively (Asari et al. 2015). The cellulose is able to be utilized in various applications such as bioethanol (Bukhari et al. 2014), adsorbents (Mahmood et al. 2018), etc. Unfortunately, isolation of the cellulose is difficult because of high lignin content in EFB. The presence of lignin around the cellulose makes chemical and enzymatic activities are restricted due to steric hindrance (Mahmood et al. 2018).

One of the biological methods which is generally known to pretreat EFB is by applying fungi. It uses low energy, is cheap, and environmentally friendly. White rot fungi are the most efficient for cellulose isolation because of their selectivity to degrade lignin, in contrast to brown and soft rot fungi where they are selectively degraded cellulose (Wang et al. 2013).

Oyster mushroom (*Pleteurotus ostereus*) is a white rot fungus that has been used previously to degrade lignocellulosic in various types of material, such as cotton husks (Li et al. 2001), and palm fronds (Metri et al. 2018).

According to literatures, it is known that the incubation time is an important factor to degrade lignocellulosic effectively. This study was conducted to determine the effect and optimum incubation time on *P. ostreatus* toward the lignocellulosic composition in EFB. Empty fruit bunch was subjected to fungal pretreatment using *P. ostreatus* at various incubation times 0th, 15th, 30th, 45th, and 60th days.

The empty fruit bunch was collected in Sungai Malaya Village, West Kalimantan. Mushroom spawn was obtained from the Mushroom Nursery in Parit Suka Maju, Pontianak, West Kalimantan. The chemicals are sulfuric acid ($H_2SO_4$), nitric acid ($HNO_3$), acetic acid ($CH_3COOH$) from Merck-Millipore, and distilled water. The study was run with five incubation times 0th, 15th, 30th, 45th, and 60th days. Each incubation time was made for 5 samples. Each sample was tested triple for lignin and cellulose contents.

EFB was washed with distilled water then dried for three days. Dried EFB was chopped and cut by 1-2 cm. The chopped EFB was put into a plastic bag and distilled water was added gradually until the moisture content reached 60% (Eq. 1). Each sample was autoclaved at 121 ˚C and 15 psi for 15 minutes. After the samples were cooled, each sample was inoculated and incubated with 0.4 grams of *P. ostreatus* for various incubation times (0th, 15th, 30th, 45th, and 60th days) with a temperature of 28±1 ˚C and relative humidity of 80%. Temperature and humidity are continuously monitored with the HTC humidity sensor. Temperature was controlled by a negative thermocouple (NTC) sensor and a soldering iron as a heater, while humidity was controlled by a mini fan and a manual sprayer.

$$\text{Moisture content} = \frac{Ww-Dw}{Ww} X100\% \quad \text{(Eq. 1)}$$

Ww: wet weight; Dw: dry weight.

Determination of cellulose was conducted by modifying Crampton and Maynard (1937) method (2). All fungal-treated EFB were separated from the *P. ostreatus* by removing mycelium. Next, treated EFBs were dried in an oven at 60 ˚C until a constant weight. A total of 0.1 grams of dried EFB from each incubation time was weighed separately in a test tube (a). Furthermore, dried EFB was added 2 mL of a solution consisting of 80% acetic acid ($CH_3COOH$) and 8.5% nitric acid ($HNO_3$). The test tube was closed and heated for 20 minutes at 100 ˚C, then 12 mL of distilled water was added. The solution was filtered through filter paper (b). The filter paper containing the cellulose residue was then washed with 95% ethanol ($C_2H_2O_5$) to clean from the remaining nitric acid. Finally, filter paper was dried in an oven at a temperature of 60 ˚C until constant weight (c). Calculation of cellulose content using Eq. 2.

$$\text{Cellulose content} = \frac{c-b}{a} x100\% \quad \text{(Eq. 2)}$$

The determination of lignin content was carried out by the Klason method (Pringle 1940). A total of 0.1 grams of dried EFB from each incubation time was weighed separately in a test tube (d). Then, the sample was added with 1 mL of 72% sulfuric acid ($H_2SO_4$) and left it for an hour at room temperature. Further, 28 mL of distilled water was added to the test tube and heated at 120 °C for 1 hour. The solution was filtered through filter paper (e). Subsequently, the filter paper containing the residue of lignin was washed with distilled water to remove the remaining sulfuric acid. The filter paper was heated in the oven at 100 °C until the constant weight (f). Calculation of lignin content using Eq. 3.

$$\text{Lignin content} = \frac{f-e}{d} x100\% \quad \text{(Eq. 3)}$$

The optimum incubation time was determined based on the ratio of the cellulose and lignin contents in EFB during the pretreatment process. The optimum incubation time is reached when C/L ratio is the highest.

Figure 1 showed growth of *P. ostreatus* from days of 15th to 60th. The first phase of fungal growth is shown from the appearance of mycelium on days 15th until 45th and the final phase was indicated by presence of basidia from 45th to 60th days.

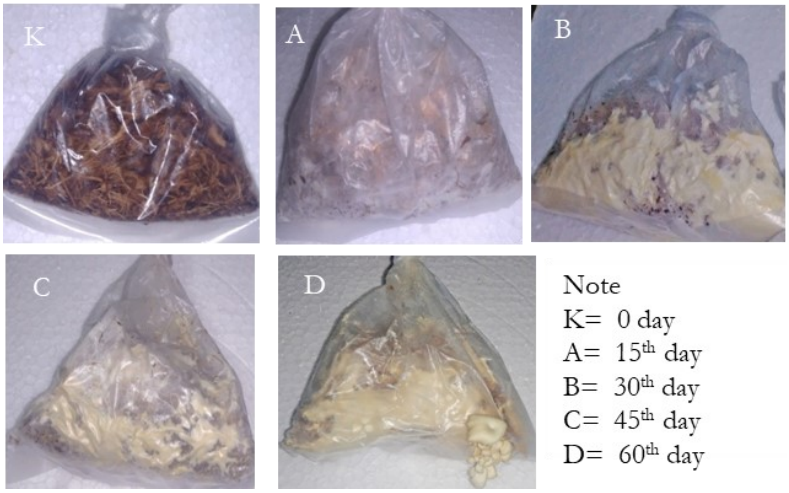


**Figure 1.** *P. ostreatus* growth in EFB.

Figure 2 showed lignin content in EFB started decreasing from 29.3% at the beginning of *P. ostreatus* growth (0th day) to 20.3% on 30th day. In contrast, the cellulose in EFB did not change significantly at early stage of incubation (days 15th until 30th). Subsequently, the cellulose began to degrade significantly from 40% on 30th day to 29.1% on the 45th day. The fungi use lignocelluloses in EFB for their daily nutrients. As a result, the lignin and cellulose composition in EFB is changeable. *Pleurotus ostreatus* degrades lignin in the beginning of their growth in order to access nutrients from carbon sources such as cellulose. Lignin binds cellulose strongly with ether and hydrogen bonds as a guard for cell resistance, a structural framework and protector (Harmsen et al. 2010).

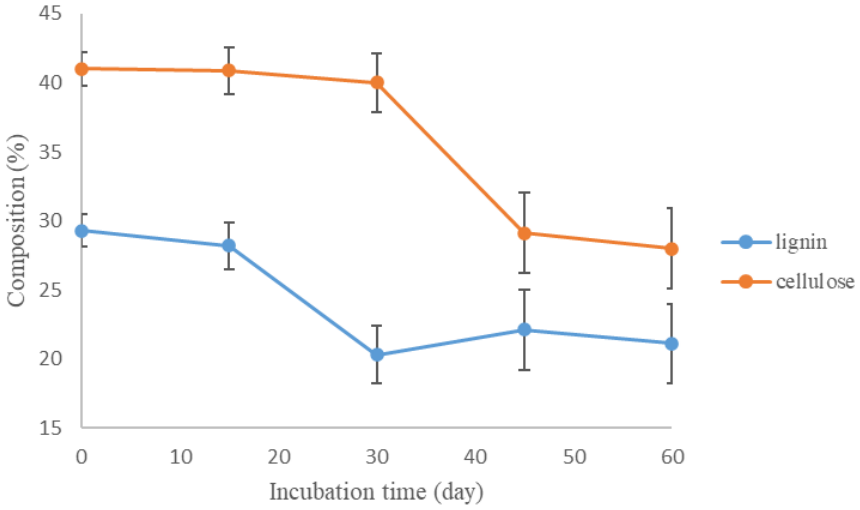


**Figure 2**. Lignocellulose composition in EFB at various incubation time.

Mustafa et al. (2016), mentioned that higher of C/L ratio indicates higher isolation yield of cellulose. Figure 3 showed that the optimum incubation time for the highest C/L ratio was 30th day. The cellulose to lignin (C/L) ratio of empty fruit bunch increased from 1.40 on 0th day to 1.99 on 30th day. Some previous studies on degradation of lignocelluloses revealed that *P. ostreatus* have also performed effectively to reduce lignin content in cottonseed husks (Li et al. 2001) and oil palm midribs (Metri et al. 2018) by giving (C/L) ratio 2.06 and 1.92, respectively.

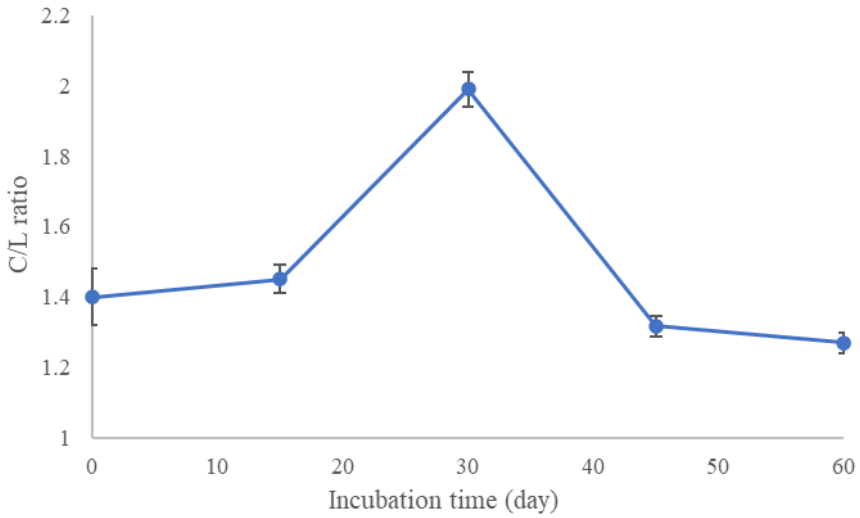


**Figure 3.** C/L changes in EFB at various incubation time.

In summary, *P. ostreatus* shows a good capability to pretreat EFB substrate by the formation of mycelium, pinning, and mushroom fruiting bodies. The incubation time of the fungus gave a significant effect on the composition of lignin and cellulose. The ability of *P.ostreatus* to degrade lignin was occurred in the early growth phase of the fungus, in contrast, highest degradation of cellulose arise in the final phase of fungal growth. The optimum incubation time was reached on 30th day.

## AUTHORS CONTRIBUTION

NRA designed, collected, and analyzed the research data, R and G.G helped, supervised all the process, and re-wrote the manuscript.

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## CONFLICT OF INTEREST

There is no conflict of interest regarding the research or the research funding.

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**Short Communication**

# Anatomical and Histological Characteristics of Gonad of Tropical Eel *Anguilla bicolor* McClelland, 1844 in Different Length Body Size

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**ABSTRACT**
The sex status of *Anguilla bicolor* McClelland is difficult to be distinguished between males and females. Thus, we evaluated 309 fishes from The Serayu River, with length and weight range around 9 - 81 cm and 0.59 - 1260 g respectively to access the anatomical and histological characteristics of their gonads for each sex. Parameters assessed were body sizes, eye diameter, fin length, and gonad weight. The results showed that body length, weight, eye index, and gonadosomatic index of the male were significantly smaller ($p<0.01$) than those of the females. Fins index was not significantly different ($p>0.05$) between males and females. The histological structure of gonad showed that the eel gonads can be classified as either indifferent gonads, testis, or ovary based on the length of body size. Collecting all the data together it can be concluded that *A. bicolor* is a gonochoric.



Anatomical and physiological parameters are essential factors in determining the maturity of eel gonads (Arai & Abdul Kadir, 2017a; Ismail et al. 2017; Ching et al. 2019). Mature male eels *Anguilla bicolor* are rarely found in freshwater (Rachmawati & Susilo 2011; Arai & Abdul Kadir 2017b; Rachmawati et al. 2017) because they prefer to live in brackish water or seawater. The immature ones, soon migrate to the sea to complete the phase of spermatogenesis (Aida et al. 2003; Tesch 2008). The length of *Anguilla japonica* males is found in fresh water and saltwater ranged between 42.5 - 63.4 cm, whereas the length of female eels is ranged between 46.1 and 85.6 cm (Sudo et al. 2013). Body length is a sex-determining factor (Sugeha et al. 2009; Arai & Abdul Kadir 2017), but body length always overlaps in different sexual statuses, thus is it challenging to distinguish the male and female eels. Previous studies reported that male eel is shorter in length of body size than that of female eel (Frisch 2004; Bark et al.2007; Tesch 2008; Côté et al. 2015). Observations of *A. bicolor's* body length was varied among researchers. Rovara et al. (2008) studied that the length of females is over 50 cm, while the lengths

of indifferent gonads or interstitial gonads were under 50 cm. Whereas, Sugeha et al. (2009) reported that the body length of male *A. bicolor* in Segara Anakan were 24.5 to 54.5 cm, while the body length of females were 33.5 to 77.8 cm. So, it is interesting to observe the anatomical characteristics of eels at various lengths body sizes in relation to their sex.

This survey was carried out from September 2015 to December 2016. *Anguilla bicolor* samples were caught from The Serayu River. Total of 309 eels with a length range between 9 - 81 cm and weight range between 0.59 - 1260 g were reared in a fibre aquarium with the size 2 x 1 x 1 $m^3$. We assessed some parameters such as body sizes, eye diameter, fin length, and gonad weight to evaluate the anatomical characteristics such as eye index, fin index, GSI (Gonadosomatic Index), and sex status. Prior to data collection, eels were anesthetized for about 15 minutes in water containing clove oil at the dose of 5 ppm (Rachmawati & Susilo 2011; Rachmawati et al. 2017). The eel was weighed with a technical scale and measured the body length with a ruler. The body length was measured using standard scale from the head to the tail. Eye index was calculated based on eye diameter measurement, using a calliper by measuring the horizontal and vertical diameter of the orbital of the eye (Yokouchi et al. 2009). Fin index was calculated based on Yokouchi et al. (2009) by measuring the length of the fin from the base to the tip of the pectoral fin. Gonads were collected from the anus to the pectoral, and weighed using an analytical scale for the measurement of GSI (Gonadosomatic Index), according to Rupia et al. (2014). Histological observation of gonads follows standard procedures: gonad was sliced 1 $cm^3$, fixed in NBF 10% for tissues processed with routine paraffin embedding, sliced approximately 6 μm in transverse sections, and stained with Haematoxylin-Eosin for histological assessment under a light microscope. Data were analyzed using one-way ANOVA, with 95% or 99% significance and qualitative data was analyzed descriptively (De Smith 2018).

The results showed that anatomically, eel gonads with a body length of 9 to 28.5 cm, looks like a thin thread that are milky white (Figure 1). They were called as indifferent gonads. Moreover, histological structure of those indifferent gonads was dominated by primordial germ cells with a large nucleus (Figure 2).

The gonad fish with length range of 22 to 42.70 cm showed anatomical structure of a lobular transparent with a round shape along the ligament and clear white like jelly appearance (Figure 3). This structure under histological assessment were showing presence of tubular containing spermatogonia (Figure 4), so they were testicles (male).

Anatomical structure of the gonad's length of eel range 24 to 81 cm was showed structure like a ribbon/lamella, milky white, folded along sides both (the right and left sides) of the abdomen (Figure 5). Histological structure of the gonad showed the structure of follicles (Figure 6), indicating ovary (female).

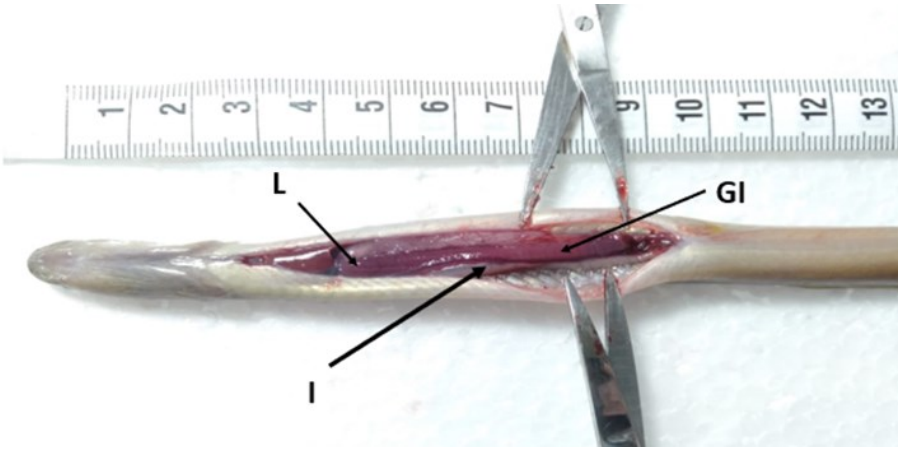


**Figure 1.** Anatomical Structure of Indifferent Gonad of Tropical Eel *Anguilla bicolor* McClelland (I: indifferent gonad; L: liver; GI: gastrointestinal).

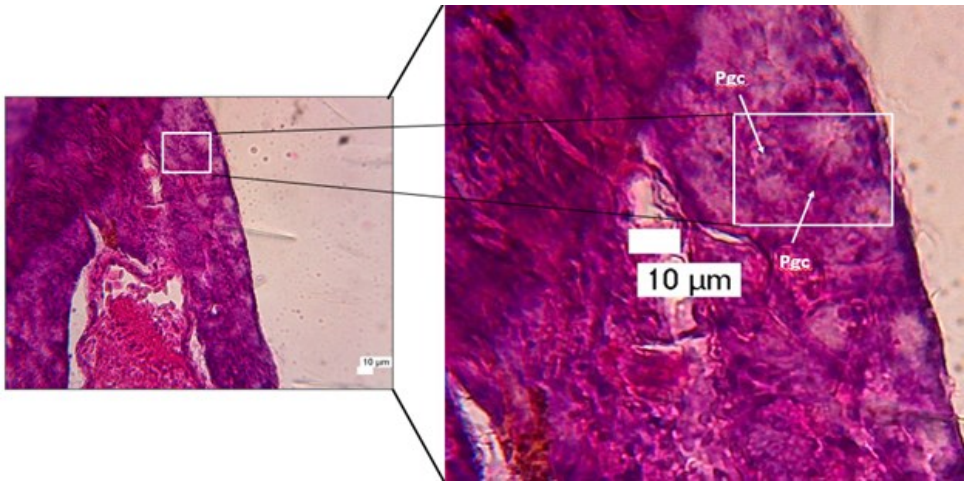


**Figure 2.** Histological Structure of Indifferent Gonad of Tropical Eel *Anguilla bicolor* McClelland (Pgc: primordial germ cell). Haematoxylin-Eosin Staining. Scale bar: 10 mm.

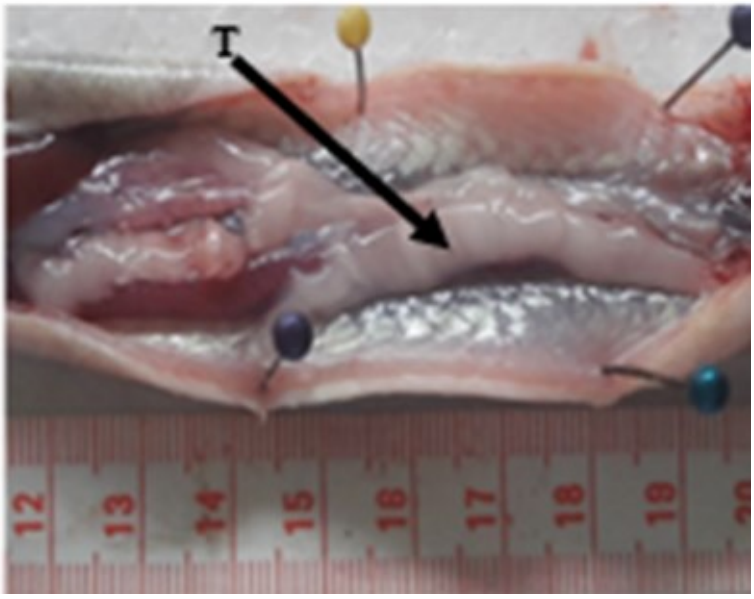


**Figure 3.** Anatomical Structure of Male Gonad (testes) Tropical Eel *Anguilla bicolor* McClelland (T: testes).

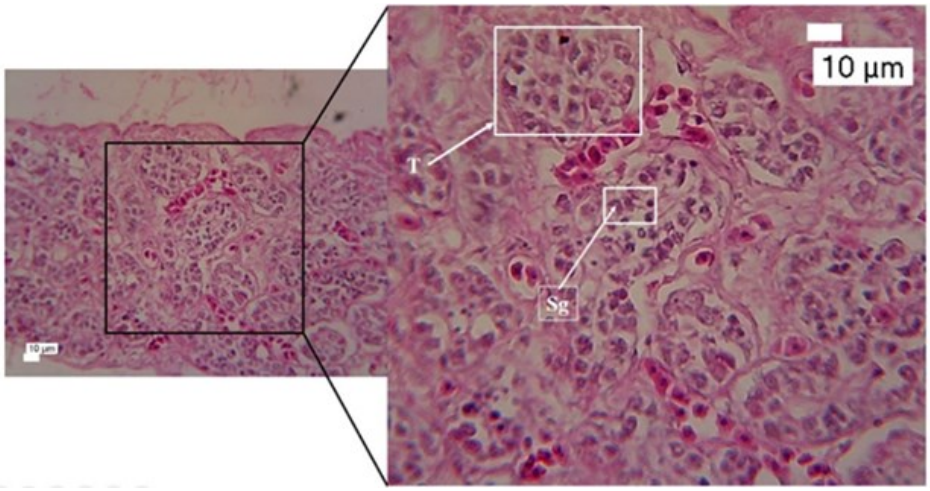


**Figure 4.** Histological Structure of Testes of Tropical Eel *Anguilla bicolor* McClelland (T: tubules; Sg: spermatogonium). Haematoxylin-Eosin Staining. Scale Bar: 10 µm.

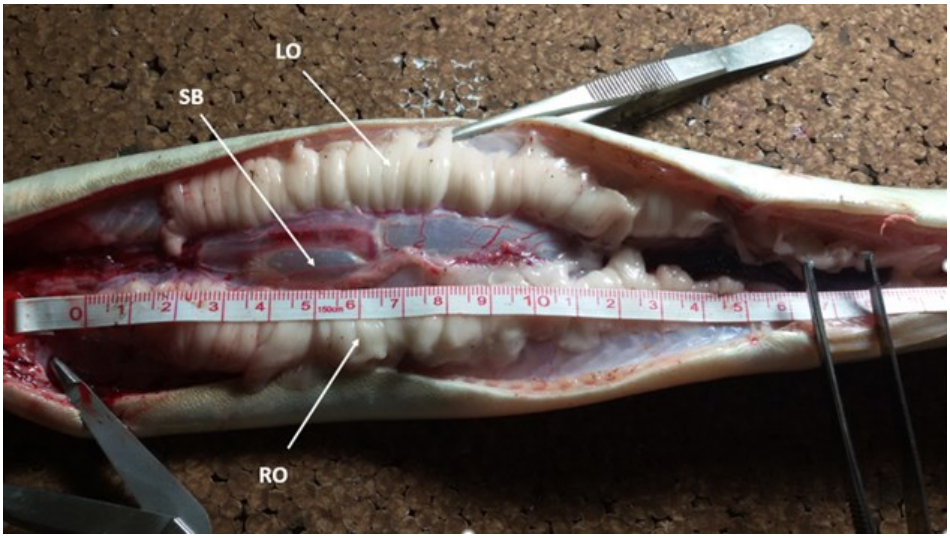


**Figure 5.** Anatomical Structure of Female Gonads (Ovaries) of Tropical Eel *Anguilla bicolor* McClelland (LO: left ovary; RO: right ovary; SB: swim's bladder).

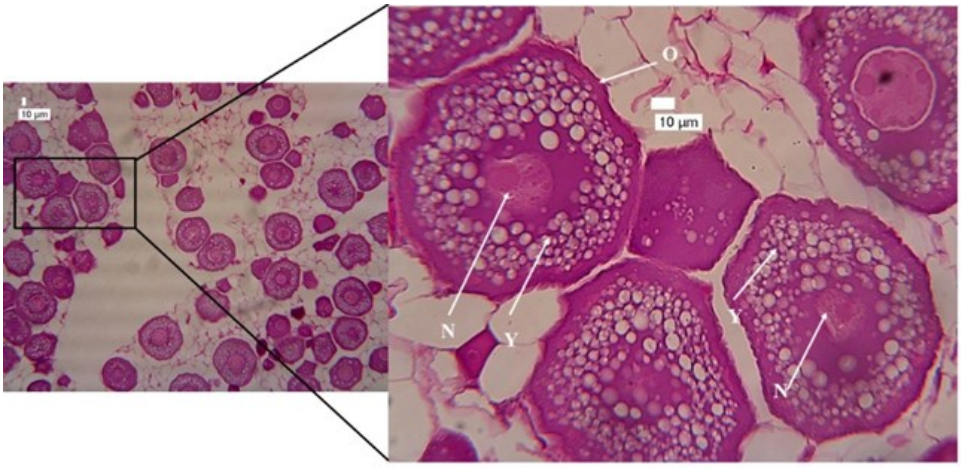


**Figure 6.** Histological structure Follicles of Female Gonad (Ovaries) of Tropical Eel *Anguilla bicolor* McClelland (O: follicle cells; N: nucleus; Y: Yolk). Haematoxylin - Eosin Staining. Scale Bar: 10 µm.

These results proved that gonads of *A. bicolor* are similar with that of *A. marmorata* (Wakiya et al. 2019), composed of three sexual characteristics:

indifferent gonad, testes, and ovary. This study differs from previous report in *A. anguilla*, namely (1) indifferent gonad, (2) interstitial gonad, (3) male gonad, and (4) female gonad (Sugeha et al. 2009). Geffroy et al. (2013) reported that indifferent gonad of *A. rostrata* developed from interstitial stages (Sirsky organs) or directly from ovaries. The Sirsky organ was an intersexual organ histologically dominated by male structures but contains a small number of oocytes (Geffroy et al. 2013). Such structure was not found in this study. Based on anatomical observations of the gonad, it is easier to distinguish testicles from ovaries on samples longer than 30 cm, however below that size, the gonad is difficult to be determined.

Histologically, gonad of eel with the length range of 9.00 to 28.50 cm (17.3514 ± 4.2799 cm) composed of cells with large nuclei (Figure 2). Thus, type of gonads was called indifferent gonad. The eel with total body length ranges between 22.00 and 42.70 cm (28.2919 ± 5.1640 cm), consists of testis tubules (Figure 4), so they were male. Whereas the eel with total body length range of 24.00 to 81 cm (47.4259 ± 19.1525 cm) was composed of follicles ovaries, thus they were females (Figure 6). Overall, the quantitative data on eye index (EI), fin index (FI), GSI, as well as body length (BL) and body weight (BW) with sex status showed that their anatomical data were overlapping not as a discrete condition (Table 1).

**Table 1.** Mean (± SD) of anatomical parameter of *Anguilla bicolor* McClelland in each sex status (indifferent, male, and female)

| Parameter | | Sex Status | | |
|---|---|---|---|---|
| | | **Indifferent** | **Male** | **Female** |
| BL (cm) | Range | 9.00 – 28,50 | 22.00 -42,70 | 24.00 – 81,00 |
| | Mean ± SD | 17.3514 ± 4.2799 | 28.2919±5.16340 | 47.4259±19.1525 |
| BW (g) | Range | 0.59 – 29,00 | 13.00 – 157,00 | 20.29 – 1062,00 |
| | Mean ± SD | 9.7018 ±7.9443 | 38.4646±34.9282 | 289.2168±289.8995 |
| EI | Range | 0.12 – 7,58 | 0.16 – 15,34 | 0.57 – 18,28 |
| | Mean ± SD | 1.4658±1.2207 | 3.5847±3.5678 | 5,5193±2,9787 |
| FI | Range | 1.74 – 6,51 | 1.60 – 6,84 | 2.88 – 6,53 |
| | Mean ± SD | 3.2939±0.8721 | 4.4802±1.2599 | 4.4380±0,8750 |
| GSI (%) | Range | 0.03 – 4,21 | 0.03 – 0,81 | 0.03- 4,37 |
| | Mean ± SD | 0.3617±0.6121 | 0.2796±0.1980 | 1,1538±1,0573 |
| N | | 147 | 74 | 88 |

Notes:
BL: body length
BW: body weight
EI: eye index
FI: fin index
GSI: gonadosomatic index

The average of eye index values of an indifferent gonad, male, and female were 1.4658, 3.8067, and 5.3617, respectively (Table 1). The mean of eye index of eel overlaps between each sex ($p < 0.01$).

Value of the fin index at each stage of gonad corresponds to the sex status ($p < 0.05$). The average of fin index among the sex status is 3.2939 ±

0.8721 (indifferent gonad); 4.4802 ± 1.2599 (male), and 4.4380 ± 0.8750 (female). The values were similar between males and females (Table 1), thus cannot be used to determine the sex. Previous report shown that FI was not related to the sex status but rather to the degree of maturity of the gonad (Durif et al. 2009; Van den Thillart & Dufour 2009; Nowosad et al. 2014). The increasing of swimming activity during the migration from freshwater to seawater can increase the fin index value (Durif et al. 2009; Van den Thillart & Dufour 2009; Nowosad et al. 2014).

The GSI value was range between 0.03 and 4.37% (Table 1). The mean of GSI value (%) in indifferent gonad, male, and female was 0.3617 ± 0.6121; 0.2796 ± 0.1980; and 1.1538 ± 1.0573 respectively (Table 1). The highest GSI value indicates female individual as compared to other sex gonadal ($p < 0.01$).

Our study showed that anatomical and histological characteristics of *Anguilla bicolor* McClelland's, such as length, body weight, eye index, and GSI can be used as a sex-determining factor for tropical eel *A. bicolor*. Based on the results, it can be concluded that *A. bicolor* has a gonochoric not hermaphrodite, with three different sex statuses which are indifferent gonad, male, and female.

Data analysis showed that anatomical parameter indices in *A. bicolor* can be used as a sex determinant ($p<0.01$). Data measurement of this study found that length of indifferent gonad was range from < 21 cm up to 33 cm, the length of male was range from 21.1- 60 cm, whereas female had body length range from > 21 cm (Figure 7). These results also proved that sex status was overlapping in *A. bicolor* (Figure 7). The results of this study confirmed previous studies in *A. japonica, A. bicolor* (Sugeha et al. 2009), and *A. rostrata* (Krueger & Oliviera 1999; Jessop 2010) which proved that the body length can be used to determine sex status.

## AUTHORS CONTRIBUTION

F.N.R. collected and analysed the data and wrote the manuscript. R.A. designed the research and supervised all the processes. Y.S. managed all the processes and the English translation.

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## CONFLICT OF INTEREST

We declared that we have no known competing for financial interests or personal relationships that could have influenced the work reported in this paper.

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Short Communications

# Java Sparrow *Lonchura oryzivora* at Bali Barat National Park: Do They Still Persist?

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**ABSTRACT**
The main purpose of the establishment Bali Barat National Park was to conserve the endemic endangered Bali Starling. However, based on data on 2004, one endangered species, Java Sparrow *Lonchura oryzivora* also resided in there. Current official report of sighting is ultimately required since it acts as reference in the management of a conservation area. We reported four sightings of Java Sparrow flocks occurred in June, 2021 at the Prapat Agung Peninsula, 28 individuals in total consisted of 13 adults and 15 juveniles, in a transition area of monsoon forest and savannah. The biggest flock sighted was eleven individuals. This study therefore confirmed that the Java Sparrow was still persisted at the Bali Barat National Park in 2021, in Prapat Agung Peninsula in particular.



Bali Barat National Park is the only National Park in Bali, situated at two regencies namely Jembrana and Buleleng. It covers area of 19.002,89 hectares, consists of 15.587,89 hectares of land and 3.415 hectares of sea waters (Mahmud et al. 2015). The park has six types of terrestrial ecosystem, namely mangrove forest, coastal forest, monsoon forest, tropical forest, evergreen forest, and savannah (Santoso et al. 2019). The main purpose of the establishment of the Bali Barat National Park was to conserve the endemic endangered species Bali Starling *Leucopsar rothschildi* based on Ministry of Forestry Decree No. 493/Kpts-II/1995. Based on data on 2004, in Bali Barat National Park resided five threatened bird species: two critically endangered species (Bali Starling *Leucopsar rothschildi* and Grey-rumped Myna *Acridotheres tertius*), two endangered species (Milky Stork *Mycteria cinerea* and Java Sparrow *Lonchura oryzivora*), and one vulnerable species (Lesser Adjutant *Leptoptilos javanicus*) (BirdLife International 2021).

Java Sparrow belongs to the order Passeriformes and family Estrildidae. It is an endemic bird of Java and its adjacent isles, as well as Bali. This

bird is characterized by white cheeks, black head, and large red beaks (MacKinnon & Phillipps 1993; Eaton et al 2016; Islam 2020). In the past, this bird can be found easily in the rice field area in a big flock. However, it is quite difficult to find them in the wild currently. In the wild, birds can be found in areas near rice fields/farms or grasslands. These grain-eating birds are often grouped and nomadic. In addition to eating seeds, especially rice grains, these birds also eat fruit and insects. The drastic decrease in the amount is mainly caused by illegal capture for pets. Its beautiful feather color makes this bird a target for collectors, even overseas (Yuda 2015; Chng et al. 2016). In addition, this bird is also considered as a pest that eats grains of rice thereby reducing crop yields. For that reason, large-scale capturing in the wild was occurred in the 1960-1970s. Another threat faced by Java Sparrow is habitat loss or habitat fragmentation due to land use change and pollution (Balen 1997; Yuda 2008). At present, Java Sparrow is protected by the Government of the Republic of Indonesia (P.106 / MENLHK / SETJEN / KUM.1 / 12/2018).

Past surveys in 1980s - 1990s, by Ash et al and van Balen, recorded the Java Sparrow at numerous sites within Bali Barat National Park (Balen 1997). However, more recent studies in 1998 by Surata (2000) and Muchtar & Nurwatha (2001), that reported the occurrence of the Java sparrow respectively at eight sites and ten other sites, were not included the Bali Barat National Park. Other survey conducted by M. Saifudin & Fathurohman in 2010 which provided other seven locality records (Yuda 2015) was also not reported the occurrence of Java Sparrow in Bali Barat National Park. Later, BirdLife International (2021) reported Java Sparrow population in Bali Barat National Park still present based on 2004 record.

To assess the extension of Java Sparrow in the Park, we conducted this survey by using the opportunistic sampling method (De Barba et al. 2010; Neyens et al. 2019). Observers were on a car, drove slowly between Lampu Merah and Prapat Agung temple on a gravel road. The car speed was approximately 20 km $h^{-1}$. When the bird was located, the car was stopped, and the observation was made. Observation was conducted using binocular Nikon Prostaff 8x42 6.3°. Photos were taken by using Nikon B700 60x optical zoom 4.3 - 258 mm (IMSW), Nikon D7100 ED AF-S Nikkor 70-300mm 1:4.5-5.6 G (GOW), and Canon 60D sigma 70-300 mm (EC), either from inside the car or outside by the car. The weather was recorded by using Accuweather application Version 14.9(3). The altitude was recorded by using compass smartphone application. Identification of plants, in where the Java Sparrow observed, referred to Steenis (2008).

During four months (June-September 2021) survey on Bali Starling population at Bali Barat National Park, we spotted the Java Sparrow at the first time on June 10$^{th}$, 2021 at the Prapat Agung Peninsula (Figure 1). There were four sightings of Java Sparrow occurred in June 10$^{th}$, 2021. The first to the third sightings were occurred along the drive on the gravel road between Lampu Merah and Prapat Agung temple, whereas the fourth was when the

authors conducted observation on Bali Starling population. The weather (12.00 – 16.00 Central Indonesia Time) recorded was partly sunny to cloudy, the air temperature was between 29 – 34°C, real feel temperature was 35-40° C, real feel shade temperature was 32-38°C, wind speed was 13-20 km $h^{-1}$, humidity was 58-66%, and cloud cover was 50 – 67%.


**Figure 1.** Map of Prapat Agung Peninsula at the Bali Barat National Park in where the Java Sparrow *Lonchura oryzivora* sighting was occurred. Source of maps were from Google Earth Pro 2021.

The first flock was sighted at 13.05 Central Indonesia Time (CIT), in a transition area of monsoon forest and savannah, located at 10 m asl. Seven birds were seen flying from coastal area direction, then perched on upper branches of a pilang *Acacia leucophloea* tree. They consisted of two adults and five juvenile individuals (Figure 2). The tree height was approximately 8 m.

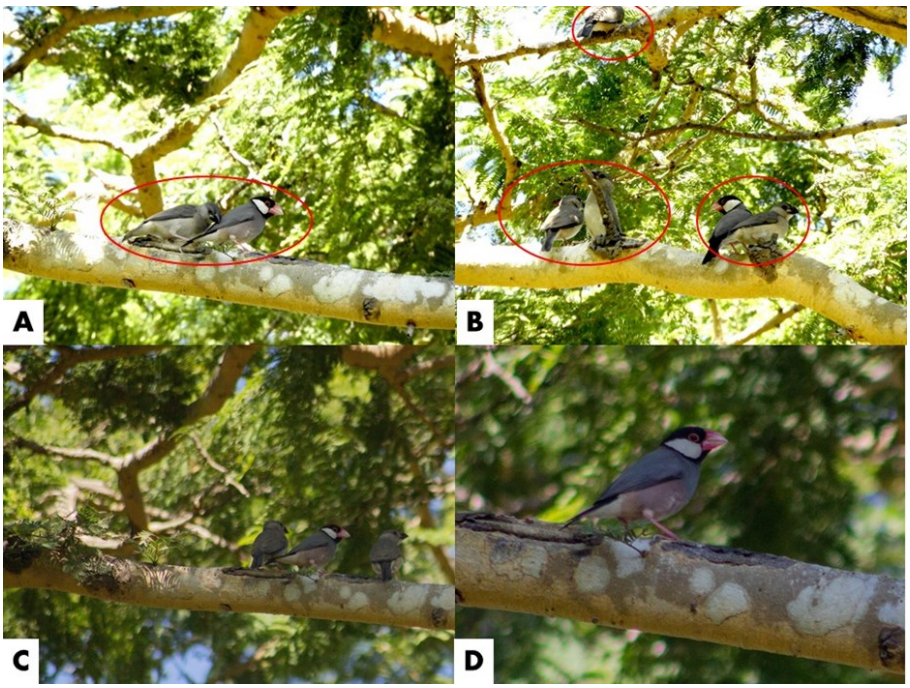


**Figure 2.** The first flock of Java sparrow *Lonchura oryzivora* sighted at Prapat Agung Peninsula, Bali Barat National Park in 10th June, 2021. Two individuals (A) were

juvenile and adult, whereas one adult and four juveniles were on picture B. One individual was having its head out of frame. Pictures C and D were the same flock of Java Sparrow taken from different angle. Photos were taken by IMSW (A,B) and EC (C,D).

The second flock was sighted at 13.15 CIT, also in a transition area of monsoon forest and savannah, located at 10 m asl. Eleven individuals were sighted, consisted of six adults and five juvenile individuals. They were initially perching on branches of pilang *Acacia leucophloea* tree, unfortunately, they were disturbed by our arrival and moved away, by several short flights among trees. The third flock was sighted at 14.13 CIT, in savannah located at 10 m asl. The birds were seen hopped out from sedges vegetation (Figure 3), *Carex* sp. (ordo Poales; family Cyperaceae) toward the gravel road. They were five individuals, consisted of four adults and one juvenile.

**Figure 3.** The sedges vegetation *Carex* sp. from where the third sighting of Java Sparrow *Lonchura oryzivora* was occurred at Prapat Agung Peninsula, Bali Barat National Park. Photo was taken by IMSW.

The fourth sighting was occurred at 14.45 CIT. Five individuals Java Sparrow were seen, consisted of one adult and four juvenile individuals. They perched on top branches of a pilang *Acacia leucophloea* tree. The tree was approximately 10 m in height. Only juvenile birds were clearly captured in photo (Figure 4).

In total we recorded 28 individuals of Java Sparrow, consisted of 13 adults and 15 juvenile individuals, in June 10$^{th}$ 2021 at Prapat Agung Peninsula, Bali Barat National Park. All those birds observed were moving from coastward to inland direction, and our car was moving constantly forward. The distance between the spot of sightings were ranging from 600 m to 900 m. We, therefore, ascertain that those birds were counted once only. No

more sightings, instead of in June 10th 2021, was occurred during our four months (from June to September 2021) observation at Prapat Agung Peninsula, Bali Barat National Park. However, one big flock, approximately 20 individuals, was observed (by MU) in mangrove forest, in muddy patches and in cave area above the Brumbun Bay- Prapat Agung Peninsula in April 2020. This nomadic species might spend their time in Prapat Agung Peninsula from April to June. There were not any current (from 2010 onwards) scholar articles on the existence of Java Sparrow in Bali (see also Yuda 2018). A survey by Rosyadi et al. (2019) in Gunung Sewu Geopark, Yogyakarta observed several flocks with number ranging from three to fifty individuals Java Sparrow per flock.


**Figure 4.** The fourth sighting of Java Sparrow *Lonchura oryzivora* at Prapat Agung Peninsula, Bali Barat National Park. Photos were taken by IMSW (A) and GOW (B).

Their occurrence could be related to their foraging behaviour. This species was found in the transition area of monsoon forest and savannah at Prapat Agung Peninsula. During dry season, savannah might provide plenty of seeds, i.e., from Poales vegetation such as *Carex* sp. for them to forage in. A study by Marone et al. (2008) in diet and seed selection patterns of seed-eating birds in desert revealed that 83% of seeds in bird stomachs were grass seeds (ordo Poales). Meanwhile, they also could use trees in monsoon forest for perching, resting, or seeking protection from the potential predator or from the harsh weather. The real feel air temperature recorded from Accuweather application on that day was 35-40°C, whereas the real feel shade temperature was 32-38°C. When endothermic animals, such as birds, were exposed to high environmental temperatures, they can adjust their behaviour (e.g., reducing activity) or their physiology (e.g., increasing the rate of evaporative water loss) in order to maintain their body temperature within tolerant

limits (Du Plessis et al. 2012). For passerines, their thermoneutral zone was from 25 to 35°C (Yuni & Rose 2005). The presence of juvenile individuals, along with the adult in each flock sighted, indicated that Bali Barat National Park might also provide sources required for their breeding.

The finding of this survey was field evidence that the Java Sparrow was still present in Bali Barat National Park. A total of 28 individuals, consisted of 13 adult and 15 juvenile individuals, was observed in a transition area of monsoon forest and savannah in June 2021. The biggest flock sighted was eleven individuals. More systematic populations and further studies, i.e., ecological and biosystematics, are suggested for the conservation of the endangered and endemic Java Sparrow.

## AUTHORS CONTRIBUTION

LPEKY, IMSW, and PY designed the research, collected and analyzed the data, and wrote the manuscript. MU, GOW, and EC collected and analyzed the data. LPEKY supervised all the process.

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## CONFLICT OF INTEREST

LPEKY, IMSW, MU, GOW, EC, and PY declare that they have no conflicts of interest.

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Short Communication

# Effectivity of *Spodoptera littoralis* Nucleopolyhedrovirus (*Spli*MNPV) and Natural Additives Mixtures against *Spodoptera litura* Fab. (Lepidoptera: Noctuidae) on Cabbage Plants

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**ABSTRACT**
Armyworm (*Spodoptera litura* Fab.) is one of the agricultural pests that can cause huge losses especially for Indonesian farmers because it is damaging various crops, especially cabbage (*Brassica oleifera* L.). *Spodoptera littoralis* nucleopolyhedrovirus (*Spli*MNPV) is one of the biological agent which is effective for the management of the *Spodoptera litura*. However, because of UV radiation it easily degraded when applied in the fields. This study was aimed to determine the effectivity of several indigenous plants for UV protectant of *Spli*MNPV for controlling armyworm at greenhouse scale. Extracts of 2% (w/v) of turmeric rhizome, red betel leaf, moringa leaf, and clove flower, were formulated with *Spli*MNPV and sprayed evenly onto two-month- old cabbages. The experiment used five replicates with six periods of sunlight exposures (0, 1, 3, 5, 7, and 15 days). A commercial product of deltamethrin was used as a comparison. The sprayed leaves were then used as a bioassay by using 25 individuals of one day old 1st larval instar by five replicates. The results showed that the turmeric additive was the most effective as a UV protectant and effectively prolonged the half-life of *Spli*MNPV to 4.12 days, while for clove, moringa leaf, and red betel was 2.48, 2.15, and 2.28 days, respectively.



Pest attacks in agricultural country such as Indonesia can cause huge losses, one of which is the armyworm (*Spodoptera litura* Fab.). Armyworms belong to the Noctuidae family and the order Lepidoptera. The armyworm is a polyphagous insect that eats many plant species. Armyworms chew large areas of the leaf and can defoliate a crop. In such cases, the larvae migrate in large groups to another field in search of food (Reddy 2015). Several plant species in tropical regions that are heavily damaged by armyworm include *Colocasia esculenta*, cotton plants, peanuts, hemp, corn, rice, soybeans, tea, tobacco, vegetables (eggplant, *Brassica*, *Capsicum*, pumpkin, *Phaseolus*, potato, *Vigna*, etc.) (Garad et al. 1984). This pest is a major problem in various areas of agricultural countries.

Pest management mostly relies on using chemical insecticides. However, there are many negative impacts caused by these chemical insecticides, both to organisms and to the environment. The negative effects of pesticides are generally divided into short-term exposure poisoning (eg. rash, headache, blurred vision, cramps, numbness, and death) and long-term exposure (cancer, reproductive disorders, immune system disorders and neurological damage). Potential damage caused by chemical pesticides will be more significant and visible in fetuses, infants, and children whose organs are still in the developing phase, than in adults (Listorti & Doumani 2001).

Due to the many negative impacts caused by chemical insecticides, an effective and environmentally friendly solution is needed to control pests. One of the most widely used bioinsecticides is a virus from the Baculovirus family. Baculovirus is widely used as a bioinsecticide because it is specific for certain pests or organisms, so that it does not have a negative impact on natural enemies or other non-target insect populations (Armenta et al. 2003). The type of baculovirus used in this study belongs to the nucleopolyhedrovirus (NPV) genus, namely *Spodoptera littoralis* nucleopolyhedrovirus (*Spli*MNPV). Baculovirus that has been swallowed by the larvae will dissolve in the intestine, then will release the virions which will stick and become one with the cells in the midgut. The nucleocapsid goes to the nucleus for replication and transcription. Furthermore, the virus will spread to the ovaries, body fat, and most endothelial cells through the tracheal system (Vialard et al. 1995). Baculovirus is capable of infecting insect cells, causing damage to the peritrophic membrane of mid gut (Lehane 1997; Engelhard & Volkman 1995).

Besides the many advantages of baculovirus as a bioinsecticide, it has one disadvantage which is easily degraded in nature due to exposure to UV light (McPartland et al. 2000). Exposure to UV-B rays with wavelengths above 280 nm can cause inactivation of baculovirus bioinsecticides. Therefore, inhibition on the inactivation of baculovirus by UV-B rays can be done by providing additives as UV protectants. Additives that have the potential as UV protectants during in vitro tests (Sukirno et al. 2019), namely moringa leaf (*Moringa oleifera* Lam.), turmeric rhizome (*Curcuma longa* L.), cloves (*Syzygium aromaticum* L.) and red betel leaf (*Piper crocatum* Ruiz & Pav.), were tested under sunlight exposures. All additives used in this study generally contain flavonoids. Flavonoids can absorb UV radiation andact as a sunscreen. In addition, there is a study that shows exposure to UV radiation can induce higher levels of flavonoids in plants (Sisa et al. 2010). After absorbing photons from UV irradiation at a certain wavelength, flavonoid molecules have different energies from the ground state through excitation. Flavonoids also have potential as sunscreens because they have chromophore groups (conjugated single double bonds) that can absorb UV-A and UV-B rays. Flavonoids are strong antioxidants as well as metal ion binders which are thought to be able to prevent the harmful effects of UV rays (Svobodová et al. 2003).

This research began with armyworm collection from cabbage plantations in Kopeng, Magelang, Central Java. The collection was done by direct collection of infested cabbage leaves, then kept in plastic jars. Armyworms were reared in the Entomology Laboratory of Faculty of Biology UGM using white bean-artificial diet (Sutanto et al. 2017; Sukirno et al. 2018). The rearing procedure was following Sukirno et al. (2018). One day old 1st larval instar of F4 were used for bioassay.

Each additive that has been mashed and frozen, weighed 100g and then made an extract at a concentration of 10% (w/v) as much as 100 mL. Furthermore, 2% extract of each additive was used to make a solution of bioinsecticide at a concentration of LC95 (8 x 106 PIB/ml) (Sukirno et al. 2019). A total 5 mL of Littovir® was suspended in 495 mL of 2% extract to make 500 ml suspension for each treatment. Deltametrin at recommended concentration (1ml/l) was used as a positive control.

This research was conducted through 2 stages; the first stage is spraying the liquid formulation of the *Spodoptera littoralis nucleopolyhedrovirus* (Littovir®), natural additives and also adhesive liquid (sticker) on cabbage leaves (*Brassica oleraceae*), while the second stage is testing on armyworm larvae by giving the collected leaves which are then placed in agar medium. The first stage was carried out with 5 treatments for the 1st instar larva of *S. litura*, namely the formulation of a bioinsecticide with four additives and Deltamethrin (Decis®) (Bayer, Indonesia) as a positive control. The formulation of bioinsecticides and additives, as well as positive controls were then sprayed onto the leaves of the cabbage plant (*Brassica oleraceae*) and the leaves would be picked according to a predetermined period. The time in the first stage is called the exposure period, namely 0, 1, 3, 5, 7, 10, and 15 days after treatment (bioinsecticide spraying). Then in the second stage, the collected cabbage leaves along with caterpillar larvae will be placed on the agar medium which will then be observed for 7 days (duration of treatment).

Table 1 showed that there were significant differences both with the addition of additives to Littovir® and positive control treatments. The period of exposure to bioinsecticides and additives at 0-5 days is effective for causing death (mortality) in caterpillars with rates ranging from 90-40%.

**Table 1**. Mortality of *Spodoptera litura* larvae to the *Spodoptera littoralis nucleopolyhedrovirus* (Littovir®) after addition of natural additives for a fifteen day exposure periods.

| **Exposure Time (d)** | **Mortality (%)** | | | | |
|---|---|---|---|---|---|
| | Turmeric | Clove | Moringa | Red Betel | Deltamethrin |
| 0 | 76.27 ± 4.26c | 90.14 ± 5.67c | 86.10 ± 4.55c | 69.70 ± 8.50b | 78.33 ± 4.64c |
| 1 | 86.26 ± 6.02c | 27.71 ± 8.92a | 34.72 ± 3.14b | 51.59 ± 4.76b | 25.96 ± 6.33ab |
| 3 | 35.42 ± 5.58b | 61.77 ± 4.05b | 35.97 ± 9.60b | 47.42 ± 4.14b | 48.08 ± 9.32b |
| 5 | 45.06 ± 9.01b | 35.28 ± 8.35ab | 34.91 ± 8.70b | 46.07 ± 10.16b | 98.89 ± 1.11c |
| 7 | 23.87 ± 3.66ab | 13.85 ± 7.07a | 32.90 ± 4.39ab | 8.39 ± 3.24a | 47.61 ± 7.65b |
| 10 | 23.18 ± 1.87ab | 11.14 ± 5.40a | 15.90 ± 3.55ab | 11.82 ± 4.33a | 34.40 ± 2.11ab |
| 15 | 7.64 ± 1.64a | 14.57 ± 4.77a | 7.69 ± 1.62a | 8.95 ± 0.63a | 9.76 ± 3.58a |

*Note: Numbers followed by the same letter in the same column showed significant difference at α: 0.05 of Tukey' HSD test.

After passing the 5th day of exposure period, mortality began to experience a drastic decline, ranging from <30%. The decrease in the effectiveness of this bioinsecticide is because Littovir® has an effective time range of about 2-4 days after treatment.

This is in accordance with research conducted by by Jha et al. (2015) in which they succeed to lyophilize adenovirus and retain its infectivity over a period of 6 months when stored at room temperature and 4°C, also there is no significant difference in the infectivity or $TCID_{50}$ titer was observed in the lyophilized virus as compared to the stock virus. Occlusion body (OB) is the infective part of Baculovirus and is quite stable under various conditions. Generally BV (Budded Virus) is less stable than OB, but can be stored for years in tissue culture media at a standard temperature of 4°C and will be even more stable at -85°C. The most detrimental factor in BV storage is light. Therefore, care needs to be taken when using light-tight boxes or when wrapping storage containers with aluminum foil (Lynn & Harrison 2016).

In addition to Littovir® treatment with four natural additives, the treatment carried out in this study was a positive control. Positive control using the chemical insecticide Decis®. The control treatment was applied to cabbage (*Brassica oleracea* L.) in the same way as the bioinsecticide treatment with additives. In the positive control, larval mortality showed a higher value than the negative control because the positive control treatment contained the chemical insecticide Decis®. The chemical insecticide Decis® or also known as Deltamethrin is included in the pyrethroid insecticide and is an insecticide with a broad scope. Pyrethroid insecticides are synthetic compounds created to mimic pyrethrins isolated from chrysanthemums. The mechanism of the chemical insecticide Decis® in infecting insects is to interfere with the function of nerve cells in sending signals by interfering with the opening and closing of small gates in the cell (NPIC 2012).

Although chemical insecticides, especially Decis®, are considered effective in insect control, Decis® has a negative impact on the environment. The use of Decis® for a long time can cause several natural insect biotypes to become resistant, not only to the chemical insecticide Decis® but also to other group 3A insecticides. Resistance can occur because of the variability in the insect's genes and result in dominance in an insect population. Furthermore, the effectiveness of the chemical insecticide Decis against resistant insects can be significantly reduced (Bayer Crop Science 2021). In addition, the negative impact is that this chemical insecticide has a half-life in the soil of 5.7-209 days, which means it takes a long time to decompose because it has a strong binding capacity to soil particles (NPIC 2012). So it is likely to disrupt the balance of the ecosystem in the soil in the long term.

Figure 1 shows that the Littovir® formulation with turmeric additive has a higher mean percentage mortality value than other natural additives. All additives showed similar effectiveness in the exposure period of day 0 to day 5. Entering the exposure period of day 7 and so on, all additives showed a significant decrease in value. The $R^2$ value of the four additives did not show

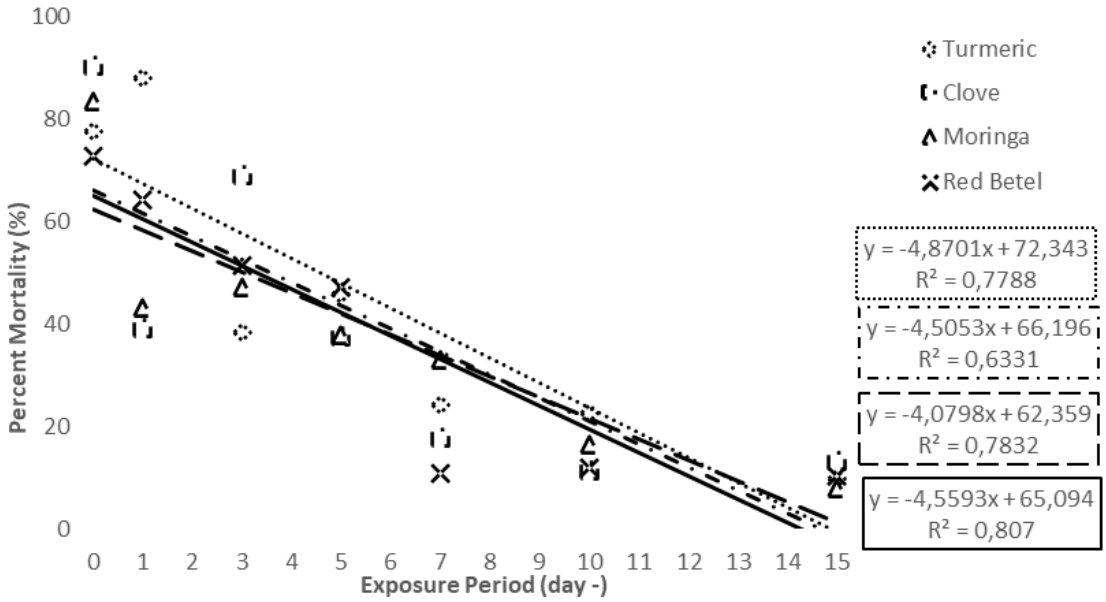


**Figure 1**. Regression correlation of all natural additives treatment on mortality of *Spodoptera litura* larvae during the fifteen day exposure period.

negative results, so it can be interpreted that all variables x (exposure period) have an effect on variable y (percent mortality).

Based on the results obtained, as described by research conducted by Bambal & Mishra (2014), which compared the formulation of turmeric rhizome extract with *Butea monosperma* flower extract, both of which were mixed with ethanol extract to determine the SPF (Sun Protection Factor) value contained. in it after being exposed to UV light with a wavelength ranging from 290-320nm (UV-B). The results of the study showed that turmeric rhizome extract got a higher SPF value than *B. monosperm* flower extract. It can be concluded that turmeric (*Curcuma longa* L.) rhizome extract showed significant activity as a sunscreen (UV-protectant).

Khan et al. (2010) revealed that the phytochemicals in *Curcuma longa* L. contain alkaloids, glycosides, flavonoids, tannins, phenolics, phytosterols, essential oils, and others. The content of flavonoids in turmeric can act as a scavenger of superoxide anion, singlet oxygen, hydroxyl radicals and lipid peroxyl radicals. Many types of flavonoids such as quercetin, luteolin which is a better antioxidant than vitamin C, vitamin E and β-carotene. Therefore, flavonoids belonging to phenolic compounds may be beneficial in preventing UV-induced formation of oxygen free radicals and lipid peroxidation (Svobodová et al. 2003).

Figure 2 shows the half-life data of Littovir® with the four additives (turmeric, clove, moringa, and red betel). The half-life is the amount of time it takes half of a compound to decay. Half-lives are generally determined in a laboratory where temperature, humidity, light, and pH, which are the determining factors, are tightly controlled. Whereas in actual use (in the field), the half-life is only useful as a reference point, which means the half-life is a variable that depends on interacting field factors such as temperature, humidity, microorganisms, light, soil pH and others.

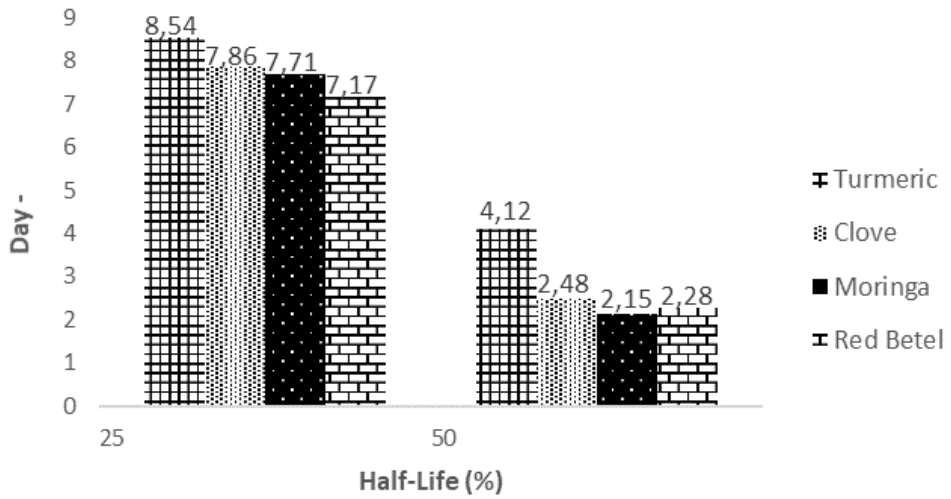


**Figure 2**. The half-life of *Spodoptera littoralis nucleopolyhedrovirus* (Littovir®) after the addition of natural additives to the leaf surface of *Brassica oleracea* L (The data from Prasetya (2021) was re-analysed).

The presence of UV light is the greatest source of energy that drives the decomposition of most pesticides. However, some chemical pesticide formulations have UV blockers, thereby reducing the amount of photodecomposition of active pesticide ingredients (Cress 1990). Meanwhile, bioinsecticides do not have a protection mechanism like chemical insecticides, so natural additives are needed that function as UV protectors. With the addition of natural additives to bioinsecticides, it is expected to increase the half-life so that it is not easily degraded when applied in the field.

The bioinsecticide formulation that has the longest half-life (50%) is with the natural additive turmeric, which is 4.12 days. Meanwhile, the other three natural additives have no significant difference in half-life. The half-life with clove additives is 2.48 days, the half-life with moringa natural additives is 2.15 days, and the half-life with red betel natural additives is 2.28 days. The bioinsecticide formulation which has the longest half-life (25%) is with natural additives turmeric, which is 8.54 days. Meanwhile, the three formulations of bioinsecticides with other natural additives did not show a significant difference in the second half-life, namely; the half-life with clove additives is 7.86 days, the half-life with moringa natural additives is 7.71 days, and the half-life with red betel natural additives is 7.17 days.Littovir® has effectiveness in controlling armyworm (*Spodoptera litura* Fab.), one of the disadvantages of bioinsecticide is that it is easily degraded when applied in the field. This degradation is caused by UV exposure from sunlight. Natural additives are needed that function as UV protectors for bioinsecticides, so that the effectiveness of bioinsecticides can be increased. Based on the research that has been done, it can be concluded that the most effective natural additive as a UV protectant for *Spodoptera littoralis* nucleopolyhedrovirus (*Spli*MNPV) (Littovir®) is turmeric (*Curcuma longa* L.). The first half-life (50%) indicated by the bioinsecticide formulation with natural additives turmeric is 4.12 days, then the second half-life (25%) will be 8.54 days.

## AUTHORS CONTRIBUTION

SS designed the experimentation and managed the research project. BAAP, ASP, and SS run the experimentation, data collection, and perform data analysis. SSu, HP, IS, SuS and RCH supervise and support the experimentation. All the authors read and have the same contributions to the submitted manuscript

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## CONFLICT OF INTEREST

All the authors declare that there is no conflict of interest.

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**Short Communications**

# Rediscovery of *Bombus rufipes* Lepeletier 1835 (Hymenoptera: Apoidea: Bombidae) on Mount Slamet

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**ABSTRACT**
*Bombus rufipes* Lepeletier 1835 (Hymenoptera: Bombidae) is the only species of Bombidae found in Java. Recent information suggests that it occurs in Java on Mounts Salak 1200 m asl., Mt. Halimun, Mt. Pangrango Gede Complexes, Mt.Cermai, (West Java) Mt.Slamet , Mt. Merapi, Mt. Merbabu, Mt Telomoyo (Central Java) and Mt Argopuro (East Java) , at altitudes above 1,500 m asl. We sought to rediscover this species on Mount Slamet by surveying natural forests at altitudes of 1,500–2,500 m asl on the eastern slopes of Mount Slamet, from August to October 2020 and August to October 2021. Descriptive and morphometric data were obtained and analyzed. The survey revealed five colonies nesting on the ground at an average depth of ca. 70 cm; the colonies contained 18–24 individuals and 22–36 cells (brood, honey, and pollen cells). Based on morphometric measurements and the description of Frison (1930), the species was identified as *Bombus rufipes* Lepeletier 1835. Therefore, *Bombus rufipes* still occurs on Mount Slamet. These results provide basic information that should aid further research on this species.



Bumble bees (*Bombus* Latreille) are important pollinators of many wild flowering plants, and are also of agricultural importance (Liang et al. 2020). Their large bodies, relatively long glossae, abundance of plumose setae, and ability to sonicate porous anthers rapidly by contracting their thoracic flight muscles make them effective pollinators (Velthuis & van Doorn 2006). Twenty different bumble bee species have been identified as significant pollinators for a variety of food crops worldwide (Kleijn et al. 2015), and several bumble bee species have been domesticated to provide pollination services to food plants (Strange 2015). Despite the value of bumble bees as pollinators, the populations of several species around the world have decreased over the past few decades.

*Bombus* (Hymenoptera, Apoidea, Bombidae) is very rare in Southeast Asia (Williams 1998; Naeem et al. 2018). Four species occur on Greater Sunda Islands, usually scattered in high mountains (Sakagami 1976). The few of *Bombus* species in Southeast Asia is due to this family being distributed in sub-tropical areas with lower daily temperatures as opposed to tropical areas.

One species occurs on the island of Java, *Bombus rufipes* Lepeletier 1835 (Sakagami 1976). In 1930, Frison discovered this species on Mounts Halimun, Cermai, and Slamet at altitudes of 1,500–2,500 m asl. Michener & Amir (1977) found *B. rufipes* only in West and Central Java; in West Java, it occurred on Mount Salak 1200 m asl., Mt Gede Pangrango complex (Puncak Pass, Telagawarna 1450-1500 m asl., Cibodas 1400-2900 m asl., Mt. Lebak 2400 m asl., Mt Pangrango 2800-300 m asl, Ciberem 1700 m asl., Cisarua 1000 m asl., Mt. Tangkuban Perahu, 1200-1300 m asl, Mt Papandayan 2200 m asl., Mt. Patuha 2450 m asl and Mt Pangalengan 1250 m asl. Recently, Kahono et al. (2009) also found *B. rufipes* in the Mount Cermai area, West Java, while in Central Java it was found on Mount Slamet 1500 m asl., Mt Telomoyo, 1900 m asl., Dieng Plateu, Kawah Sibanteng 2400 m asl., Telaga Warna 2090 m asl., and Mt Merapi, the Dieng Plateau, and Mount Merapi at altitudes of 1,500–2,400 m asl. Kato et al. (1992) found colonies of *Bombus rufipes* in Cibodas (West Java) at an altitude of 1400-1600 m asl, and in Lake Talang (West Sumatra) at an altitude of 1200-1300 m asl. Based on this information, it appears that the findings of Kato et al. (1992) are the most comprehensive information about this species. The time span of discovery and information about *Bombus rufipes* in Java occurs in a very long period of time.

According to Williams et al. (2020), *Bombus rufipes* Lepeletier 1835 has five synonyms: *Bombus rufipes* Lepeletier de Saint-Fargeau, [1835], *Bombus flavipes* Handlirsch, 1888, *Bombus rufipes* var. [subsp.] *obscuripes* Friese, 1914: *Bombus rufipes* var. [subsp.] *intermissus* Friese, 1918, *Bremus rufipes* var. [subsp.] *richardsi* (Frison 1930). *Bombus rufipes* and *B. eximius* are now considered to belong to the subgenus *Melanobombus*. Morphologically, the black wings, black hair of the male face, they usually (but not always) have orange mid and hind tibiae (taxon *obscuripes*), while the individuals from Java usually have predominantly black mid and hind tibiae.

This study sought to rediscover *Bombus rufipes* Lepeletier 1835, on Mount Slamet, and to obtain additional important information on this species. This study was conducted in natural forests on the eastern slopes of Mount Slamet (7°14'44"S, 109°14'42"E), BKPH (Sub District Forest Management), Gunung Slamet Timur KPH (District Forest Management), East Banyumas, at altitudes of 1,500–2,500 m asl. Sampling was conducted from August to October 2020 and 2021 using a random sampling method with line transects at altitudes of 1,500–2,800 m asl. Nesting sites were sought by observing individual bumble bees visiting flowers. When bees were found, a bee nest on the ground was sought. The depth of the nest and numbers of individuals and cells (including brood and honey cells and pollen cells) in the nest were determined. Adult individuals were captured for measurement and morphological observations. From each colony, two adult bees were taken.

During the study from August to October 2020, we found five colonies scattered in primary forest at elevations of 1,500–2,500 m asl. and from August to October 2021 at the same place we found seven colonies. In Southeast Asia, bumble bees tend to nest in primary or protected highland

forests (Koch & General 2019). Nests are found on the ground under higher trees, at depths of ca 68–70 cm. This discovery turned out to be nothing new and specific because in general the *Bombus* nests in the ground, only specific species has found at surface nester. All Megalobombus (including *Bombus rufipes*) nest in the ground (Sakagami 1976). The entrance is disguised with moss or dry leaves and the nest materials include moss and dry leaves (Figure 1 a,b,c and d,e,f). This finding was in line with Kato et al. (1992) some nests have been found at abandoned underground nest of small mamals, the entrance nest was covered with shrubs, but the deep of the nest was 40 cm, shallower than what was found in this study. This finding is in line with Hines et al. (2018) who stated that bumble bee nest in the ground with varying sizes and depths.

The nest of *B. ruficeps* found at Mt. Slamet consisted of 8 to 12 brood cells, 5 to 8 honey cells, and 5 to 6 pollen cells (Figure 2), and differ from the findings of Kato et al. (1992) which showed the number of colonies found to be up to 46 cells. *B. rufipes* has small colonies compared to other *Bombus* species in Southeast Asia (Liang et al. 2020).


**Figure 1.** Nest structure: a) nest hollow, b) nest material with moss, c) worker in the nest d) location, e) entrance, and f) nest composition.


**Figure 2.** Nest composition: a) brood cell, b) honey cell, and c) pollen cells.

Morphological observations showed that the body is black, the wing tips are brown, the wings reflect a metallic blue. forelegs, middle and back parts of the tibia and tarsus are orange (Figure 3a and 4a). Mid legs, femur, tibia and tarsus are lights brown (Figure 3b and 4b) and the hind legs femur, tibia and tarsus are brown (Figure 3c and 4 c). Front of the heads is black (Figure 4d.). However, our weakness is that we are not able to distinguish between working queens and males, so the data is displayed without any information on caste or gender. Sample body length ranged from 19 – 22 mm (table 1).

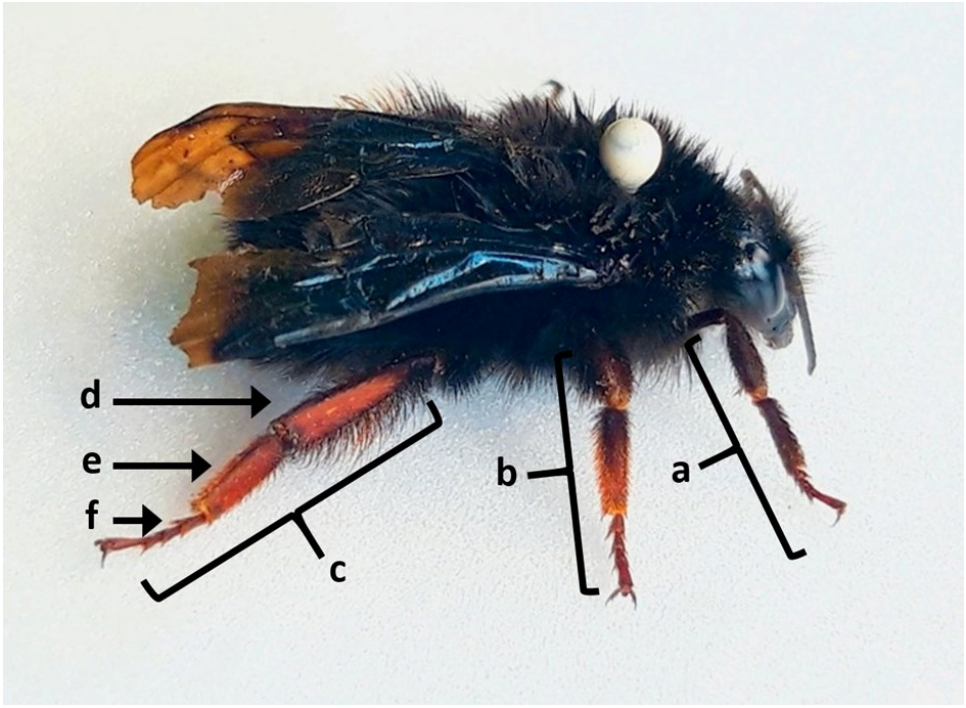


**Figure 3.** a. font legs, b. mid legs, c. hind legs, d femur, e. tibia, f. tarsus.

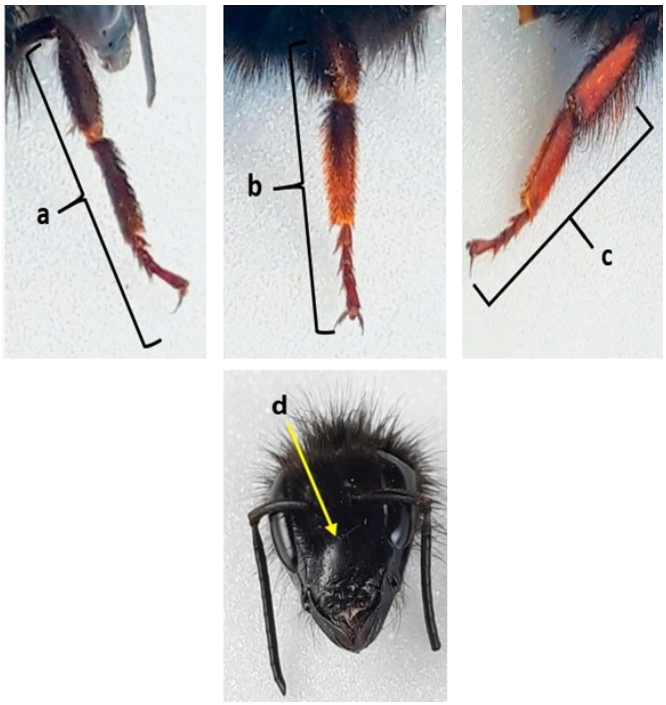


**Figure 4.** a. fore legs, b. mid legs, c. hind leg, and d. front of *Bombus rufipes*.

Based on the observed morphological character seems to be in accordance with the character characterized by Williams et al. (2020). Queens have very large body length 23–30 mm, workers 13–20 mm. They can be distinguished by their combination of wings yellow with the vein orange (cf. *B. rufipes*), the hair and integument of mid and hind tibiae and of all barsitarsi

**Table 1.** Morphometrics character from Bombus rufipes from My Slamet.

| No | Measurements (mm) | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Length of the body | 19 | 20 | 19 | 19 | 20 | 22 | 22 | 22 | 19 | 21 | 20,3 |
| 2 | Width of head | 5,44 | 5,35 | 4,59 | 4,5 | 4,63 | 4,45 | 5,34 | 4,6 | 4,65 | 4,56 | 4,811 |
| 3 | Length of head (Clypeal apex-vertex) | 5,16 | 5,5 | 4,57 | 4,39 | 4,63 | 4,41 | 5,07 | 4,75 | 4,65 | 4,5 | 4,763 |
| 4 | Length of compound eye | 2,88 | 3,43 | 3,05 | 2,88 | 2,89 | 2,83 | 3,38 | 2,87 | 2,78 | 2,67 | 2,966 |
| 5 | Width of compound eye | 1,01 | 1,2 | 1,06 | 1,13 | 1,26 | 0,87 | 1,28 | 1,17 | 1,05 | 1,09 | 1,112 |
| 6 | Alveolorbital distance | 0,79 | 0,99 | 0,64 | 0,73 | 0,87 | 0,82 | 0,6 | 0,64 | 0,82 | 0,51 | 0,741 |
| 7 | Alveolacellar distance | 1,65 | 2,08 | 1,8 | 1,53 | 2,02 | 1,75 | 1,79 | 1,67 | 1,86 | 1,71 | 1,786 |
| 8 | Alveolar Diameter | 1,49 | 1,17 | 1,08 | 1,08 | 0,87 | 1,14 | 1,43 | 1,63 | 1,26 | 1,31 | 1,246 |
| 9 | Length of clypelus | 1,58 | 1,6 | 1,65 | 1,57 | 1,93 | 1,35 | 1,56 | 1,7 | 1,59 | 1,5 | 1,603 |
| 10 | Maximum width of clypelus | 2,24 | 2,49 | 2,38 | 2,32 | 2,85 | 2,22 | 2,55 | 1,99 | 2,25 | 2,44 | 2,373 |
| 11 | Intertentorial distance, width of clypelus | 1,63 | 1,7 | 1,24 | 1,4 | 1,49 | 1,21 | 1,37 | 1,22 | 1,51 | 1,38 | 1,415 |
| 12 | Length of malar space | 1,12 | 0,97 | 0,77 | 0,99 | 0,98 | 0,83 | 0,81 | 0,75 | 0,83 | 0,87 | 0,892 |
| 13 | Length of scape | 2,08 | 2,34 | 2,05 | 1,94 | 1,99 | 1,91 | 1,98 | 1,91 | 2,05 | 2,17 | 2,042 |
| 14 | Length of mandible | 2,47 | 2,34 | 1,27 | 1,37 | 1,45 | 1,8 | 2,29 | 1,62 | 1,4 | 1,31 | 1,732 |
| 15 | Width of mandible | 0,88 | 0,89 | 0,69 | 0,61 | 0,75 | 0,58 | 0,84 | 0,8 | 0,93 | 0,81 | 0,778 |
| 16 | Tegula | 1,23 | 1,04 | 1,1 | 0,93 | 0,95 | 0,91 | 1,14 | 1,02 | 0,81 | 0,96 | 1,009 |
| 17 | WL2 | 3,21 | 0 | 3,39 | 3,77 | 4,28 | 3,32 | 4,51 | 3,88 | 3,8 | 4 | 3,416 |
| 18 | Hamuli | 22 | 0 | 15 | 16 | 25 | 23 | 25 | 24 | 25 | 23 | 19,8 |
| 19 | Length of mesoscutum | 4,24 | 4 | 3,85 | 3,73 | 4,17 | 3,97 | 4,17 | 3,81 | 3,48 | 4,11 | 3,953 |
| 20 | Width of mesoscutum | 4,6 | 4,59 | 4,38 | 4,72 | 4,5 | 4,56 | 4,68 | 4,24 | 4,35 | 4,71 | 4,533 |
| 21 | Width of scutellum | 0,89 | 0,55 | 0,63 | 0,45 | 0,9 | 0,67 | 0,74 | 0,6 | 0,57 | 0,61 | 0,661 |
| 22 | Length of scutellum | 3,83 | 4,42 | 3,81 | 3,27 | 3,96 | 3,24 | 3,61 | 3,4 | 3,24 | 3,21 | 3,599 |
| 23 | Length of tibia III | 4,71 | 5,65 | 5,04 | 5,1 | 5,44 | 4,65 | 5,48 | 5,12 | 4,52 | 4,59 | 5,03 |
| 24 | Width of tibia III | 1,51 | 1,96 | 1,63 | 1,54 | 1,77 | 1,69 | 1,68 | 1,77 | 1,35 | 1,77 | 1,667 |
| 25 | Length of Basitarsus III | 3,07 | 3,63 | 3,37 | 3,21 | 3,44 | 2,81 | 3,66 | 3,17 | 3,23 | 3,39 | 3,298 |
| 26 | Width of Basitarsus III | 1,13 | 1,65 | 1,06 | 1,34 | 1,52 | 1,31 | 1,34 | 1,51 | 1,43 | 1,55 | 1,384 |

orange (cf. some *B. rufipes*). In terms of morphology, the black wings, black hair of the male face, *Bombus* found at Mt Slamet are more in line with individuals from Sumatra, which usually (not always) have the mid and hind tibiae orange (taxon obscuripes), compared to the individuals from Java, which more usually have the mid and hind tibiae predominantly black (taxon rufipes s. str.).

Based on the matching nesting and morphological characteristics, we conclude that the *Bombus* species found was *Bombus rufipes*, and that it still occurs on Mount Slamet.

## AUTHORS CONTRIBUTION

IW designed the study, analyzed the data, and wrote the manuscript. TH, EP, EY and ES collected data and wrote the manuscript.

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## CONFLICT OF INTEREST

The authors report no conflicts of interest regarding this research.

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Research Article

# Restriction Mapping of *MC4R* Gene on Bali Cattle (*Bos sondaicus*) as Genetic Marker for Breeding Program in Compared to *Bos taurus* and *Bos indicus*

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## ABSTRACT
*MC4R* is a gene that has potential effects on growth traits such as body weight and feed intake. The usage of single nucleotide polymorphism (SNPs) in *melanocortin-4 receptor* (*MC4R*) as selection markers could help achieve effectiveness in the breeding program. This study aimed to analyze the restriction mapping based on SNPs in the *MC4R* gene for Bali cattle (*Bos sondaicus*) compared to various breeds of cattle. Partial *MC4R* gene was amplified using a primer (F: 5'-ACC AAT GTC AGT GAG TCC CC- 3' and R: 5'-CTT CAT GTT GGC GCC CTG-3') with a polymerase chain reaction (PCR) method. Genotype and allele frequencies were calculated using Chi-Square test and analyzed with Hardy–Weinberg law. Restriction enzyme was analyzed using Nebcutter V.2 to see the association between SNPs and the recognition site of restriction enzyme. The result showed four SNPs g.554 T>C, g.634 G>T, g.673 C>T, and g.742 G>A were found in the exon region. SNP g.742 G>A was found as a heterozygote genotype and the rest are SNP g.554 T>C, g.634 G>T, and g.673 C>T were found as homozygote genotypes. All SNPs were synonymous which did not change the amino acid translated. Three restriction enzymes were identified as *MmeI*, *TspRI*, and *BsrI* which attach to SNPs g.554 T>C, g.634 G>T, and g.742 G>A respectively. SNPs found notably g. 742 G>A can be used as genetic markers associated with growth traits for further research on Bali cattle.



## INTRODUCTION
Melanocortin receptor is a family of five 7-transmembrane G protein-coupled melanocortin receptors (Yang 2011). It is consists of five subtype genes, those are *MC1R*, *MC2R*, *MC3R*, *MC4R*, and *MC5R* which are located on autosomes. The *MC4R* gene has been studied extensively due to its function in regards to energy control. The association of human *MC4R* to obesity initiated studies on the relationship between fat tissue accumulation in livestock (Switonski et al. 2013).

*MC4R* gene has been proven to have an important role in regulating feed intake, energy homeostasis, obesity, and controlled eating behaviour (Kurniawati et al. 2021) which plays in sympathetic nerve activity, adrenal and thyroid function, as well as mediates the duty of leptin on energy

homeostasis. A study has been conducted to see the affiliation between polymorphism in the *MC4R* gene and some aspects involved in growth traits such as feed intake, feed conversion ratio, average daily gain, and weaning weight. It shows a significant association between SNP found and the weaning weight, weaning body length, digestibility matter (DM), organic matter (OM), and total digestible nutrient (TDN) of Bligon goats (Latifah et al. 2020).

Single Nucleotide Polymorphism (SNP) is a difference in unit of a base in DNA chain found in unalike individuals. Usage of SNP in order to mark the specific base differences in genes has been used widely to determine gene markers concerning improvement in the livestock selection program. SNP's position could be obtained by comparing with reference and DNA sequencing, while SNP emergence can cause changes in proteins translated (Albakri & Hartatik 2021). SNPs exist throughout the entire genome both within coding regions as well as outside of coding sequences. Those within coding regions are categorized into synonymous SNP and nonsynonymous SNP. Nonsynonymous SNP is a coding-region SNP that alters the transcribed codon such that different amino acid is incorporated. Synonymous SNP within coding regions do not lead to a change in the amino acid incorporated at the site of their occurrence (Hunt et al. 2009).

Restriction enzyme usage for genotyping is a cost-effective method and it is a fundamental way of how PCR-RFLP works. RFLP is a technique in which individual SNPs are differentiated using analysis of patterns derived from cleavage of their amplified DNA. Sample with different nucleotides in the same site of SNP would differ in distance between sites of cleavage of a particular restriction endonuclease which makes the length of fragments produced would differ. Restriction endonuclease is an enzyme that cleaves DNA molecules when specific nucleotide sequences are recognized. Sites of recognition are usually four to six base pairs in length (Chuang et al. 2008). Therefore the aim of this study was to identify SNP found and related to the restriction enzymes based on *MC4R* gene sequences which could be utilized for further research related to a growth trait potential gene on Bali cattle.

## MATERIALS AND METHODS

### Sample Collection

Blood samples used in this study were obtained using Vacutainer with EDTA as anticoagulant from 10 Bali cattle (*Bos sondaicus*) which collected from BPTU HPT Denpasar with ID number 3B2013-50, 3B2013-61, 3B2013-77, 3B2013-113, 3B2013-126, 3B2013-150, 3B2013-153, 3B2013-159, 3B14-8, and 3B16-3; 3 Belgian Blue cross cattle (*Bos taurus*) Id number 3B630, 3P648, and 3B635; a Brahman cross cattle (*Bos indicus*) Id number 3116; and 2 Wagyu cross cattle (*Bos taurus*) ID number 3P637 and 3W507 respectively through intravenous injection. Belgian Blue cross, Wagyu cross, and Brahman cross blood samples were obtained from PT. Widodo Makmur Perkasa. All blood samples were collected in August 2019. Blood samples were then isolated

and extracted using SYNC™ DNA Extraction Kit (Geneaid, Taiwan). An *MC4R* gene reference was acquired from the NCBI GenBank nucleotide database, with accession numbers EU366351.1.

### DNA amplification

PCR was performed on a total of 16 samples using target primers *MC4R*.III (F: 5'ACC AAT GTC AGT GAG TCC C 3'; R: 5'CTT CAT GTT GGC GCC CTG 3') with reagent set up in a 25 µL reaction volume containing 2 µL of DNA with total 20 ng, 0.5 µL both forward and reverse primers with the concentration of 10 pmol/µL, 12.5 µL of PCR kit (KAPA BIOSYSTEMS, USA), and 9.5 µL Double Distilled Water (DDW). The result of PCR was 654 bp in length covering coding section (exon) of the *MC4R* gene in cattle. The procedure was conducted as follows, pre-denaturation phase at 94°C for 3 minutes, denaturation at 94 °C for 30 seconds, annealing at 63 °C for 30 seconds, elongation at 72 °C for 30 seconds, and post elongation at 72 °C for 10 minutes. The PCR processing were repeated for 35 cycles.

### DNA sequencing and SNP identification

The 25 µL samples of PCR product and 10 µL of primers forward were sent to LPPT UGM, Yogyakarta, Indonesia for sequencing. Sixteen samples were aligned along with GenBank reference EU366351.1 using Bioedit v.7.2.5 software. Clustal W Multiple alignment was applied to all samples with 1000 bootstrap NJ Tree and Full multiple alignment. The alignment result was then identified as the SNPs emerged. Amino acid changes were identified in sites where SNPs were found.

### Genotype and allele analyzing

The electroforegram for each sample was analyzed using Bioedit v.7.2.5 to recognize if SNPs found were include homozygote or heterozygote form of genotype. Electroforegram on each SNP result was identified by its double peak. Allele frequencies were calculated using Chi-square test and reevaluated with Hardy-Weinberg equilibrium law.

### Restriction enzyme

Restriction mapping was conducted using the NebCutter V2.0 website. Enzymes used were all available from NEB (New England Biolabs). An enzyme detected around or at the cutting site where SNPs occur was analyzed for its specificity, type of enzyme, and site where cutting occurs. The amount of cutting sites displayed was analyzed with 1, 2, and 3 cutters for samples and GenBank reference respectively.

## RESULTS AND DISCUSSION

### Results

A total of sixteen samples were compared along with GenBank reference (Accession no. EU366351.1) for alignment sequence analysis of *MC4R* gene

in the whole coding sequence (exon) section. Coding sequence (CDS) of *MC4R* EU366351.1 starts at 278 bp up to 1276 bp with sizes of 999 bp. PCR result using a primer in this study has sizes of 654 bp which can be found in EU366351.1 sequence, located in 350 bp until 1003 bp within an exon. The result showed 4 identified SNPs which are shown in Figure 1 with clear electroforegram interpretation, those are g.554 T>C, g.634 G>T, g. 673 C>T, and g. 742 G>A. All SNPs were found in Bali cattle (*Bos sondaicus*) samples. Out of 4 SNPs found, one was a heterozygote genotype and the rest were homozygote genotypes. At SNP g. 742 G>A which is a heterozygote, two alleles were identified with GG, GA, AA genotypes. The Chi-square analysis as represented in Table 1 shows that SNP g.554 T>C, g.634 G>T, and g. 673 C>T have $X^2$ count > $X^2$ table result, which indicates that these SNPs deviate from the Hardy-Weinberg law. Conversely, SNP g. 742 G>A has $X^2$ count < $X^2$ table result which indicates that it has fits with the Hardy-Weinberg law. Five genotypes of Bali cattle determined via sequence analysis were submitted to NCBI's GenBank with ID number 2521876 (Genbank access. number OL623708-OL623717).

A study conducted by Albakri and Hartatik (2021) reported that SNPs found in the CDS of the *MC4R* gene were g.316Y>C, g.811G>R, g.1133C>S and, g.1266G>R. A previous study was reported that two SNPs g.1108 C>T and g.1133 C>G in Madura cattle which have significant associate with shoulder height at yearling age (Prihandini et al. 2019). Another research by Maharani et al. (2018) showed SNP g. 1133 C>G found in Kebumen Ongole cattle has affected high birth body length with GG genotype. Meanwhile, research conducted by Fathoni et al. (2020) shows that SNP g. 1133 C>G has no significant association with growth traits in Sumba Ongole cattle. Liu et al. (2009) found in their research that 2 SNPs g.-129

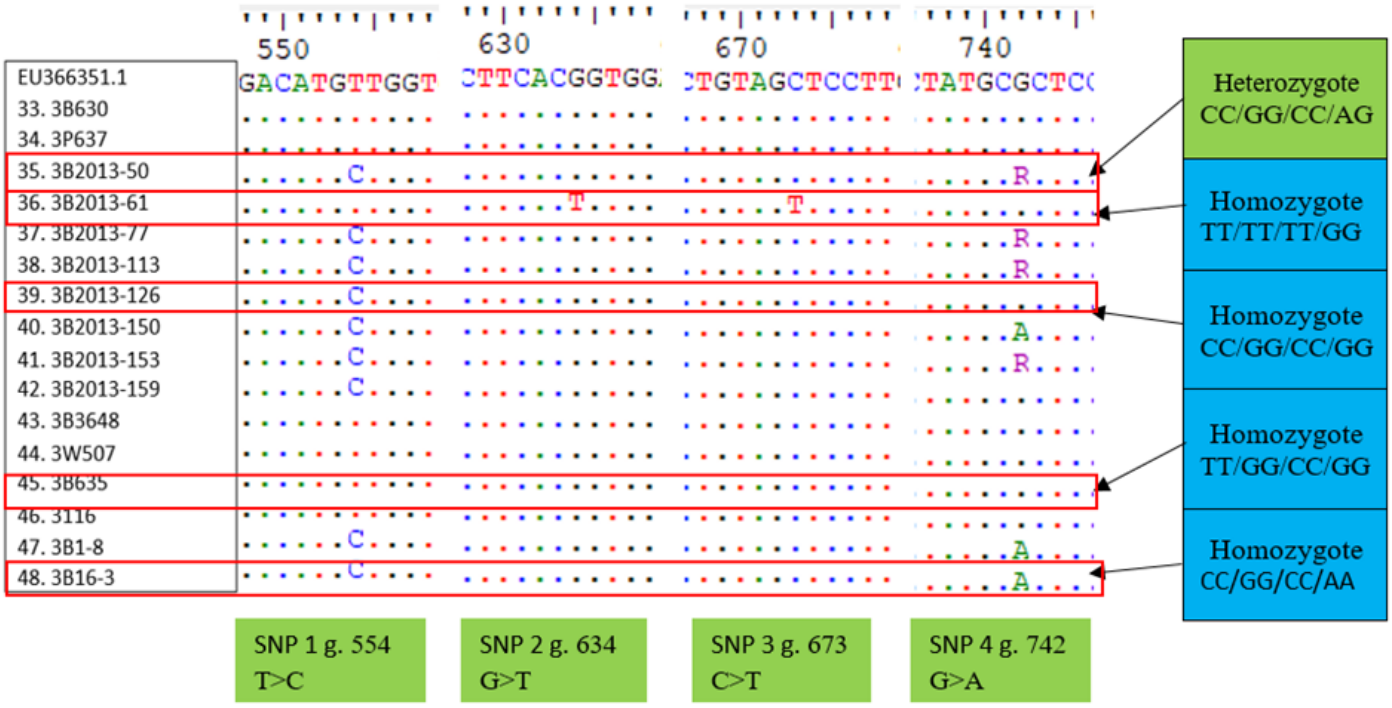


**Figure 1.** Alignment recap on SNPs at g.554 T>C, g.634 G>T, g. 673 C>T, g. 742 G>A.

A>G which has a significant effect on live weight and g.1069 C>G has significantly associated with live weight, carcass weight, backfat thickness, and marbling score in cattle.

**Table 1**. Chi-Square analysis result on Bali cattle (*Bos sondaicus*).

| No | SNP | Data | Genotype Frequency | Allele Frequency | Chi-square test value | |
|---|---|---|---|---|---|---|
| | | | | | $X^2$ Count | $X^2$ Table |
| 1 | 554 T>C (Exon) | TT | 0.1 | T = 0.1 C = 0.9 | 10 | 3.84 |
| | | TC | 0 | | | |
| | | CC | 0.9 | | | |
| 2 | 634 G>T (Exon) | GG | 0.9 | G = 0.9 T = 0.1 | 10 | 3.84 |
| | | GT | 0 | | | |
| | | TT | 0.1 | | | |
| 3 | 673 C>T (Exon) | CC | 0.9 | C = 0.9 T = 0.1 | 10 | 3.84 |
| | | CT | 0 | | | |
| | | TT | 0.1 | | | |
| 4 | 742 G>A (Exon) | GG | 0.3 | G = 0.5 A = 0.5 | 0.4 | 5.99 |
| | | GA | 0.4 | | | |
| | | AA | 0.3 | | | |

SNPs are base nucleotide differences found within DNA sequence which could affect the translation of amino acid. Thymine (T) transform into Uracil (U) in the mRNA which goes down into the process of amino acid translation. The occurrence of SNPs could cause a change in a codon which could change the type of amino acid, this type of mutation is called nonsynonymous SNP. On the other hand, SNPs occur and cause a change in a codon, but do not change the type of amino acid and still have the same translation protein called synonymous SNP. A change of amino acids in CDS could deliver a sufficient impact on the phenotype. As shown in Table 2, all SNPs found in this study show a synonymous SNP type, which shows no change in amino acid translated in CDS. Synonymous SNP may be not related directly to the protein translation that occurs in the DNA, but it might have an impact on DNA and RNA regulatory and replication. Hunt et al. (2009) stated that synonymous SNPs have influences in generating ectopic mRNA splicing, silence the effects of a deleterious mutation, intricateness of infectious disease, and impact mRNA stability and translation. A study conducted by Koren et al. (2012) shows there was a strong association between DNA replication timing and the specific types of point mutation (SNPs) observed such as transitions and transversions mutation. Transition mutations is a change of base nucleotide from pyrimidine (C or U) to another pyrimidine or from purine (A or G) to another purine, whilst transversion mutation is a change of base nucleotide from purine to pyrimidine or vice versa. The result shows g.554 T>C, g.742 G>A, and g.673 C>T belonged to transitions mutation type and g.634 G>T was included in transversions mutation type.

**Table 2.** Mutation type and Amino acid changes.

| SNP | Codon | Amino acid | Mutation |
|---|---|---|---|
| g.554 T>C | UUG | Leucine | Synonymous |
| | CUG | Leucine | |
| g.634 G>T | ACG | Threonine | Synonymous |
| | ACU | Threonine | |
| g.673 C>T | AGC | Serine | Synonymous |
| | AGU | Serine | |
| g.742 G>A | GCG | Alanine | Synonymous |
| | GCA | Alanine | |

Restriction mapping is a method used to analyze if there are restriction enzymes related to SNPs found which could be used as a tool in order to utilized for genetic marking. Characteristic of restriction enzyme is cut at the specific site of the DNA alignment. Results displayed in Table 3 present 3 enzymes that have the cutting site in or around a point where SNPs emerge. *MmeI* cuts in g. 554 T>C, *TspRI* in g. 634 G>T, and *BsrI* in g. 742 G>A. *MmeI* and *TspRI* are 3 cutter types whilst *BsrI* is 1 cutter type. The amount of cut type could be used as consideration when using a restriction enzyme for genetic marking as 1 cutter type enzymes could have more relevant cut than 2 or 3 cutters. Based on Table 3, *TspRI* has the shortest fragment size which is 15 bp and *MmeI* has the longest fragment size which is 415 bp.

Determining restriction enzyme that could be used as a recommended gene marker and be used for genotyping are based on two criteria, first the range of enzyme sites must not too numerous and not too short, at least more than 100 bp. Another criterion for enzyme selection is the price of the enzyme (Kurniawati et al. 2021). *BsrI* has moderate fragment size compared to *MmeI* and *TspRI*, moreover, *BsrI* has 1 cutters site which can be more specific.

## CONCLUSION

A total of three restriction enzymes were found in three different SNPs *MmeI* in g. 554 T>C, *TspRI* in g. 634 G>T, and *BsrI* in g. 742 G>A. The *BsrI* enzyme is recommended as a tool for genotyping for further cattle selection programs, especially on Bali cattle. The SNP g. 742 G>A which is recognized by *BsrI* enzyme can be utilized as marker candidate for growth traits. Hence, further research should be addressed to investigate the association between the SNP g. 742 G>A and growth traits on Bali cattle.

**Table 3.** Restriction mapping related to 3 SNPs in this study.

| SNP | Enzyme | Recognizing site | Site on CDS (bp) | Amount of cut | Site of cut | Fragment Size |
|---|---|---|---|---|---|---|
| g. 554 T>C | *MmeI* | TCCRAC | 277 | 3 cutters | 275, 158, 311 | 86, 117, 36, 415 |
| g. 634 G>T | *TspRI* | CASTG | 357 | 3 cutters | 357, 87, 576 | 15, 270, 219, 150 |
| g. 742 G>A | *BsrI* | ACTGG | 465 | 1 cutters | 466 | 393, 261 |

## AUTHORS CONTRIBUTION

T.H. designed the research, collected samples, and supervised all the processes, Y.C.P. analyzed the data and wrote the manuscript.

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## CONFLICT OF INTEREST

We certify that there is no conflict of interest with any organization or third party regarding the material discussed in this research.

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Research Article

# How to link: Plasmid Curing and Lead Tolerance Ability of *Pediococcus pentosaceus*

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## ABSTRACT
*Pediococcus pentosaceus* has a high level of resistance to heavy metals, making it one of the biological alternatives for dealing with heavy metal contamination in the environment. The current study sought to identify the genetic factors responsible for this ability by curing the plasmid of these bacteria using various curing agents (Acridine orange and Sodium Dodecyl Sulfate). The findings demonstrate that both curing agents had perfect curing ability. The bacteria were able to tolerate a wide range of Lead concentrations (50-2000 ppm). This capacity was reduced when the plasmid was removed, but it did not disappear, implying additional resistance genes on the chromosomes. The antibiotic susceptibility observations supported the significance of plasmid genes in lead resistance ability, the findings revealed differences in the pattern of antibiotic resistance between wild and cure plasmid bacteria, the wild one had different antibiotic MIC values for Nitrofurantoin and Trimethoprim/Sulfamethoxazole (<=16 and <=10 μg/mL) respectively), on the other hand for the same antibiotics, the MIC results for plasmid-cured bacteria were 64 and 80 g/mL. Based on the findings, we can conclude that plasmid genes play a significant role in *Pediococcus pentosaceus* to resist lead, and there is a strong correlation between antibiotic resistance and lead resistance.



## INTRODUCTION
Heavy metal contamination threatens all living organisms by consuming contaminated food and water. For example, lead (Pb) is one of the most dangerous heavy metals and major environmental pollutants, ranking second and seventh on the list of the most dangerous materials (Jaafar 2020). Bioremediation tactics have recently been employed to eliminate heavy metal contamination in the environment, but the most important ones from these techniques, which rely on bacteria's usage (Jaafar 2020).

Bacteria have used a range of techniques to remove heavy metals, the most well-known of which is the discharge of heavy metals outside the cell. Heavy metal resistance genes have been detected in both plasmids and chromosomes (Marzan et al. 2017). Moreover, according to research, there's a link between antibiotic and heavy metal resistance when they are both present on the same plasmid (Samanta et al. 2012).

Lactic acid bacteria LAB is a category of bacteria that reacts positively to gram stains. They have a rod or coccus shape under the light microscope, and their DNA contains less than 55% mol of G+C (Axelsson 2004). Plasmids are self-replicating genetic materials found in lactic acid bacteria, and their presence has drawn a lot of attention because of the numerous roles they are linked to (Cui et al. 2015). Even though the presence of plasmids in bacteria is not required for their survival at this time, they do provide several benefits, the most notable of which is the ability to withstand various stress conditions, such as antibiotic resistance and heavy metal contamination, which allows the organism that carries it to have a more remarkable ability to survive in the event of competition with other organisms in the same environment (Wegrzyn & Wegrzyn 2002). All these capabilities may be due to the plasmids confer adaptive advantages, improving the growth and behavior of their host cells.

Plasmids can be found in a variety of lactic acid bacteria, including *Bifidobacterium*, *Brevibacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, and *Weissella*. The plasmids in LAB bacteria have different sizes (0.87 kb to more than 250 kb), copy number (from 1 to more than 100 plasmids per cell), and phenotypes conferred to their hosts (Schroeter & Klaenhammer 2009; Mills et al. 2006; Ainsworth et al. 2014; Qin et al. 2012).

*Pediococcus sp*. mainly, *Pediococcus pentosaceus* and *Pediococcus acidilactici* have different types of plasmids, ranging in size from 1.82–190 kb. Some of these plasmids encrypt a different function, such as the utilization of raffinose and sucrose (Gonzalez & Kunka 1986), resistance toward antibiotics (Tankovic et al. 1993), as well as production of bacteriocin and immunity (Cui et al. 2012). It was also discovered that certain strains had multiple plasmids, allowing the bacteria to ferment sugars such as raffinose, melibiose, and sucrose, and produce bacteriocins (Alegre et al. 2005; Teresa Alegre et al. 2009).

So, this study aims to determine the effects of plasmid-removing compounds on *Pediococcus pentosaceus* resistance to various Lead concentrations. The objectives include isolation of naturally occurring bacteria from fish aquaculture, genetic identification of *Pediococcus pentosaceus*, screening of their Pb heavy metal resistance, comparison of resistance after plasmid curing using two curing methods, and selection of an effective curing agent.

## MATERIALS AND METHODS

### Isolation and identification of *P. pentosaceus*

Three fishponds on the Al- Garma campus of Basrah University, Marine Science Center, were used to collect water samples using 500 mL glass sterile bottles. The ponds' coordination's are shown in Table 1; in this project, a serial dilution approach was used with sterile physiological saline and plated on MRS Agar; the plates were incubated at 30 °C for 72 to 96 hours. Then pure colonies were identified using some morphological and also employed biochemical characteristics (H2S formation, Catalase, Nitrate reduction, Ure-

ase, Indole Production) (Bergey 1994), VITEK II (Biomerieux, USA), a bacterial identification tool. The 16S rRNA gene was amplified and compared to NCBI reference strains for further emphasis.

**Table 1.** The Coordination of fish ponds.

| Sampling ponds | Latitude | Longitude |
|---|---|---|
| 1 | 30° 33' 39.91"N | 47°44'28.34"E |
| 2 | 30° 33' 35.37"N | 47°44'25.31"E |
| 3 | 30° 33' 38.47"N | 47°44'30.20"E |

## Amplification of 16S rRNA gene

16S rRNA gene was targeted for PCR amplification. The following primers were used: Forward- 27F: 5'- AGAGTTTGATCCTGGCTCAG - 3' and reverse – 1492R: 5'- GGTTACCTTGTTACGACTT - 3'. Each PCR reaction tube contained 25 µL mixtures of Bioneer master mix, 2 µl of purified DNA (50 ng/µL), 3 µL of forward and reverse primer (62.5 µmol/L) and $H_2O$ to complete the volume to 50 µl. Thermocycler (3Prime, UK) with the following thermal profile; initial denaturation step at 94°C for 3 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min, and ending it with a final extension at 72°C for 10 min. To visualize the amplicons, a 1% agarose gel was run (Miyoshi et al. 2005).

## The sequencing and analysis of DNA

Eighteen µl of PCR product (forward and reverse) were prepared and sent to MACROGEN/Korea for DNA purification and sequencing. The F and R sequences obtained were first processed with SnapGene software. After sequencing, the 16S rRNA sequences results of the bacteria were revised then aligned with the NCBI data bank to compare with the other resemble bacteria.

## Preparation of plasmid cured bacteria

Overnight, *P. pentosaceus* was cultured in nutrient broth. Two tubes (each one contains five mL of nutrient medium) were prepared, one containing 1 mg/mL acridine orange and the other one containing 1 mg/mL sodium dodecyl sulfate (SDS), bacteria were then sub-cultured in the prepared media and incubated at 37°C for four days, and after that 0.5 mL of the growth was cultured on two separate nutrient agar plates, and the colonies that were able to grow were classified as cured bacteria, which succumbed to profile the plasmid to approve the cure of plasmids (GG & OH 2016).

## Plasmid profiling

The plasmids were isolated according to the Qiagen kit's instructions, and the loss of the plasmids (plasmid profiling) was confirmed using gel electrophoresis.

The following were the expected outcomes:

A. There are no bands on the gel, indicating that the plasmids were lost, so the plasmid curing technique was successful.
B. The presence of plasmids and failure of the plasmid curing technique is shown by the formation of bands in the gel.

### Antibiotic susceptibility test

The VITEK II was used to test bacterial resistance to several antibiotics (for both wild and plasmid cured bacteria). The tests are run likewise on cards which have of diminution of antimicrobials to appoint the breaking point, which mentioned to the minimum inhibitory concentration (MIC) of antibiotics, as well as the Advanced Expert System (AES) was used in conjunction with the VITEK II automated antimicrobial susceptibility test system to ascertain the beta-lactamase in tested bacteria.

### Heavy metal tolerance assays

#### Preparation of heavy metals concentrations

To make stock solutions from lead, the exact weight of Pb $(NO_3)_2$ was dissolved in sterile deionized distilled water. Different concentrations of Pb (II) were then prepared using an appropriate serial dilution of these stock solutions (Jaafar 2020).

### The Minimum Inhibitory Concentration of wild and plasmid cured *P. pentosaceus*

The MIC test was usually used to determine the lowest concentration of lead required to suppress *P.pentosaceus* growth. The following is how the test was carried out; on a Nutrient agar plate, 0.1 mL of a pure broth culture of *P. pentosaceus* bacteria grown overnight at 37°C in MRS broth (MRS, Hi medium) was spread aseptically, then, in the center of the plates, 6mm diameter paper discs saturated with various concentrations of lead (25, 50, 100, 250, 500, 1000, 1500, 1800, and 2000 ppm) were placed. The plates (including the control) were then incubated for 24 hours at 37 °C, the diameter of the zone around the disc was calculated (Raghad et al. 2016). This experiment was done in triplicate; acridine orange-cured bacteria, SDS-cured bacteria, and wild-type bacteria.

## RESULTS AND DISCUSSION

### Isolation and identification of *P. pentosaceus*

For further diagnosis, we used an isolate that gave positive gram stain and negative catalase reactions in the current study. The bacteria was initially identified as *P.pentosaceus* based on its morphological, cultural, and biochemical properties (Table 2). The colony characteristics of these bacteria were investigated by handling the colony aseptically and transferring it to the selective medium (MRS) to monitor the isolates' growth patterns. Initially, the colonies were thought to belong to the genus *P. pentosaceus* because they were

creamy white, circular, low convex, and had a complete rim (Table 2).

**Table 2.** *P. pentosaceus* morphological feature

| **Biochemical tests** | |
|---|---|
| H2S formation | - |
| Catalase | - |
| Nitrate reduction | - |
| Urease | - |
| Indole Production | + |
| **Colony** | **Morphology** |
| Arranging | Round |
| Shape of cell | Round |
| Motility | Non motile |
| Color of colony | White Creamy |
| Gram stain | Positive |
| Outward | Mucoid |

## Genetic identification

The agarose gel electrophoresis was carried out for five replicates to check the PCR results of the amplified 16S rRNA gene. The bands of about 1450bp were observed on the gel, as shown in (Figure 1).

The amplified 16S rRNA gene sequencing results were revised and analyzed using the Basic Local Alignment Search Tool (BLAST) to search for a similar sequence in the national center for biotechnology information database. The result was diagnosed as *P. pentosaceus*.

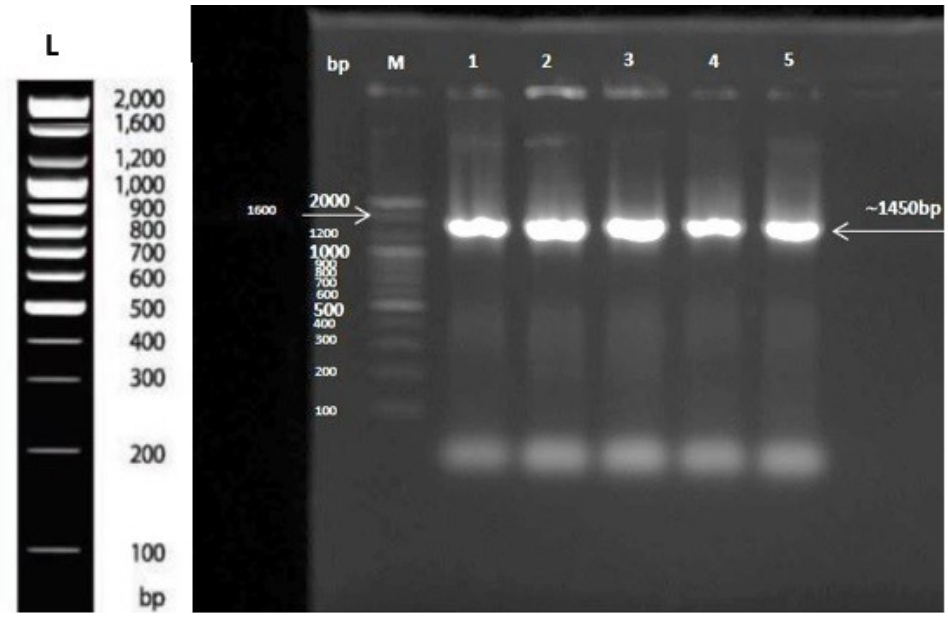


**Figure 1.** The analysis of 1% agarose gel electrophoresis of extracted DNA from pure bacterial colonies amplified by universal 16S rRNA primer: M: 100bp marker; 1, 2, 3, 4, 5: five replicates of isolated bacteria; standard 100bp ladder.

## Plasmid profiling of wild and cured bacteria

Acridine orange and SDS have indicated a positive curing results, and that agree with (Sulochana M.B., Arunasri R 2014; Haque 2017). The current work focuses on the relationship between *P. pentosaceus*' ability to tolerate different concentrations of Lead and the loss of the plasmids and identifying an effective curing agent.

In contrast to the wild type, which exhibited a single band in lane (1), the two materials employed to remove plasmids from P. pentosaceus had a good effect, as no bands formed during the gel electrophoresis process in both lanes (2) and (3) (Figure 2), the single band in lane (1) representing a size of about 10kbp appeared as a result of the plasmid extraction from it, and this proves the success of extraction process. Species *of Pediococci*, mostly *P. pentosaceus* and *P. acidilactici*, have plasmids, extending in size from 1.82–190 kbp encode different functions (Cui et al. 2015).

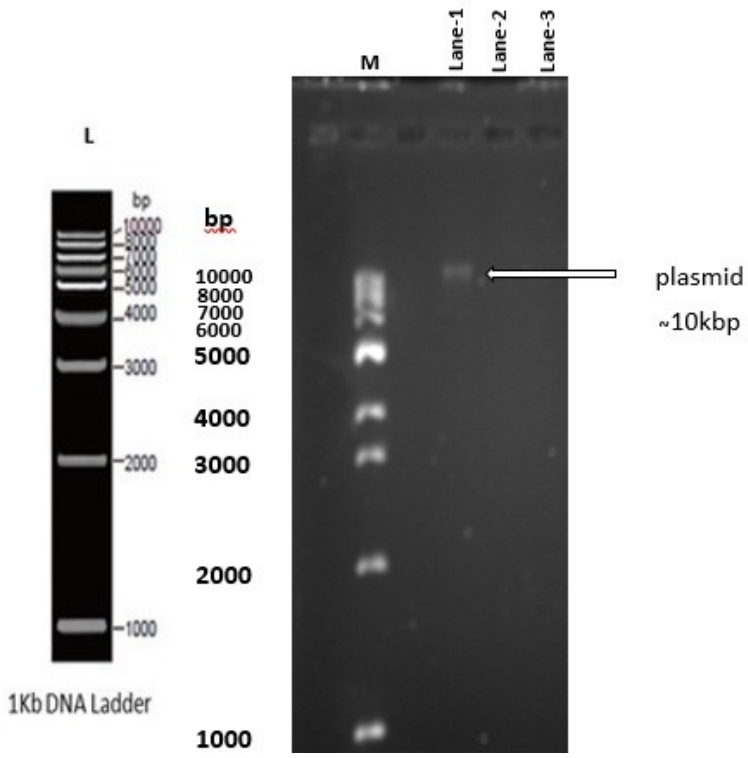


**Figure 2.** Left (standard 1kb DNA ladder); Right (M:1Kb DNA Ladder, lane-1: plasmid profile of wild-type *P. pentosaceus*, lane-2: plasmid profile of cured-type bacteria with 1mg/mL of SDS, lane-2: plasmid profile of cured-type bacteria with 1mg/mL of acridine orange.

## Antibiotic susceptibility in the wild, and plasmid curing

### *P. pentosaceus*

Table 3 demonstrates the susceptibility pattern of *P. pentosaceus* (wild and plasmid cured) to various antibiotics, based on the MIC value, which indicates whether the bacteria are sensitive, mild opponents, or opponents to antimicrobials. The current study's findings show that the wild kind of bacteria tested was responsive to a wide range of antibiotics with varying MIC values. The plasmid curing bacteria by both of acridine orange and SDS

**Table 3.** The results of the antibiotic sensitivity pattern to the various antibiotics using VITEK II.

| | Wild | | Plasmid Cured by SDS | | Plasmid Cured by acridine orange | |
|---|---|---|---|---|---|---|
| Antimicrobial | MIC | interpretations | MIC | interpretations | MIC | interpretations |
| Beta-lactamase | POS | + | NEG | - | NEG | - |
| Levofloxacin | <=0.12 | S | <=0.12 | S | <=0.12 | S |
| Erythromycin | 1 | I | 2 | I | 4 | I |
| Linezolid | 1 | I | 1 | S | 1 | S |
| Teicoplanin | <=0.5 | S | 2 | S | 2 | S |
| Vancomycin | <=0.5 | S | 4 | S | 4 | S |
| Tetracycline | <=1 | S | <=1 | S | <=1 | S |
| Tigecycline | <=0.12 | S | <=0.12 | S | <=0.12 | S |
| Nitrofurantoin | <=16 | S | 64 | I | 64 | I |
| Trimethoprim/ Sulfamethoxazole | <=10 | R | 80 | R | 80 | *R |

*=AES modified, POS: positive, NEG: negative, S: sensitive, I: intermediate, R: resistant.

agent have differed sensitivity manner, they were resistant to some type of antibiotic (Nitrofurantoin, Trimethoprim/Sulfamethoxazole), with MIC value, 64 and 80 µg/mL respectively. The fluctuated result may be due to the elimination of plasmids harboring antibiotic resistance genes.

Beta-lactams are the most widely used group of antimicrobial agents in human bacterial disease treatment; a high rate of mutations characterizes BL-encoding genes. Moreover, these genes are usually found in mobile genetic elements (plasmids, transposons), which facilitates their rapid spread among infectious bacteria, as well as bacteria inhabiting the natural environment (water, soil) (Rozwandowicz et al. 2018).

As shown in Table 3, beta-lactamase was indicated as a positive result in the wild *P. pentosaceus*; while this enzyme wasn't detected in both cured bacteria, and that may be due to the loos of plasmid carrying the beta-lactamase coding genes, these results demonstrate the loss of Plasmids during the curing process.

## MIC of wild and plasmid cured *P. pentosaceus*

The MIC of (1000, 1500, and 1800) ppm was the clearest different inhibition results so that we add it in the (Figure 3) which shows the effect of the Minimum inhibitory concentration of Pb on the *p. pentosaceus*, the wide diameter of the inhibition zone related to the wild bacteria comparing to the narrow zone of the cured one with SDS and acridine orange, so that the removal of plasmids has a detrimental effect, resulting in reduced bacterial resistance to Pb concentrations. On the other hand, the bacterial resistance did not decline permanently, and that may be due to the chromosome-borne genes responsible for metals resistance as well as these harboring on plasmids, and that agree with (Carattoli 2003) that suggested that these genes can exchange between plasmid (s) and the bacterial chromosome.

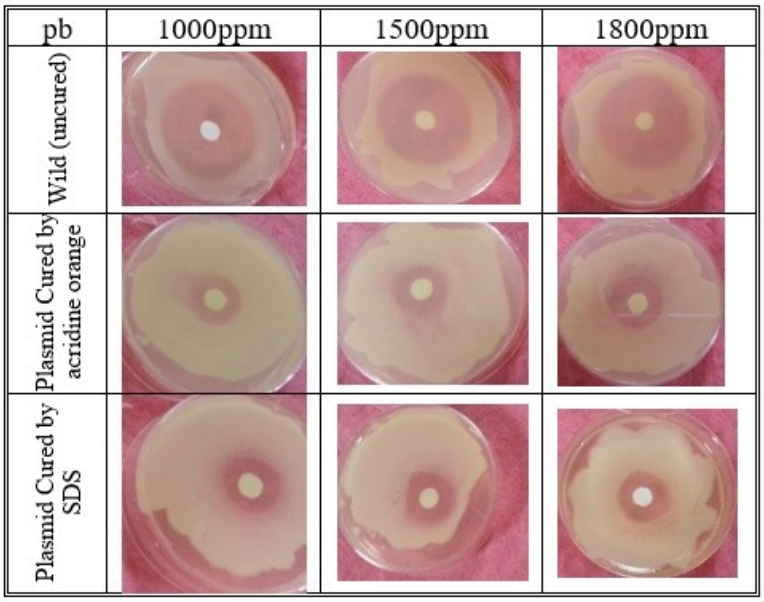


**Figure 3.** Selected MIC effect of (1000, 1500, 1800) ppm on the wild and cured *P. pentosaceus*

Figure 4 illustrates that both materials (acridine orange and SDS) employed in the plasmid curing technique had a good effect; the effect was nearly similar, also the figure demonstrated that the diameter of the inhibition zone increased gradually as a result of an increase in the Pb concentration, and that due to the increment the harsh and toxic effect of the heavy metal on the bacterial metabolic activity (Sobolev & Begonia 2008).

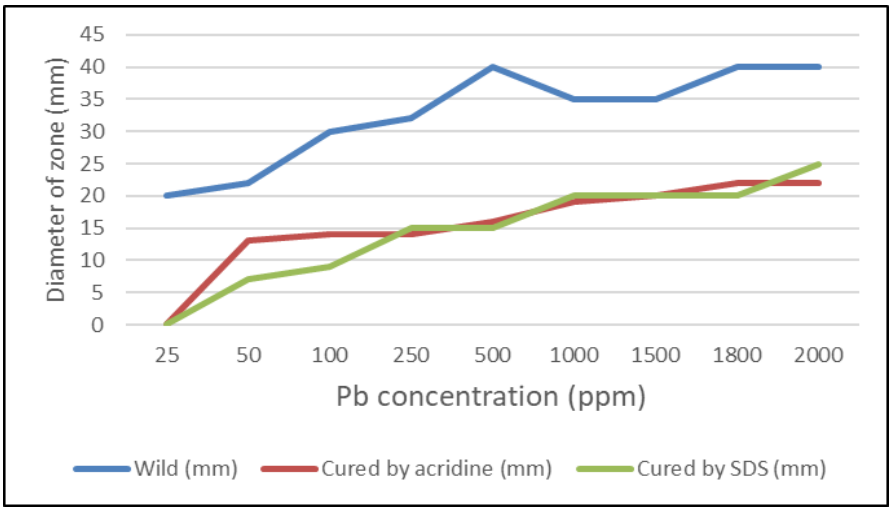


**Figure 4.** Concentrations of Pb and the diameter of inhibition zones in wild and cured *P. pentosaceus*

## CONCLUSION

Based on the MIC value, the fluctuated result may be due to the elimination of plasmids harboring antibiotic resistance genes. Beta-lactams are the most widely used group of antimicrobial agents in human bacterial disease treatment, Beta-lactamase was indicated as a positive result in the wild *P. pentosaceus*; while this enzyme wasn't detected in both cured bacteria, and that

may be due to the loos of plasmid carrying the beta-lactamase coding genes.

The removal of plasmids leads to reduced bacterial resistance to Pb concentrations. However, the bacterial resistance did not decline permanently, and that may be due to the chromosome-borne genes responsible for metals resistance as well as these harboring on plasmids; on the other hand, both acridine orange and SDS have a positive effect.

## AUTHORS CONTRIBUTION

All authors contributed to the data conception, design, analysis, and interpretation, as well as authoring or critically editing the article for key intellectual content.

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## CONFLICT OF INTEREST

On behalf of all authors, the corresponding author confirms that there have been no involvements that could raise questions about bias in the work reported or in the conclusions, implications.

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Research Article

# New Record of Arbuscular Mycorrhizal Fungi (AMF) Association with Kebar Grass (*Biophytum petersianum* Klotzsch.) in the Grassland Area of Kebar, Tambrauw Regency, West Papua, Indonesia

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## ABSTRACT
Arbuscular mycorrhizal fungi (AMF) are an important form of symbiosis between fungi and plants in an ecosystem. One of the medicinal plants used by the people in West Papua is kebar grass (*Biophytum petersianum* Klotzsch.). This study aims to determine the AMF association in the rhizosphere of *B. petersianum* in grasslands. Survey method was used in this study. The presence of AMF was observed by examining root colonization and spore diversity. The results showed that the percentage of AMF colonization in roots was between 46.7–90.0% with an average of 71.66%. Meanwhile, the number of spores found in the plant rhizosphere averaged 119.8 spores per 10 grams of soil sample. There were 18 species of AMF dominated by the genus *Glomus* (7 species), *Acaulospora* (3 species), while the genus *Claroideoglomus, Entrophospora, Gigaspora,* and *Scutellospora* were dominated each with 2 species. This finding is the first record on the presence of AMF on *B. petersianum* in West Papua.



## INTRODUCTION
Indonesia has a great wealth of biological resources (Kartikasari et al. 2012; von Rintelen et al. 2017) and a diverse forest ecosystem that influences the presence of other organisms (Kartikasari et al. 2012), including fungi (Suharno et al. 2014). In Papua, the condition of the forest and its content are even better than in other areas with a fairly large variety of fungi (Suharno et al. 2014). Mycorrhizae are a form of mutual symbiosis (mutual benefit) between fungi and plants.

Arbuscular mycorrhizal fungi (AMF) is one of the mycorrhizal groups classified as large in membership and symbiotic in most groups of vascular plants (Souza 2015; Suharno et al. 2020). This association involves the root system of plants with fungi belonging to the phylum Glomeromycota (Smith & Read 2008; Souza 2015). Furthermore, this symbiosis plays an important role in nutrient cycling and uptake by plant roots (Zhang et al. 2021). Moreo-

ver, mycorrhizae can associate around 80–90% of plant species (agriculture, forestry, and plantations), including natural plants (Giovannetti et al. 2006; Smith & Read 2008; Suharno et al. 2020) such as halophytic, hydrophytic, and xerophytic plants (Souza 2015) and also helps in increasing the efficiency of nutrient uptake (especially phosphorus) on some marginal lands (Souza 2015; Tuheteru et al. 2019; Suharno et al. 2021).

In addition to the development program of medicinal ingredients (including traditional medicine), the role of fungi in increasing plant growth is very important. The potential of indigenous fungi is very useful and has the potential to be developed. Subsequently, the most important thing is to develop the function of fungi and medicinal plants as superior products (Suharno et al. 2018). However, supporting factors are necessary for optimal production, and not just only the physical and chemical factors of the soil, but the most important is also the involvement of microorganisms that supports plant growth. AMF is one of the multifunctional microorganisms in symbiosis with plants.

AMF plays a role in the growth of medicinal plants. In China, several *types* were found in symbiosis with the roots of medicinal plants such as ginseng (*Panax* spp.), *Datura stramonium*, *Atractylodes macrocephaly*, and other types of medicinal plants (Wang & Shi 2008). Furthermore, it is also found to be associated with traditional medicinal plants in India (Kumar et al. 2021). Under the salt stress condition, some AMF are also able to rise proline dan phenol (Duc et al. 2021). AMF plays important role in facilitating secondary metabolite synthesis as a protection from several pathogen (Sun et al. 2021). While in Indonesia, research on AMF in medicinal plant is still limited. Inoculation of AMF on *Centela asiatica* was increased asiaticoside (0.1–0.6%) (Trisilawati et al. 2019). In Papua, the medicinal plant Wati (*Piper methysticum*) is found to be associated with AMF (Suharno et al. 2018).

Kebar grass (*Biophytum petersianum* Klotzsch.) is widely known in Indonesia as a traditional medicinal plant for women's fertility (Unitly & Inara 2011; Sembiring & Darwati 2013). It is also used for the immune system, inflammation, fever, and wound healing (Inngjerdingen et al. 2006; Inngjerdingen et al. 2008; Kayadoe et al. 2012). Besides that, *B. petersianum* is also used for the treatment of malaria (Giovannetti et al. 2010; Kayadoe et al. 2012), hypertension (Mouzou et al. 2009), thrush medicine, an antidote to snake bites, and laxative for children (Unitly & Inara 2011).

The distribution of this herbaceous plant is quite wide in tropical regions such as Africa, Madagascar, and Asia (Sambodo et al. 2018), including Papua, New Guinea. In Indonesia, it is found in several areas such as Java and Papua (Tambrauw and Manokwari) (Sembiring & Darwati 2013). It belongs to the family of Oxalidaceae (Inngjerdingen et al. 2006; Sambodo et al. 2018) and has a synonymous name *B. sensitivum* (L.) DC (Inngjerdingen et al. 2006). Although it is not included in the grass family (Poaceae), its stature looks like grass and is often found among weeds (*Imperata cylindrica*), creates a perception for local people to call it "grass" kebar. In Papua, *B. petersianum* is

commonly found in Kebar District, Tambrauw Regency, especially in grassland areas. The relationship between AMF and plants in absorbing nutrients to improve plant growth is very important. Therefore, it is necessary to study the association of *B. petersianum* with AMF in grassland areas.

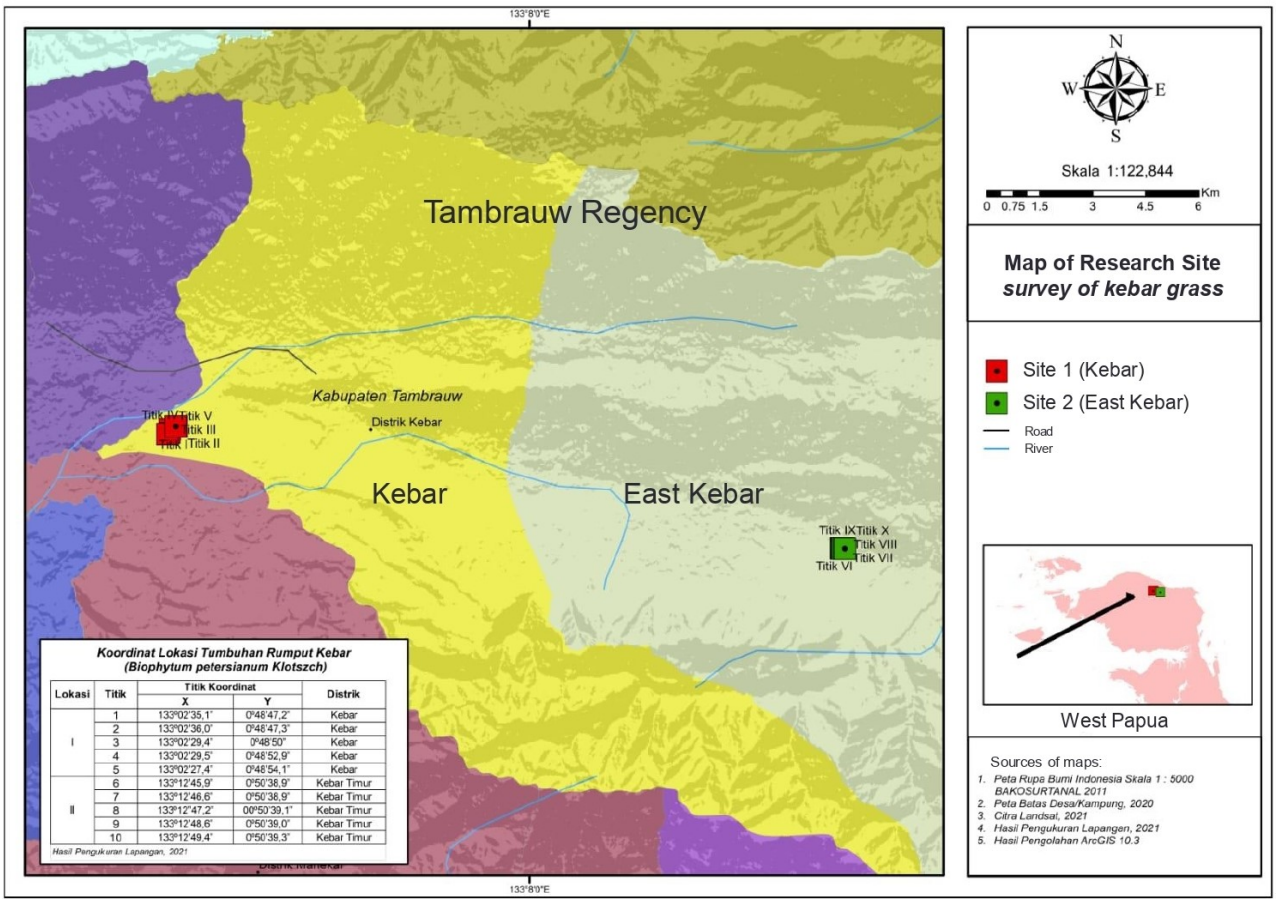
## MATERIALS AND METHODS

### Study Area

This study was conducted in 2 sub-districts, Kebar and East Kebar in Tambrauw Regency, West Papua. The selection of the location was based on information about the existence of kebar grass from the local community. The Tambrauw regency is located in the bird's head area of Papua (Figure 1). Furthermore, the total area is 11,529,179 km$^2$ and consists of 29 sub-districts. Moreover, Kebar and East Kebar are savanna grassland areas in Tambrauw Regency, West Papua.

### Environmental Condition

The environmental condition such as altitude, temperature, and humidity of the study site were measured. Soil properties of the *B. petersianum* rhizosphere such as pH, organic C, N total, C/N ratio, available P, kalium, Ca, Na, Mg, Cation Exchange Capacity (CEC), and soil texture were tested. Soil samples were only taken in three locations, i.e.: Kebar (DK1), Kebar (DK4), and East Kebar (DKT2), because of the similar condition. The analysis of the soil samples was carried out at the Seameo-Biotrop Bogor laboratory.




| Lokasi | Titik | Titik Koordinat X | Titik Koordinat Y | Distrik |
|---|---|---|---|---|
| I | 1 | 133°02'35,1" | 0°48'47,2" | Kebar |
| | 2 | 133°02'36,0" | 0°48'47,3" | Kebar |
| | 3 | 133°02'29,4" | 0°48'50" | Kebar |
| | 4 | 133°02'29,5" | 0°48'52,9" | Kebar |
| | 5 | 133°02'27,4" | 0°48'54,1" | Kebar |
| II | 6 | 133°12'45,9" | 0°50'38,9" | Kebar Timur |
| | 7 | 133°12'46,6" | 0°50'38,9" | Kebar Timur |
| | 8 | 133°12'47,2" | 00°50'39,1" | Kebar Timur |
| | 9 | 133°12'48,6" | 0°50'39,0" | Kebar Timur |
| | 10 | 133°12'49,4" | 0°50'39,3" | Kebar Timur |

**Figure 1.** Sampling location in Tambrauw regency, West Papua.

### Survey of *B. petersianum* Presence

A survey of kebar grass was carried out in 10 locations in the districts of Kebar and East Kebar. These locations were chosen because they are kebar grass producer areas according to the local community. The initial survey showed that *B. petersianum* was not found in the primary and secondary forest areas, while it was abundant in the grasslands. Therefore, the grassland area was the main focus of the observation. Furthermore, five observation sites were located in each district, then 10 plots (1 x 1 $m^2$) were randomly distributed with 20 meters distance between plots to have totally 100 plots. The presence of kebar grass was observed and the number of individuals in each plot was counted.

### Survey of AMF Presence

The survey of AMF existence in the rhizosphere of *B. petersianum* was carried out by observing the AMF spores; the method used was the extraction of spores by wet sieving. Then, 1 kg of soil samples was taken and the AMF was isolated using the wet sieving method with *sucrose centrifugation technique* (Vierheilig et al. 2005). Meanwhile, the soil samples (100 g) was filtered using a stratified sieve with a mesh size of 250, 100, and 30 µm. Furthermore, the solution containing the filtered spores was centrifuged (2,500 rpm, 10 minutes) with the addition of 50% sucrose. The separated spores were washed on filter paper and then observed and counted under a stereo microscope (30x).

### AMF Association in *B. petersianum*

The AMF association with *B. petersianum* was determined by observing the infection and colonization of AMF in the plant root system. The method used in this observation is root staining (Brundrett et al. 1996). Plant roots was taken randomly at two district locations, each at five different locations (Table 1). To determine fungal colonization, the slide method was used (Brundrett et al. 1996; Vierheilig et al. 2005) by observing 30 of 1 cm root slices, while staining was carried out with tryphan blue (Kormanik & Mc.Graw 1984; Vierheilig et al. 2005; Sun & Tang 2012). The status of AMF is known through symbiotic characteristics, such as the presence of intraradical and extraradical hyphae, vesicles, arbuscules, as well as the possible presence of intraradical spores in root tissue.

### AMF Type Diversity

The AMF diversity was identified based on the morphological characteristics of the spores. The morphological identification was based on spore characteristics (spore shape, color, hyphae stalk attachment, wall, and reaction of spore contents with Melzer's solution). Meanwhile, identification of genus to species used several literatures from Schenck and Perez (1990), Brundrett et al. (1996), and Suharno et al. (2020).

### Analysis Data

Observational data are displayed in the form of tables and figures.

## RESULTS AND DISCUSSION

### Environmental conditions

Observations showed that the study site is at an altitude of 572–619 m above sea level (Table 1) which was quite high during the day, with a humidity of 28 to 68%. Moreover, soil characteristics in this area vary, ranging from dusty clay to loamy sand (Table 2).

**Table 1.** Location, coordinate, and environmental condition of the study site in Kebar Sub-district, Tambrauw, West Papua.

| No | Location (code) | Coordinate | Altitude (m asl.) | Temperature (°C) | Humidity (%) |
|---|---|---|---|---|---|
| 1 | East Kebar (DKT2) | S : 00°50'38,9" E : 133°12'45,9" | 619 | 38 | 31 |
| 2 | East Kebar (DKT7) | S : 00°50'38,9" E : 133°12'46,6" | 604 | 36 | 30 |
| 3 | East Kebar (DKT9_1) | S : 00°50'39,1" E : 133°12"47,2" | 603 | 32 | 28 |
| 4 | East Kebar (DKT9_2) | S : 00°50'39,0" E : 133°12'48,6" | 606 | 33 | 29 |
| 5 | East Kebar (DKT10) | S : 00°50'39,3" E : 133°12'49,4" | 604 | 32 | 31 |
| 6 | Kebar (DK1) | S : 00°48'47,2" E : 133°02'35,1" | 574 | 22 | 68 |
| 7 | Kebar (DK3) | S : 00°48'47,3" E : 133°02'36,0" | 575 | 28 | 42 |
| 8 | Kebar (DK4) | S : 00°48'50" E : 133°02'29,4" | 573 | 27 | 46 |
| 9 | Kebar (DK5) | S : 00°48'52,9" E : 133°02'29,5" | 573 | 33 | 38 |
| 10 | Kebar (DK6) | S : 00°48'54,1" E : 133°02'27,4" | 572 | 32 | 32 |

The analysis of the soil sample shows that the soil quality in the Tambrauw area was acidic, with a soil pH value of 4.8 to 6.4 (pH analysis ($H_2O$) classified as acidic. Meanwhile, the organic C content is between 1.56-4.01 % (low–high), with an average of 2.65% including the medium category. The total N content ranged from 0.19-0.49% with an average of 0.31% including the medium category. Then, the C/N ratio between 8.00-9.00 with an average of 8.33 (low) (Table 2). According to Sorensen (1993) and Warren et al. (2017), the high and low C content are associated with the carbon stock value in an area. This result shows that under low acidity soil and low to high organic carbon condition, AMF still could be found with the presence of 18 species of AMF on the *B. petersianum* rhizosphere. AMF could grow at pH 2.7 – 9.2 (Aguilera et al. 2015). *Acaulospora* is one of AMF that could well adapted to the acid soil condition.

The available P content (available $P_2O_5$) ranged from 10.7–16.2 ppm, with an average of 14.0 ppm (high). Meanwhile, the K content ranged from 0.19–0.79 cmol.kg$^{-1}$ with an average of 0.38 cmol.kg$^{-1}$ (low), Ca averaged 2.42 cmol.kg$^{-1}$ (low), and Mg 0.97 cmol.kg$^{-1}$ (low). Subsequently, the cation exchange capacity (CEC) of soil is between 9.55–21.22 cmol.kg$^{-1}$ with an average of 14.55 (low), with a base saturation (BS) level of around 16.82–45.65 %, with an average of 29.79% (low). Its category of soil properties were based on Eviati and Sulaeman (2009). Furthermore, the soil analysis shows the area overgrown with Kebar grass has a loamy sand texture to dusty clay. Carrenho et al. (2007) reported that host plant species and soil texture were influenced to the level of AMF colonization. Host plant species is associated with the compatibility of plant-fungus symbiosis. Some AMF only have association with specific host plant. Colonization of AMF were also influenced by the nutrient content and its availability which related to the soil texture. In some condition, soil texture also influenced the availability of water which would have impact on the spore production in the rhizosphere.

The soil characteristics in several locations in Sub-district of Kebar are quite suitable for agricultural land, which has long been known as a producer of peanuts and corn. Although some investors seem to be developing corn cultivation in grasslands, this land is located in a plain area lower than the topography of the higher plains, hills, and surrounding mountains.

**Table 2.** Soil physicochemical characteristics in the Kebar grass area in West Papua.

| Soil physicochemical characteristics | | Location and code | | | Average |
|---|---|---|---|---|---|
| | | Kebar (DK1) | Kebar (DK4) | East Kebar (DKT9_1) | |
| pH ($H_2O$) | | 6.4 | 5.3 | 4.8 | 5.5 |
| pH ($CaCl_2$) | | 4.9 | 4.8 | 4.1 | 4.6 |
| Organic C | (%) | 2.37 | 1.56 | 4.01 | 2.65 |
| N total | (%) | 0.26 | 0.19 | 0.49 | 0.31 |
| C/N Ratio | | 9.00 | 8.00 | 8.00 | 8.33 |
| $P_2O_5$ available | (ppm) | 15.1 | 10.7 | 16.2 | 14.00 |
| K-dd | (cmol.kg$^{-1}$) | 0.20 | 0.19 | 0.76 | 0.38 |
| Ca-dd | (cmol.kg$^{-1}$) | 4.24 | 1.58 | 1.45 | 2.42 |
| Na-dd | (cmol.kg$^{-1}$) | 0.22 | 0.21 | 0.25 | 0.27 |
| Mg –dd | (cmol.kg$^{-1}$) | 1.21 | 0.59 | 1.11 | 0.97 |
| CEC | (cmol.kg$^{-1}$) | 12.88 | 9.55 | 21.22 | 14.55 |
| Base saturation | (%) | 45.65 | 26.91 | 16.82 | 29.79 |
| *Al-H$_{dd}$KCl 1N:* | | | | | |
| Al-dd | (me.100g$^{-1}$) | 0.00 | 0.00 | 2.56 | 0.85 |
| H-dd | (me.100g$^{-1}$) | 0.10 | 0.35 | 0.65 | 0.37 |
| Soil texture | | | | | |
| Sand | (%) | 89.4 | 84.4 | 2.9 | 58.90 |
| Dust | (%) | 5.3 | 9.4 | 45.4 | 20.03 |
| Clay | (%) | 5.3 | 6.2 | 51.7 | 21.07 |
| | | Loamy sand | Loamy sand | Dusty clay | Sandy loam |

Note: Soil analysis was carried out at Seameo-Biotrop soil laboratory, Bogor.

The land in the form of grassland is a suitable location for livestock food. According to public information, this area was once a center for cattle breeding and development. However, it continues even after the cattle population has decreased. These are provided by the government in form of assistance to the community capital to develop a cattle farming business that often consumes kebar grass. According to Xu et al. (2017), land use change will have impact on the diversity of AMF. AMF diversity was significantly higher in grassland than in forest or arable land. Significant differences in AMF community composition were found among different land use types.

## The population of *B. petersianum*

The results show that the kebar grass distribution is not evenly distributed throughout the Kebar and East Kebar Districts. The average population of *B. petersianum* is 11.2 per m$^2$. However, Kebar grass was not found in site 3 (East Kebar) site 6, dan 7 (Kebar) (Figure 2). Moreover, when the survey was conducted, Kebar grass was not found in primary and secondary forest areas. This is because the habitat and other plant associations do not allow it to grow and to develop. Furthermore, forests with higher plant species can cause land cover, and kebar grass which is less than six inches in height cannot compete for light for growth.

The small kebar grass habitus (< 15 cm) grows in association with other plants in the grassland dominated by plant species from the *Poaceae* family. Meanwhile, the one grassland in Kebar (DK4) is different, but weeds (*Imperata cylindrica*) are widely distributed throughout the savanna area. Kebar grass is known to be most commonly found in association with weeds (*Imperata cylindrica*) in Kebar Sub-district area. Moreover, it is also associated with "road" grass *(Themeda triandra*), melastoma (*Melastoma malabathricum*), *tekian* (*Cyperus* spp.), *Heteropogon* spp., and other types of grass in East Kebar Sub-district.

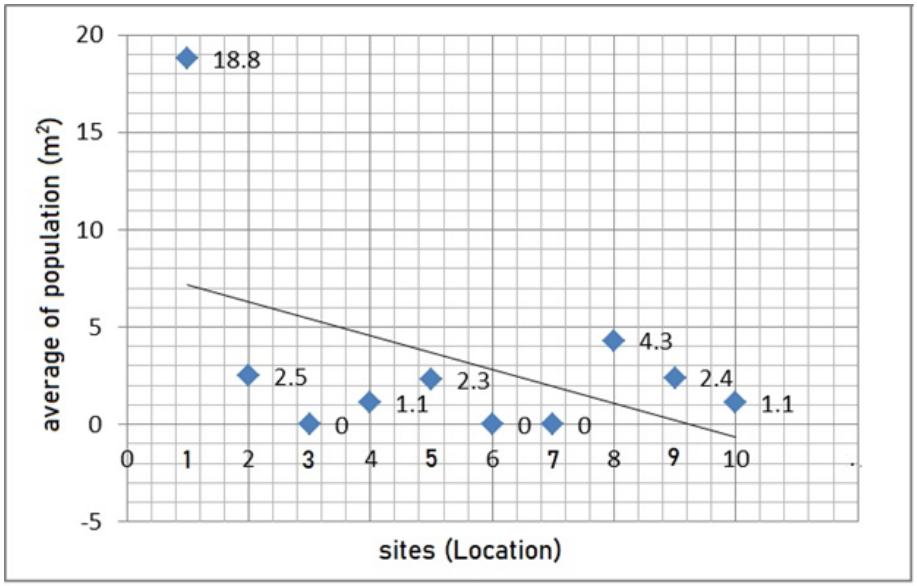



**Figure 2.** Distribution and trend of the kebar grass population in Tambrauw Regency, West Papua. Observation locations 1 – 5 in East Kebar, and 6 – 10 in Kebar.

## Presence and Colonization of AMF

The presence of AMF in this area is known from spores found in the rhizosphere of kebar grass (Table 3). The results show the average number of spores found is 119.8 spores per 10 g (1,198 spores per 100 g) of soil sample. Furthermore, Tao et al. (2004) found an average of 1,530 spores per 100 g in a savanna field in southwest China. The number of spores found depends on the depth of the soil. In general, it is very high to a depth of 20 cm, but it decreases with the depth of the soil and depends on the type and composition of the plant (Cuenca & Lovera 2010).

**Table 3.** The number of spores and the colonization of AMF in the rhizosphere of *B. petersianum* in Tambrauw Regency, West Papua.

| No | Sample code | Location (sub-district) | Number of spores (10 g soil sample) | AMF colonization (%) |
|---|---|---|---|---|
| 1. | DK1 | Kebar | 124.5 | 86.7 |
| 2. | DK3 | Kebar | 160 | 90 |
| 3. | DK4 | Kebar | 100 | 90 |
| 4. | DK5 | Kebar | 113 | 70 |
| 5. | DK6 | Kebar | 132 | 66.7 |
| | Average | | 125.9 | |
| 6. | DKT2 | East Kebar | 116 | 76.7 |
| 7. | DKT7 | East Kebar | 120.5 | 46.7 |
| 8. | DKT9_1 | East Kebar | 108 | 60 |
| 9. | DKT9_2 | East Kebar | 113 | 63.3 |
| 10. | DKT10 | East Kebar | 111 | 66.7 |
| | Average | | 133.7 | |
| | Total average | | 119.8 | |

The results show that there is an AMF colonization in the root system of *B. petersianum* (Figure 3). Meanwhile, the AMF colonization rate is in the range of 46.7–90.0%, with an average of 80.0% (high category) (Table 3). Furthermore, Suharno et al. (2018) found the level of AMF colonization in the medicinal plant wati (*Piper methysticum*) originating from the lowlands of Merauke between 38.46–83.3%. According to Suharno et al. (2020), the percentage of AMF colonization is included in the large category. Moreover, Casazza et al. (2017) and Zhang et al. (2021) suggested that the intensity and diversity of AMF colonization levels varied for each plant species and habitat differences. Several factors influence the content of the soil water (Cuenca & Lovera 2010), pH (Coughlan et al. 2000), temperature, and altitude (Zhang et al. 2021). However, AMF colonization rates are lower at high altitudes in different mountainous environments (Zhang et al. 2021).

The level of AMF colonization is very important in the symbiotic process with plants (Smith & Read 2008; Suharno et al. 2021). The intraradical mycelium will influence the ecosystem processes indirectly through the nutrition and performance of host plants, while the extraradracal mycelium is directly related to the ecosystem function (Barceló et al. 2020). Therefore, the infection and colonization of AMF in the ecosystem are very important.

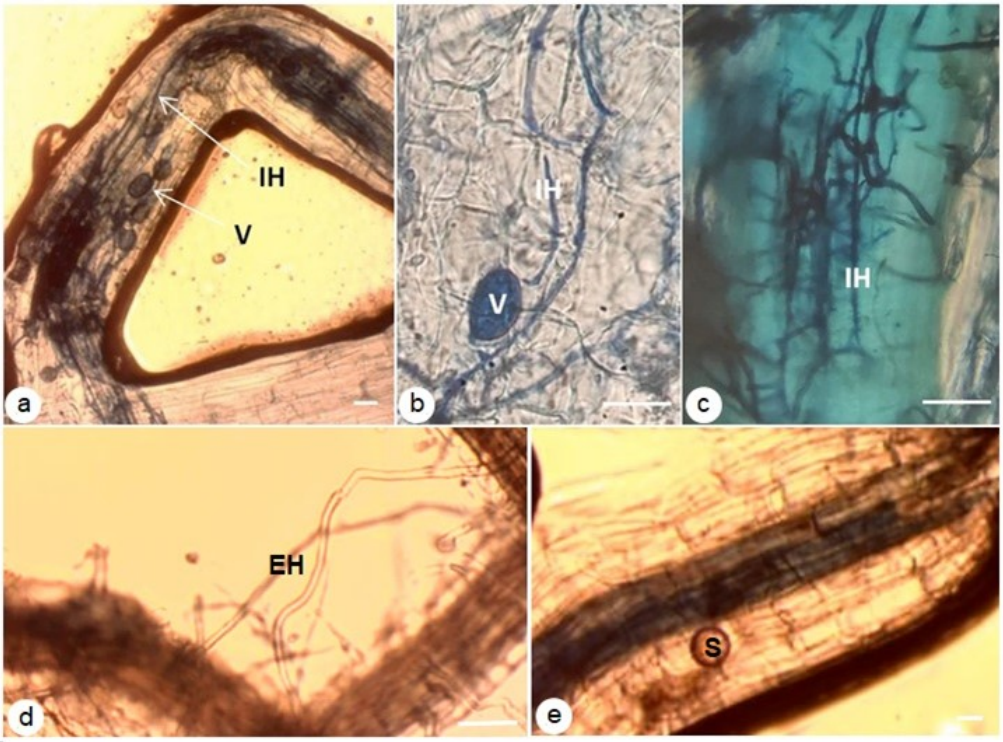



**Figure 3.** The presence of AMF on the root system of kebar grass (*B. petersianum*). a. intraradical hyphae and vesicules in the root, b. vesicules c. intraradical hyphae, d. extraradical hyphae on the root hairs, e. intraradical spore. (v: vesicula, IH: intraradical hyphae, EH: extraradical hyphae, S: intraradical spores, scale bar: 100 μm).

Moreover, AMF hyphae proliferation and spore production can also contribute to mycorrhizal activity and function related to ecosystems and environmental variables (Furrazola et al. 2015). In this study, on low pH soil (Table 2) AMF colonization was high and may be maximal if pH would be increased. Rohyadi et al. (2004) reported that under acid condition, AMF colonization also low, but it will improve in line with the increasing of pH. The increment of AMF colonization has positive correlation with the host plat growth.

## AMF Diversity

Based on the characteristics of the spore, there are 18 species of AMF (Table 4; Figure 4). These species belong to six genera, including *Glomus* (7 species of AMF), *Acaulospora* (3 species), and the genus *Claroideoglomus*, *Entrophospora*, *Gigaspora*, *Scutellospora* with two species each (Table 4; Figure 4). Some spore could not been identified yet since the identification process was only based on morphological characteristics.

In Papua, a study on AMF diversity based on the morphological characteristics of the medicinal plant Wati (*Piper methysticum*) found around 10 species (Suharno et al. 2018), while nine species were found in the pokem plant (*Setaria italica*) nine types (Suharno et al. 2015). *G. etunicatum*, *A. foveata*, *Glomus* sp. DK1-1., and *Scutellospora* sp. DK4-2., were widely distributed, while *Gigaspora* sp. DKT9-2, *Gigaspora* sp. DK9, *Claroideoglomus lamellosum*, and *Acaulospora* sp. DK4. have limited distribution. Suharno et al. (2018), reported that *G. etunicatum* and *A. foveata* are AMF which have broad distribution in

**Table 4.** The diversity of AMF types found in the rhizosphere of *B. petersianum* in Kebar, Tambrauw Regency, West Papua.

| No | Species of AMF | Location (sub-district) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Kebar | | | | | East Kebar | | | | |
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| 1 | *Acaulospora* sp. DK4 | - | - | - | + | - | - | - | - | - | - |
| 2 | *Acaulospora* sp. DK1-3 | + | - | + | - | - | + | - | - | - | - |
| 3 | *Acaulospora foveata* DK2-3 | + | + | - | + | - | + | - | + | - | - |
| 4 | *Clariodeoglomus etunicatum* DK8 | - | + | - | + | - | - | + | - | + | + |
| 5 | *Clariodeoglomus lamellosum* DKT10 | + | + | - | - | + | - | - | - | - | + |
| 6 | *Entrophospora* sp. DKT6-2 | - | - | - | - | - | + | + | + | - | - |
| 7 | *Entrophospora* sp. DKT9-3 | - | - | - | - | - | - | - | - | + | + |
| 8 | *Gigaspora* sp. DK9 | - | - | - | - | + | - | - | - | - | - |
| 9 | *Gigaspora* sp. DKT9-2 | - | - | - | - | - | - | - | - | + | - |
| 10 | *Glomus agregatum* DKT2 | - | - | + | - | - | - | + | - | - | - |
| 11 | *Glomus* sp. DK1-1 | + | - | + | - | + | + | - | + | + | + |
| 12 | *Glomus* sp. DK2-4 | + | + | - | - | - | - | - | - | - | + |
| 13 | *Glomus* sp. DK3-2 | - | + | - | + | - | - | - | - | - | - |
| 14 | *Glomus* sp. DKT6-1 | - | - | - | + | - | + | + | - | - | - |
| 15 | *Glomus* sp. DKT6-4 | - | - | - | + | - | - | + | - | - | - |
| 16 | *Glomus* sp. DKT8-1 | - | - | - | - | - | - | + | + | + | - |
| 17 | *Scutellospora* sp. DK2-2 | + | - | + | - | - | + | - | - | - | - |
| 18 | *Scutellospora* sp. DK4-2 | - | + | + | + | - | + | - | + | - | + |

Notes: + = present, - absence

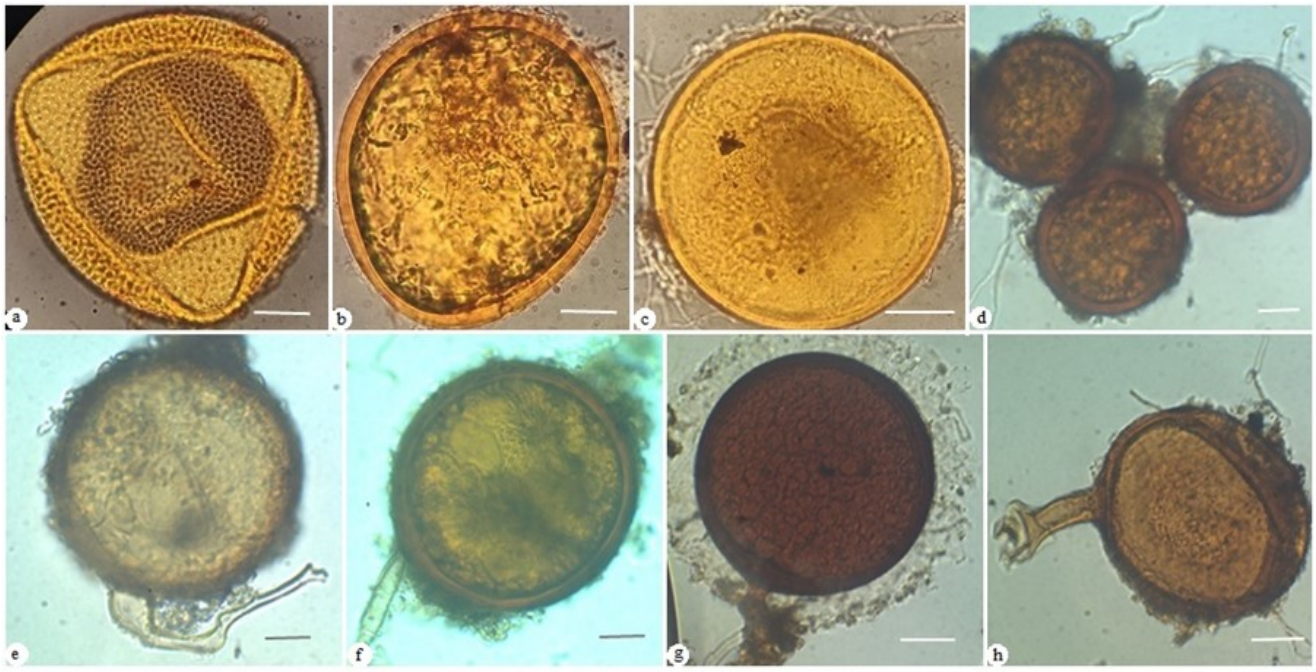



**Figure 4.** Diversity of AMF spores found in the rhizosphere of the kebar grass plant, in Tambrauw, West Papua. a. *Acaulospora foveata*, b. *Glomus* sp. DK2-4, c. *Glomus* sp. DK3-2, d. *Glomus aggregatum*, e. *Gigaspora* sp. DK9, f. *Claroideoglomus etunicatum*, g. *C. lamellosum*, h. *Entrophospora* sp. DKT9-3 (scale bar: 50 μm).

various types of habitat, while *C. lamellosum* often found in sandy soil habitat (Suharno et al. 2016). The physicochemical characteristics of the soil condition in the study site were similar, such as acidic soil, low-high C organic, medium N, low K, therefore the AMF distributed widely in that grassland.

*C. etunicatum* is capable of symbiosis with medicinal plants such as *Aloe vera*, *Artemisia nilagrica*, and *Withania somnifera*. *G. aggregatum* has symbiosis with *Coleus aromaticus*, and *Acaulospora foveata* associated with *Paris polyphylla* (Sun et al. 2021). However, *C. lamellosum* which has symbiosis with Kebar grass is not known to have symbiosis with other medicinal plants.

The results showed that the *Glomus* (38.89%) and *Acaulospora* (16.67%) were the most dominant genus found in the area. Among the other genera, the genus *Glomus* has the most species (Schüßler & Walker 2010; Souza 2015). There are approximately 76 types (56.72%) of AMF from the genus *Glomus*, and 37 types (27.61%) from the genus *Acaulospora,* which have been identified based on morphological and molecular characteristics. A total of approximately 134 AMF types have been identified (Schüßler & Walker 2010).

*G. aggregatum*, *A. foveata*, *C. etunicatum*, *C. lamellosum* and some other species were found to be associated with *B. petersianum* in the study site at 572 –619 m asl, temperature of 22–38 $^{o}$C, and 30–68% humidity. Moreover, the distribution of AMF is derived from the distribution of individual plant biomes and climatic factors. However, dispersal limitations, local environmental conditions, and interactions between AMF taxa also determine local diversity and global distribution (Kivlin et al. 2011).

## CONCLUSIONS

Kebar grass is in symbiosis with the AMF in a grassland area, Tambrauw Regency. The high number of AMF spores and the level of its colonization indicate that the kebar grass has a very close symbiotic form. Several species such as *G. aggregatum*, *A. foveata*, *C. etunicatum*, *C. lamellosum* and others found to be associated with kebar grass. The information of this study show new record on the AMF association with *B. petersianum*. Therefore, there is a need to conduct a compatibility test on the AMF diversity, including the possibility of obtaining the species that plays the most effective role in the growth of various other plants.

## AUTHORS CONTRIBUTION

S. designed the research and supervised all the process, I.R. collected and analyzed the data and wrote the manuscript, R.H.R.T and S.S. supervised this manuscript.

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## CONFLICT OF INTEREST

We confirm that there is no conflict of interest associated with this publication.

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**Research Article**

# Growth and Physiological Attributes of Rice by the Inoculation of Osmotolerant Rhizobacteria (*Enterobacter flavescens*) under Drought Condition

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## ABSTRACT
Rice (*Oryza sativa* L.) has mechanism for morphological, physiological, and biochemical self-defense in response to drought conditions. The ability of osmotolerant rhizobacteria to develop association with plants suggests that it could be used as an inoculum to support plant growth under drought stress. The purpose of this study is to determine the response of 'IR64' and 'Situ Bagendit' to the inoculation with osmotolerant rhizobacteria under drought conditions. The experiment had 3 treatment factors: 2 rice cultivars ('IR64' and 'Situ Bagendit'), 3 drought treatments (25%, 50% and 100% field capacity), and 2 types of rhizobacteria treatments (without inoculation and with inoculation using osmotolerant rhizobacteria (*Enterobacter flavescens*). Plant growth was measured in terms of plant height, number of leaves, number of tillers and panicles, and percentage of filled grain. Physiological and biochemical parameters, namely chlorophyll, carotenoids, proline, superoxide dismutase (SOD) peroxidase (POX) and ascorbate peroxidase (APX) were measured. The inoculation of osmotolerant rhizobacteria enhanced 'IR64' and 'Situ Bagendit' growth (plant height, number of leaves, tillers and panicles) and increased the percentage of grains in 'IR64' cultivar. Proline content, SOD, and APX activities were all increased by osmotolerant rhizobacteria inoculation, however, carotenoid content was decreased. Plant growth, physiological and biochemical responses of both cultivar to drought were enhanced by inoculation with osmotolerant rhizobacteria.



## INTRODUCTION
Water availability is crucial in sustaining growth and development of plant. Climate change has reduced the availability of water for agriculture. Drought can affect various cellular, biochemical, and physiological attributes in plant (Bouman & Tuong 2001; Mundree et al. 2004). This stress can cause metabolic changes in plant by controlling osmotic pressure and creating free radicals as ROS (reactive oxygen species) (Bhattacharjee 2005; Halliwell 2006). Plants develop non-enzymatic or enzymatic oxidative defense system in response to free radicals (Miller 2008). The non-enzymatic defense system can take the form of antioxidant production, while enzymatically it can take form

of enhanced SOD, POX and APX biosynthesis (Wang et al. 2005).

Rhizobacteria are microbes living around plant roots and playing a crucial role in plant growth. Several species of rhizobacteria have been identified as having a role in promoting plant development and crop yield (Loon 2007; Elango et al. 2013). Changes in water availability and environmental circumstances will have an impact physiologically of the soil microbial population, which will alter cell osmolarity and osmotic pressure on rhizosphere microorganisms (Miller & Janet 1996). Some rhizospheric microorganisms have developed osmotolerant systems for survival. They are also able to synthesize organic compounds in the cytoplasm that act as osmoregulators or osmoprotectants during osmotic stress or drought stress by producing proline compounds and/or glycine betaine (Csonka 1989). According to (Munro et al. 1989; Kunin & Rudi 1991), microorganisms and plants produce glycine betaine, an osmolyte that helps them endure osmotic stress. Osmoprotectants help rhizobacteria to adapt and to survive under salt and drought stress by keeping the osmotic potential of cell greater than that of their surroundings.

The ability of rhizobacteria to develop association with plants in the root system of plants opens its potential as an inoculum for plant cultivation. In a drought stressed environment, such an association, is likely to improve plant development. The addition of rhizobacteria isolates is known to stimulate plant growth by increasing P availability and nitrogen fixation (Gholami et al. 2008). It is also suggested that betaine produced by rhizobacteria reduced on the root surface is able to the root solute potential, resulting in a flow to the rhizosphere in a way that the rhizobacteria can survive under drought stress conditions. Glycine betaine is an osmolyte that is not found in all higher plants, but is produced by a variety of microbes and plants to help them withstand osmotic stress. Some bacteria, such as *Cyanobacteria*, *Eschericia coli*, *Salmonella typhimurium*, *Klebsiella pneumoniae,* and *Azospirillum sp.* generate glycinebetaine, a significant osmoprotectant (Yuwono 2005). *Enterobacter flavescens* is one of the rhizobacteria that can create an osmoprotectants, such as proline and/or glycine betaine compounds, in response to osmotic stress or drought stress (Csonka 1989). Osmoprotectants help rhizobacteria adapt and survive in environments that are stressed by salt or drought by maintaining the cell's osmotic potential higher than its surroundings. This is accomplished in *Escherichia coli* via osmoregulatory processes that result in a cytoplasm that has an appropriate osmotic pressure and is conducive to enzyme action. *E. coli* cells accumulate potassium ions and activate system for the transport and synthesis of several organic osmolytes compatible with metabolism in response to osmotic stress and a decrease in cell turgor pressure, preventing cell dehydration and stabilizing enzyme activity in high ionic strength solutions (Munro et al. 1989). Plants accumulate glycinebetaine and proline during osmotic stress suggest that these two compounds have the potential to counteract the inhibitor effects of osmotic stress in bacteria. Proline and glycinebetaine interact with proteins in a unique way that protect

them from denaturation in the presence of high electrolyte concentration. These two chemicals are simply inert compatible solutes that help cells maintain turgor in high-osmolarity environment (Csonka 1989).

In Indonesia, rice is the most widely consumed food. However, rice productivity is decreasing due to problems such as salinity and drought stress. Currently, there are many superior rice cultivars with increased higher productivity compared to local cultivars. In the face of drought, the use of suitable and adaptable superior cultivars could be a viable option for rice production. In specific growing condition, superior variety seeds have a high purity, high growth percentage, and high yield potential. 'IR64' is a lowland rice cultivar that can be planted in irrigated or swampy rice fields, while 'Situ Bagendit' is a rice cultivar that can be grown on dry land or paddy fields (Suprihatno et al. 2009). 'Situ Bagendit' is known as upland rice which demonstrated resistance to drought stress compared to rice 'IR64'.

In response to decreasing availability of freshwater, alternative sources are required by improving plant adaptability in drought condition. For drought survival, several rhizospheric bacteria have developed osmotolerant system. Rhizobacteria's ability to form associations with plants in the root system of plants makes it a viable inoculum for plant cultivation. 'IR64' and 'Situ Bagendit' were used in this study to determine the plant response under drought conditions with the application of osmotolerant rhizobacteria. This study aims at establishing the response of 'IR64' and 'Situ Bagendit' to the inoculation using osmotolerant rhizobacteria under drought condition.

## MATERIALS AND METHODS

### Materials

In this work, osmotolerant rhizobacteria *E. flavescens* was taken from Faculty of Agriculture, Universitas Gadjah Mada, Yogyakarta, while two rice cultivar (*Oryza sativa* L.) 'IR64' and 'Situ Bagendit' were collected from the Indonesian Center for Rice Research (ICRR), Indonesia.

### Methods

#### Osmotolerant rhizobacteria inoculation and application of drought treatment

The study was carried out in the Greenhouse of the Sawitsari Research Station, Faculty of Biology, Universitas Gadjah Mada, Indonesia between July 2019-February 2020. The experiment involved three factors and was conducted in a completely randomized design (CRD). The first factor was the rice cultivar with two rice cultivar ('IR64' and 'Situ Bagendit'), the second factor was inoculation using osmotolerant rhizobacteria (*E. flavescens*) and without inoculation, and the last factor was three levels of drought at 25%, 50%, and 100% of field capacity.

Seed sterilization was accomplished by soaking the seeds in 70% (v/v) ethanol for 5 minutes, followed by submersion in 0,2 percent $HgCl_2$ for 4 minutes. The seeds were rinsed six times with sterile distilled water following surface sterilisation. Rice seeds were inoculated with osmotolerant rhizobac-

teria inoculants in a volume of 5 mL ($10^8$ cfu/mL) prior to planting. During 21 days, the soil's water-holding capacity was sustained at field capacity. Following that, the soil moisture was changed to achieve 25%, 50%, and 100% field capacity. Each treatment's field capacity is maintained by watering every three days till 12 weeks.

### Plant Growth Parameters

Plant height, number of leaves, tillers, and panicles were measured weekly during treatment. The distance between the longest leaf tip and the plant above the soil surface was used to determine plant height.

### Physiological Responses Measurement

Physiological characteristics were measured using the (Harborne 1984) method with a few adjustments on chlorophyll and carotenoid content. Cold acetone solution (3 mL of 80%) was used to homogenize a 0.3 grams leaf sample pulverized in a mortar. The spectrophotometer (Thermo Scientific GENESYS 10 UV Scanning) was used to analyze chlorophyll content at 470 nm, 645 nm, and 664 nm multi-wavelengths and the results were expresses in $mg.g^{-1}$ FW (fresh weight).

Proline content analysis was measured using (Bates et al. 1973) method. Leaf samples of 0.25 grams were pulverized and homogenized in 5 ml containing 3% sulfosalicylic acid solution. The sample was mixed with ninhydrin reagent (ninhydrin, acetic acid, and phosphoric acid) and glacial acetic acid in a 1:1:1 ratio, and then heated in a water bath (Memmer GmbH + Co.KG.WNB-7) at 95 $^oC$ for 60 minutes. The solution was cooled to 25$^o$ C and reacted with toluene to form two layers. The absorbance of the solution at 520 nm wavelength was compared to the standard proline curve to measure proline levels.

The activity of superoxide dismutase (SOD) was measured using the (Marklund & Marklund 1974) procedure. Fresh leaf samples (0.5 grams) were frozen in liquid nitrogen and crushed, then homogenized with 0.01 M phosphate buffer (pH 7.0), 1 mM EDTA, and 1% PVP, then centrifuged for 20 minutes at 4° C at 15000 rpm. A reaction mixture was prepared, consisting of 2 mL Tris-HCl Buffer at pH 8.2, 0.5 mL 2 mM pyrogallol, and 2 ml ddH20 were combined with 0.5 mL supernatant. The generated test mixture was compared to a blank solution) containing pyrogallol) at 325 nm at 3 minutes intervals by spectrophotometer (Thermo Scientific GENESYS 10 UV Scanning). The oxidation data for pyrogallol were gathered every minute for 3 minutes and utilized to determine auto-oxidation of 100%. The results are given in units per milligram of protein (1 unit is the amount of enzyme used to inhibits 5 percent of pyrogallol oxidation per minute).

Ascorbate peroxidase activity measurements were carried out following the (Nakano & Asada 1981) protocol with modifications. Enzyme extract (100 µL), EDTA 0.1 mM (400 µL), 0.05 mM sodium phosphate buffer at pH 7.0, 0.05 mM ascorbic acid solution (400 µL) and $ddH_20$ (1.5 mL) were

mixed together. The mixed solution was then added with 400 µL of 3% $H_2O_2$ solution followed by incubation for 60 seconds. Furthermore, a spectrophotometer was used to detect the decrease in absorbance at wavelength of 290 nm with a time interval of 3 minutes (Thermo Scientific GENESYS 10 UV Scanning). APX enzyme concentrations were calculated using the exclusion coefficient (€ = 2.8 $mM^{-1}cm^{-1}$). The amount of APX that oxidizes one nmol per mL of ascorbate per minute is defined as one unit (U). The activity of the APX enzyme is measured in $U.mg^{-1}$ protein. A mixed solution without enzyme extract is used as a control.

The determination of peroxidase (POX) activity was performed using the modified (Kar & Dinabandhu 1976) protocol. Sodium phosphate buffer solution (500 µl of 0.05 mM) at pH 7.0, pyrogallol solution (10 µL), and 250 µL of 5 mM $H_2O_2$ solution were put into the tube, followed by 500 µL of enzyme extract and incubated at $25^0$C for 15 minutes in a water bath. The tube was filled with 5% $H_2SO_4$ solution (250 µL) and gently shaken. The purpurogallin absorbance was measured at 420 nm with spectrophotometer (Thermo Scientific GENESYS 10 UV Scanning). The same process was used to make a blank solution, but no enzyme extract was added. The activity of the POX enzyme was measured at 420 nm ($A_{420}$).

### Data Analysis

Homogeneity and normality of the data were analyzed using one-way ANOVA and followed by Duncan's Multiple Range Test (at 95% confidence level) using IBM-SPSS version 16.0.

## RESULTS AND DISCUSSION

### Results

#### Growth Performance

Treatment under different water availability (100%, 50%, and 25% field capacity) resulted in significantly different plant heights in the two rice cultivars treated without and with the inoculation of osmotolerant rhizobacteria (*E. flavescens*) (Figure 1). The rise plant height during the vegetative phase, and the plant does not tend to grow taller after it enters the generative phase. According to Figure 1, plant height increased until the 5th week in rice 'IR64' and 'Situ Bagendit' grown at 100% field capacity, and it remained steady until the 12th week. The increase in plant height remained until the 6th week in the 50% field capacity treatment, whereas in the 25% field capacity treatment, plant height constant at 8th week. Plant development is more ideal at 100% field capacity because the water needs are more adequate than at 25% field capacity. This situation also permits plants to perform better in their growth phase and enter their reproductive phase more quickly. The establishment of tillers and panicles was faster with 100% and 50% field capacity than with 25% field capacity treatment.

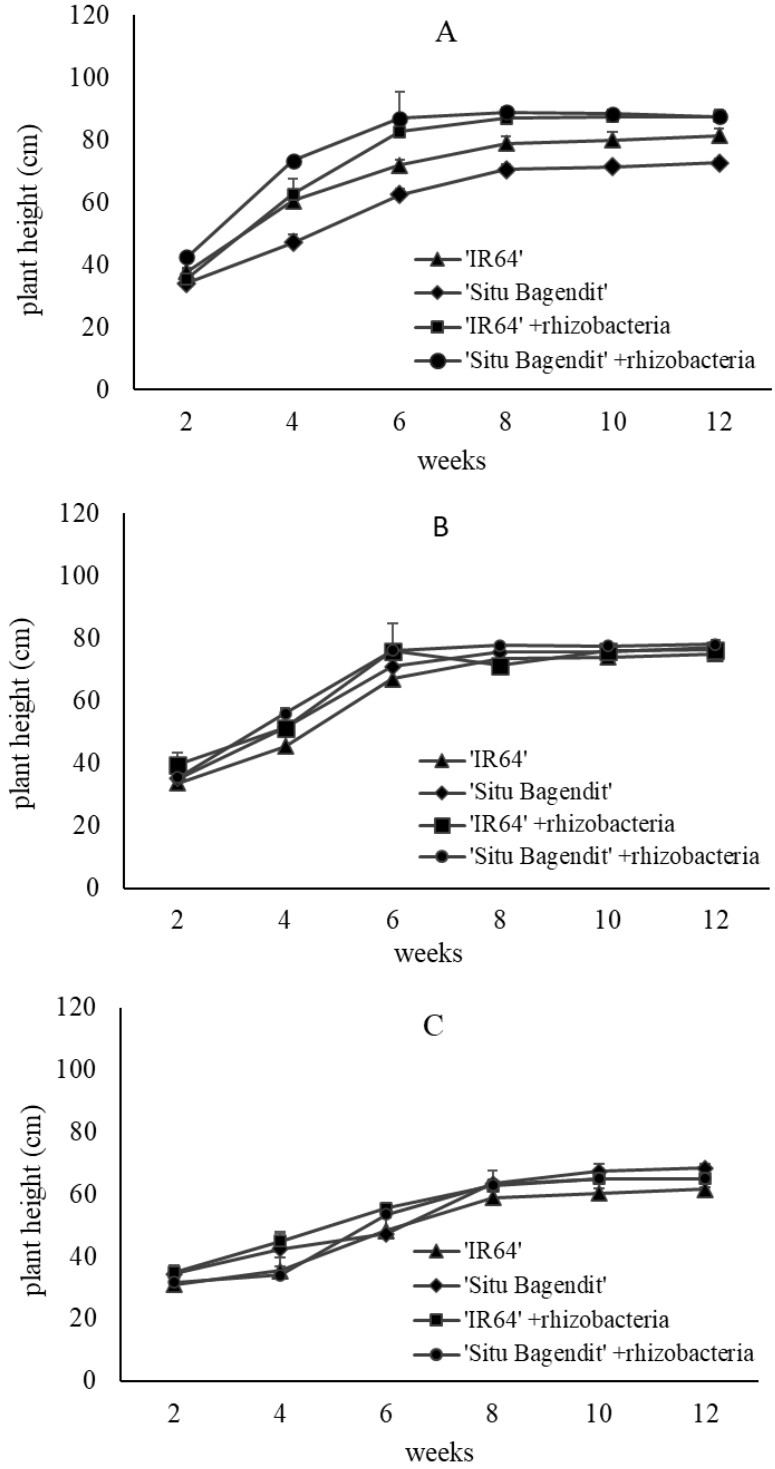


**Figure 1.** Effect of rhizobacteria inoculation on plant height of rice 'IR64' and 'Situ Bagendit' for 12 weeks under drought condition A: 100% field capacity, B: 50% field capacity, and C: 25% field capacity.

According to Figure 1, the highest increase in plant height was found in 'Situ Bagendit' with inoculation using osmotolerant rhizobacteria in the 100% field capacity treatment, while the treatment without inoculation using osmotolerant rhizobacteria resulted in the lowest plant height. In comparison to 'IR64' with and without inoculation using osmotolerant rhizobacteria at 50% field capacity treatment, 'Situ Bagendit' with osmotolerant rhizobacteria inoculation produced the maximum plant height. 'Situ Bagendit' produced higher plant height than rice 'IR64' at 25% field capacity treatment.

Based on the data obtained during 12 weeks of planting, it is observed that 'Situ Bagendit' plants produced higher plant height compared to 'IR64' (Table 1). Treatments of water availability (100%, 50% and 25% field capacity) resulted in considerably varied plant heights in two rice cultivars, both with and without rhizobacteria inoculation. Under 25% field capacity, 'Situ Bagendit' plants without rhizobacterial inoculation produced higher plant height compared to 'IR64'. This proves that 'Situ Bagendit' is more resistant to drought than 'IR64'. The inoculation of rhizobacteria to each rice cultivar gave maximum results in supporting plant growth, both at 100%, 50%, and 25% field capacity.

The number of leaves was significantly different in rice 'IR64' and 'Situ Bagendit' between treatments with and without osmotolerant rhizobacterial inoculation. The number of leaves in two cultivars differed significantly between treatments of 100% field capacity with 50% and 25% field capacity, while there was no significant difference between treatments of 50% and 25% field capacity. The highest number of leaves was found under 25% of field capacity with osmotolerant rhizobacteria inoculation to 'IR64' and 'Situ Bagendit', while the least number of leaves was found in 'Situ Bagendit' rice at 50% and 25% field capacity without rhizobacterial inoculation (Table 1).

Different level of water availability also resulted in different number of tiller and panicle in the two rice cultivars treated without and with the inoculation of osmotolerant rhizobacteria (Table 2). 'IR64' at 25% field capacity without rhizobacteria inoculation had lowest average number of tillers. Both cultivars of rice generated the same number of tiller in each field capacity, regardless of whether they were inoculated with rhizobacteria or not. "Situ Bagendit" produced the most tillers at 100% field capacity without rhizobacteria inoculation treatment (with average 5.33 tillers), which was not substantially different from the treatment with inoculation. When comparing treatments with 50% and 25% field capacity, the number of tillers 'IR64' and 'Situ Bagendit' at 100% field capacity with or without rhizobacteria inoculation resulted in the highest number of tillers, while the treatment with 25% field capacity resulted the lowest number of tillers.

**Table 1**. Plant height and leaf number of rice (*Oryza sativa* L.) 'IR64' and 'Situ Bagendit' at week 12 at 100%, 50%, and 25% field capacity.

| Parameter | Field Capacity | 'IR64' | | 'Situ Bagendit' | |
|---|---|---|---|---|---|
| | | Without Rhizobacteria | With Rhizobacteria | Without Rhizobacteria | With Rhizobacteria |
| Plant Height (cm) | 100% | 79.50 $^{b}$ | 87.33 $^{a}$ | 85.33 $^{a}$ | 87.33 $^{a}$ |
| | 50% | 75.00 $^{c}$ | 76.33 $^{cb}$ | 77.00 $^{cb}$ | 78.00 $^{cb}$ |
| | 25% | 61.67 $^{e}$ | 65.00 $^{ed}$ | 68.33 $^{d}$ | 65.00 $^{ed}$ |
| Leaf Number | 100% | 11.00 $^{p}$ | 8.00 $^{q}$ | 11.00 $^{p}$ | 8.00 $^{q}$ |
| | 50% | 8.67 $^{q}$ | 11.00 $^{p}$ | 7.67 $^{q}$ | 8.67 $^{q}$ |
| | 25% | 9.00 $^{q}$ | 11.67 $^{p}$ | 7.67 $^{q}$ | 11.67 $^{p}$ |

Values having same letter (s) in a row and column of each parameter was not significantly different at ($p \leq 0.05$) level of significant by DMRT.

**Table 2**. Number of tiller, panicle, and percentage of filled grain of rice (*Oryza sativa* L.) 'IR64' and 'Situ Bagendit' at week 12 at 100%, 50%, and 25% field capacity.

| Parameter | Field Capacity | 'IR64' | | 'Situ Bagendit' | |
|---|---|---|---|---|---|
| | | Without Rhizobacteria | With Rhizobacteria | Without Rhizobacteria | With Rhizobacteria |
| Number of Tiller | 100% | 3.67 edc | 4.67 cba | 5.33 a | 4.33 dcba |
| | 50% | 3.33 edcb | 4.00 edcb | 3.00 fe | 4.33 dcba |
| | 25% | 2.33 f | 3.67 edc | 3.00 fe | 3.33 fed |
| Number of Panicle | 100% | 3.67 lk | 4.67 ji | 5.33 i | 4.67 ji |
| | 50% | 3.33 kj | 4.67 ji | 3.00 ml | 4.33 kj |
| | 25% | 2.33 m | 3.00 ml | 3.00 ml | 3.00 ml |
| Percentage of Filled Grain (%) | 100% | 65.72 q | 78.62 p | 32.48 s | 61.02 q |
| | 50% | 47.12 r | 46.90 r | 17.47 ut | 34.80 s |
| | 25% | 19.54 t | 46.25 r | 10.80 u | 21.32 t |

Values having same letter (s) in a row and column of each parameter was not significantly different at ($p \leq 0.05$) level of significant by DMRT.

'Situ Bagendit' produced the most panicles with 100% field capacity treatment without osmotolerant rhizobacteria inoculation with average of 5.33 panicles. The results were similar to those obtained with rice 'IR64' at 100% and 50% field capacity by inoculation with rhizobacteria, as well as with 'IR64' at 100% and 50% field capacity by inoculation with rhizobacteria. The lowest average number of panicles was found at 25% field capacity (2.33 panicles) was recorded in 'IR64', which was not significantly different from the inoculation treatment with rhizobacteria, and with 'Situ Bagendit' at 25% and 50% field capacity without rhizobacteria inoculation and 25% field capacity with rhizobacteria inoculation. The average number of tillers and panicles produced by both rice cultivars was about the same. The number of panicles produced by 'Situ Bagendit' at 100% field capacity treatment with rhizobacteria inoculation was higher than the number of tillers, whereas the number of tillers was higher in 'IR64' and 'Situ Bagendit' at 25% field capacity with rhizobacteria osmotolerant in comparison to number of panicles.

'IR64' under 100% of field capacity inoculated with rhizobacteria resulted in the highest percentage of filled grain (78,62%), while the lowest percentage was found in 'Situ Bagendit' under 25% of field capacity without the inoculation of rhizobacteria. Water availability at 25% of field capacity resulted in the lowest percentage of grain compared to 100% treatment and 50% of field capacity. The osmotolerant rhizobacteria inoculation also gave a significant difference in the resulting grain compared to the treatment without rhizobacteria, both in rice 'IR64' and 'Situ Bagendit' on treatment of 100%, 50% and 25% of field capacity.

According to Salsinha et al. (2021), with the increase in drought stress level, the proportion of growth parameters (plant height, and number of tillers) decrease significantly ($p<0,05$). Salsinha et al. (2021) also shows that drought-tolerant cultivar (Boawae 100 Malam and Padi Merah Kuatnana) was significantly different from Ciherang cultivar. In this study, the analysis of growth shows that 'Situ Bagendit' is drought-tolerant cultivar with high sus-

ceptibility to drought stress. Drought stress affects food crop development and yield. Due to a lack of water, the development process from vegetative to reproductive phase has also been slowed that also damage cell membranes, perhaps leading to cell death. Plant morphological growth, like height, number of tillers, panicles and grains was reduced in the presence of drought stress (Farooq et al. 2009). Drought during vegetative phase can inhibit the growth of leaves and roots. Drought stress during flowering and grain filling reduced grain yield considerably as compared to the control. The decrease in yield at the flowering stage is largely due to the decrease in the number of grains per panicle. Stress during the growth stage can decrease assimilation translocation to seed which decreases seed weight and increases the percentage of empty seeds. The success of seed formation depends on the availability of assimilates translocated to the seeds. The source of assimilates for seed formation comes from the flag leaves. Flag leaves contribute 45% assimilation for the formation of rice seeds (Abou-Khalifa et al. 2008).

## Physiological responses

Plant uses photosynthetic pigments primarily for light gathering and the creation of reducing power. Although chlorophyll a and b are susceptible to soil drying, carotenoids play a complementary role in helping plants endure drought (Farooq et al. 2009). The highest chlorophyll content was found in rice 'IR64' under 50% field capacity with rhizobacterial osmotolerant inoculation. 'Situ Bagendit' under 100% field capacity resulted in the lowest chlorophyll content compared to the 50% and 25% field capacity treatment, as well as with the 'IR64' plant treatment (Table 3). Drought stress altered the ratios of chlorophyll a and b, as well as carotenoids. Drought stressed cotton (Massacci et al. 2008) and sunflower plants (Kiani et al. 2008) were found to have reduced chlorophyll content. As a relative water content and leaf water potential diminish, the foliar photosynthesis rate of higher plants is known to decrease. According Salsinha et al. (2021), drought tolerance is higher in rice cultivars with lower chlorophyll reduction percentage (Hare Tora and Boawaw 100 Malam with 9.37% and 7.56 % respectively), however Pak Mutin, Gogo Jak and Padi Putih Maumere had the biggest fall in chlorophyll levels indicating a high drought sensitivity. As water content and leaf water potential fell in higher plants, the photosynthetic rate is decreased. The measurement of chlorophyll a and b shows that when the amount of water in treatment was lowered, the amount of photosynthetic pigments was similarly reduced, affecting the morphological and physiological processes of the plants (Usman et al. 2013).

Drought can cause carotenoid damage due to free radical activity. According to Martinez-Ferri et al. (2004), Jaleel et al. (2009); Du et al. (2010) and Anjum et al. (2011), enhanced biosynthesis and free radical destructive activity, as well as increased conversion of carotenoid pigment into other chemicals such as ABA, might cause a decrease in pigment content, which is essential to create plant adaptation strategies in response to drought stress.

**Table 3**. Chlorophyll, Carotenoid and Proline Content of rice (*Oryza sativa* L 'IR64' and 'Situ Bagendit') at week 12 under 100%, 50%, and 25% field capacity.

| Parameter | Field Capacity | 'IR64' | | 'Situ Bagendit' | |
|---|---|---|---|---|---|
| | | Without Rhizobacteria | With Rhizobacteria | Without Rhizobacteria | With Rhizobacteria |
| Chlorophyll (mg.g$^{-1}$ FW) | 100% | 3.55 $^{cba}$ | 3.40 $^{cba}$ | 2.56 $^{c}$ | 2.67 $^{cb}$ |
| | 50% | 3.34 $^{cba}$ | 3.98 $^{a}$ | 3.00 $^{cba}$ | 3.86 $^{ba}$ |
| | 25% | 3.15 $^{cba}$ | 3.13 $^{cba}$ | 3.15 $^{cba}$ | 3.42 $^{cba}$ |
| Carotenoid (mg.g$^{-1}$ FW) | 100% | 0.85 $^{i}$ | 0.16 $^{kj}$ | 0.14 $^{k}$ | 0.32 $^{kj}$ |
| | 50% | 0.59 $^{kji}$ | 0.23 $^{kj}$ | 0.52 $^{kji}$ | 0.33 $^{kj}$ |
| | 25% | 0.41 $^{kji}$ | 0.31 $^{kj}$ | 0.62 $^{ji}$ | 0.33 $^{kj}$ |
| Proline (µmol g$^{-1}$ FW) | 100% | 0.105 $^{r}$ | 0.109 $^{r}$ | 0.137 $^{rq}$ | 0.107 $^{r}$ |
| | 50% | 0.117 $^{r}$ | 0.123 $^{r}$ | 0.147 $^{rq}$ | 0.161 $^{rq}$ |
| | 25% | 0.125 $^{r}$ | 0.199 $^{rq}$ | 0.223 $^{q}$ | 0.344 $^{p}$ |

Values having same letter (s) in a row and column of each parameter was not significantly different at ($p \leq 0.05$) level of significant by DMRT.

The highest carotenoid content was found in 'IR64' with 100% field capacity treatment without rhizobacterial osmotolerant inoculation. Rhizobacterial osmotolerant inoculation treatment resulted in lower carotenoid content compared to the treatment without rhizobacteria osmotolerant inoculation, both in 'IR64' and 'Situ Bagendit in under the three field capacity treatments (Table 3).

Drought has also been linked to changes the metabolism of soluble carbohydrates, a group of molecules that can act as suitable solutes and anti-oxidants. When there is a lack of water, there are chemicals which are tend to rise. Free amino acids are another type of molecules that may be impacted by lack of water. Water stressed leaves had higher levels of proline and total free amino acids (Pinheiro et al. 2004; Van Heerden 2002). A distinctive plant response to environmental challenges, notably drought stress, is the accumulation of defensive solutes like proline and soluble sugar in the leaf (Sakamoto 2002). During stress, proline and soluble sugar, both operate as osmoprotectans (HongBo et al. 2005; Reddy et al. 2004). Proline may also operate as an antioxidant. Proline content in 'Situ Bagendit' rice at 25% field capacity treatment resulted in the highest proline content. The inoculation using rhizobacteria also increases the proline content produced. The increase of the proline content in stressed plants is an adaptation to deal with stressful conditions (Table 3). Under severe situations, proline builds up and provides energy for growth and survival (Chandrashekar & Sandhyarani 1996). Proline, sucrose, glycinebetaine, and other substances accumulation in the cytoplasm have a function in osmotic adjustment, which can improve the rate of water absorption (Shehab et al. 2010; Usman et al. 2013). Proline biosynthesis is stimulated directly during stress as a drought resistance strategy. Proline has been shown to scavenge ROS and other free radicals in studies. However, exogenous proline at high concentrations (40-50 mM) has little effect on rice plants under abiotic stress (Hayat et al. 2012). As a result of the induced drought stress in the roots, plants' proline levels rise. Drought tolerant rice

(such as Padi Hitam Mumere, Shintara, Padi Merah Noemuti, and Gogo Sikka) showed the highest levels of proline from control to severe drought curcumtances (Salsinha et al. 2021).

## Biochemical properties

Drought stress causes a plant's reaction to be complex, involving the synthesis of polyamines and the emergence of a new group of proteins whose function is unknown. Abscisic acid is crucial to the reaction because it causes closing stomata, limiting water loss while also reducing $CO_2$ available for photosynthesis, which can lead to electron production in the photosystem (Arora et al. 2002). Plants have highly efficient scavenging systems for reactive oxygen species, which protect them from oxidative processes that harmful to them. These defenses are not only prevalent within the cell, but also in the apoplast to a lesser extent. Plants evolved cellular adaptive responses as a result, such as up-regulation of oxidative stress protectors and the accumulation of protective solutes. Superoxide dismutase (SOD), ascorbate peroxidase (APX), and peroxidase (POX) are antioxidant defense enzymes that help to reduce superoxide and hydrogen peroxide concentrations. The dismutation of superoxide into oxygen and hydrogen peroxide is catalyzed by superoxide-dismutase (SOD). Peroxidases which comprise both enzymic and non-enzymic $H_2O_2$ degradation, remove $H_2O_2$ (Peltzer et al. 2002). The activity of ascorbate peroxidase has mostly been found in chloroplasts and cytosols. SOD and Ascorbate Peroxidase (APX) enzymes are found in both soluble and thylakoid-bound forms in chloroplasts. The activity of APX had higher activity drought stress at 25% field capacity and 50% field capacity compared to 100% field capacity in both rice cultivars. Ascorbate peroxidase is another key antioxidant enzyme. APX participates in the oxidative chain reaction that transforms $H_2O_2$ into $O_2$ and $H_2O$ with absorbic acid as one of the electron suppliers (Refli & Yekti 2016).

The catalytic alterations in the detoxification of peroxide radicals into water and oxygen are catalyzed by peroxidase (POX) enzymes (Hiraga et al. 2000). This enzyme is also involved in plant adaptations such as lignification, suberization, and auxin metabolism simulation (Lagrimini et al. 1997). POX activity in rice 'IR64' and 'Situ Bagendit' experienced a decrease in rice drought rate of 50% and 25% in field capacity compared to 100% (Table 4). Peroxidase are important for scavenging $H_2O_2$ toxicity. Under adverse situation such as drought stress, the combined activity CAT and SOD transforms the deadly superoxide radical ($O_2$) and hydrogen peroxide ($H_2O_2$) to water and molecular oxygen, preventing cellular damage (Noctor et al. 2000; Chaitanya et al. 2002). Drought-tolerant wheat, coffee, rice, and caper cultivars had higher antioxidant system than susceptible cultivars, according to (Guo et al. 2006; Lascano et al., 2001; Lima et al. 2002; Ozkur et al. 2009).

The results of this study indicate that drought stress can simulate changes in antioxidant enzymes activity (SOD, APX, and POX) of rice leaves in both rice cultivars, 'IR64' and 'Situ Bagendit'. The change in enzyme activ-

**Table 4**. Oxidative Enzyme Activity of rice plant (*Oryza sativa* L 'IR64' and 'Situ Bagendit') at 12 weeks under 100%, 50%, and 25% field capacity.

| Parameter | Field Capacity | 'IR64' | | 'Situ Bagendit' | |
|---|---|---|---|---|---|
| | | Without Rhizobacteria | With Rhizobacteria | Without Rhizobacteria | With Rhizobacteria |
| SOD (U/L) | 100% | 0.640 $^{a}$ | 0.914 $^{a}$ | 1.145 $^{a}$ | 1.527 $^{a}$ |
| | 50% | 0.868 $^{a}$ | 1.295 $^{a}$ | 1.091 $^{a}$ | 1.517 $^{a}$ |
| | 25% | 0.985 $^{a}$ | 1.100 $^{a}$ | 0.997 $^{a}$ | 0.969 $^{a}$ |
| APX (U/L) | 100% | 2.336 $^{j}$ | 4.403 $^{ji}$ | 2.166 $^{j}$ | 3.182 $^{ji}$ |
| | 50% | 3.734 $^{ji}$ | 5.041 $^{ji}$ | 3.760 $^{ji}$ | 3.272 $^{ji}$ |
| | 25% | 3.801 $^{ji}$ | 5.764 $^{i}$ | 5.092 $^{ji}$ | 4.990 $^{ji}$ |
| POX ($A_{420}$) | 100% | 0.210 $^{p}$ | 0.361 $^{p}$ | 0.318 $^{p}$ | 0.300 $^{p}$ |
| | 50% | 0.202 $^{p}$ | 0.239 $^{p}$ | 0.283 $^{p}$ | 0.298 $^{p}$ |
| | 25% | 0.164 $^{p}$ | 0.228 $^{p}$ | 0.228 $^{p}$ | 0.174 $^{p}$ |

Values having same letter (s) in a row and column of each parameter was not significantly different at ($p \leq 0.05$) level of significant by DMRT.

ity plays a role in suppressing the destructive activity of free radicals. The activity of these oxidative enzymes tends to be more active in 'Situ Bagendit' (drought resistant) rice plants than in 'IR64' (Table 4). The high activity of oxidative enzymes indicates the development of a better oxidative defense system compared to drought rice.

## CONCLUSION

Under stress condition, the mechanism plants defense was increased to ensure the tolerance of plant in responding to stress. The rice plants (*Oryza sativa* L.) 'IR64' and 'Situ Bagendit' are becoming increasingly stunted as drought stress increases. Inoculating osmotolerant rhizobacteria help accelerate plant growth, as indicated by increased plant height, number of leaves, tillers, and panicles in both rice cultivars, as well as a higher percentage of filled-grain in 'IR64'. The biochemical and physiological response of 'IR64' and 'Situ Bagendit' to drought were enhanced by inoculation with osmotolerant rhizobacteria, as evidenced by an increase in proline content, SOD and APX enzyme activity, while the carotenoid level reduced. The rhizobacteria osmotolerant inoculation showed the increased POX activity at IR64 cultivar. Inoculation of osmotolerant rhizobacteria can be used to increase the cultivation of rice plants under drought stress.

## AUTHORS CONTRIBUTION

TY and DR designed the research and supervised all the process, HDK collected and analyzed the data and prepared the publication. All authors read and approved the final version of the manuscript.

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## CONFLICT OF INTEREST

There are no conflicts of interest declared by the authors.

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**Research Article**

# Utilization of Coffee Pulp Waste Composted with Cellulolytic Actinomycetes to Enhance Chili Plant Growth

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## ABSTRACT

The abundant volume of coffee bean pulp as a by-product of the post-harvest processing is an important source of soil organic matter if it is properly handled. The alternative way to use coffee bean pulp waste to reduce the impact of environmental pollution is composting. This study aims to determine the ability of actinomycetes to degrade coffee pulp, to identify the physical and chemical characteristics of coffee pulp compost, and to evaluate the effect of coffee pulp compost on chili plant growth. The results showed that 7 isolates of actinomycetes were able to hydrolyze coffee pulp *in vitro* with a hydrolytic index of 1.7-3.81. The treatment of coffee pulp compost with the addition of a starter of cellulolytic actinomycetes (P2) at the end of the three-week incubation period showed the highest organic N (25 mg/kg), P (7.05 mg/kg), and K (33 mg/kg), t compared to other treatments. The effect of giving coffee pulp compost towards the growth of chili plants shows that the coffee pulp composted with zeolite 5% (w/w) increased the height of the chili plants by 37.6%, while in coffee pulp composted by cellulolytic actinomycetes 5% (v/w) increased the number of leaves by 96% and plant biomass by 25%. Based on the results of this research, coffee pulp compost has the potential to be used as biological fertilizer to increase plant growth, both composted by zeolite and cellulolytic actinomycetes.



## INTRODUCTION

Coffee (*Coffea* sp.) is one of the export commodities which plays important role in generating foreign exchange. Based on BPS data, the number of coffee exports was 412.370,3 tons in 2016 and 464.198,3 tons in 2017 (BPS 2018). Increased demand for coffee consumption comes from abroad as well as the domestic market. Unfortunately, the coffee processing industry posses a problem in the utilization of coffee pulp waste into economically valued products. According to previous data, raw coffee cherries processing generated approximately one ton of fruit waste from two tons of raw coffee (Brunerová et al. 2019). Thus, the disposal of coffee pulp waste must be well managed to reduce negative effects on the environment as well as public health.

Coffee pulp waste obtained during the dry and wet coffee processing stages which are wet fruit skin, liquid waste containing mucus, dry logs, and dry shells. Coffee fruit has a moisture content between 60-65%. Coffee cherries are still protected by the outer skin or pericarp, pulp, pectin, endocarp, and silver skin when they are harvested (Klingel et al. 2020). The results of the mass balance analysis showed that 100 kg of processed dry coffee cherries generated 29 kg (29%) of dry logs which consisted of 15.95 kg of coffee beans (55%) and 13.05 kg of dry logs (45%). The dominant composition of dry logs are shell, mucus, and fruit skin. Besides that, the dry logs also contain several compounds such as reducing sugars, non-reducing sugars, pectin compounds, protein, and crude fiber (Widyotomo 2013). Therefore, it is necessary to process coffee pulp waste which has economic value for reducing the impact of environmental pollution.

Composting coffee pulp is an alternative way to reduce the environmental negative impacts caused by coffee bean pulp waste. The abundant volume of coffee hulls as post-harvest processing product are important source of organic matter for soil as long as they are composted properly. After composting process, the coffee pulp waste contains 1.0-2.3 of nitrogen and 8 of C / N ratio to be utilized by plants (Setyorini 2006). The decomposition process of plant residue i.e. coffee pulp waste by decomposer microorganisms produces nutrients that can be easily absorbed by plants. Several types of decomposer microbes have a role in plant residues decomposition i.e. bacteria, fungi and actinomycetes groups. Actinomycetes are a Gram-positive bacteria group which is able to decompose organic materials by producing various types of hydrolytic enzymes such as cellulases, xylanases, pectinases, and proteases. Actinomycetes species are dominated by *Streptomyces* spp. (Soeka et al. 2019).

The role of *Streptomyces* spp. in coffee pulp waste decomposition was investigated in this research. The coffee pulp waste composted by adding *Streptomyces* spp. producing cellulase enzymes as decomposer microbes. These microbes have been previously studied as cellulase and chitinase producers, phosphate solvent, nitrogen providers, and IAA- phytohormones producers (Fatmawati et al. 2019). This study aimed to determine the best nutrient content from coffee pulp composting by *Streptomyces* spp. as a decomposer microbe. In addition, this research aimed to obtain the best compost formulation which can be used as biological fertilizer for increasing the growth of chili plant.

## MATERIALS AND METHODS

### Materials

In this study, there are several equipments for composting process, namely: polyethylenebuckets with lids 30L in capacity, PVC tube with the diameter of 3 cm, gardening towel, weight scales, pH meters, thermometers, and hand sprayers. In addition, the materials that are used in this study were coffee pulp waste from wet and dry processing (from Banyuanyar, Boyolali, Central

Java), soil, ISP2 medium, actinomycetes isolates namely ASR 45, ASR 47, ASR 49, ASR 58, ASR 67, ASR 69, and ASR 71 (collection of Microbiology Laboratorium Universitas Sebelas Maret), EM4, zeolite, chili seeds as a plant for testing, and water.

## Methods

### Preparation of coffee pulp

The coffee bean pulp waste was prepared and weighed. Ten grams of samples were used for proximate analysis, including sugar content using the Luff Schroll method (Taufik & Guntarti 2016), fiber and moisture content using the gravimetric method.

### Hydrolytic assay of coffee pulp by actinomycetes strain *in vitro*

10 g of coffee pulp powder are added to the ISP4 agar medium (10 g coffee pulp powder, 1 g $K_2HPO_4$, 1 g $MgSO_4$, 1 g NaCl, 2 g $(NH_4)_2.SO_4$), 2 g $CaCO_3$, 0.1 ml $MgCl_2.7H_2O$, 0.1 ml $FeSO_4$, 0.1 ml $ZnSO_4$, agar 18 g, 1 L distilled water). Afterwards, 4 plugs of 7 days old actinomycetes culture on the ISP2 agar medium were inoculated on ISP4 agar containing coffee pulp powder 1% (v/w) medium and incubated at 28° C for 5 days. After 5 days of incubation, the hydrolytic activity of actinomycetes cellulolytic enzymes was observed by dripping 1% Congo Red solution on the actinomycetes colony. The diameter of the clear zone and the hydrolytic index (HI) value were then measured (Ferbiyanto et al. 2015).

### Composting coffee pulp

The composting procedure of coffee pulp waste is as follows: 5 kg of coffee pulp waste were prepared from each wet processing, dry processing, and a mixture of both processes. The coffee pulp waste is then chopped into smaller sizes to facilitate the decomposition process and mixed with manure in a 4: 1 ratio. Each mixture was separated into three parts for four treatments which consist of: (1) EM4 5% (v/w), (2) cellulolytic actinomycetes 5% (v/w) with $10^{-8}$ CFU/ml, and (3) inorganic activator (Zeolite) 5% (w/w), and (4) combination of cellulolytic actinomycetes 2.5% (v/w) and zeolite 2.5% (w/w). The materials were put into the bucket and tightly covered with plastic lid for a week to permit the fermentation process. In addition, biomass homogenization is carried out by stirring along the fermentation process. The ripened compost was marked by maturity parameters such as compost texture, pH, temperature, and color that were measured and observed at the end of fermentation. Apart from physical parameters, analysis of nutrient content was also carried out involving organic N, P, K, C, and moisture content (Setyorini 2006).

### Growth promoter assay of coffee bean pulp compost on chili plants

A greenhouse experiment was conducted to determine the effect of coffee pulp compost on chili plant growth. The experiment was conducted using a

completely randomized design in three replication. The five experimental groups consist of planting medium amended with: (1) coffee pulp composted with EM4 5%, (2) coffee pulp composted with cellulolytic actinomycetes 5%, (3) coffee pulp composted with zeolite 5%, (4) coffee pulp composted with combination of cellulolytic actinomycetes 2.5% + zeolite 2.5%, and (5) soil without compost for the control. Planting chilies begin with sowing chili seeds in planting medium pot containing 3 seeds in each pot. Each replication consisted of 3 pots. The planting medium consists of compost of coffee bean pulp waste and soil with a ratio of 1: 1. The chili plants were then placed in a greenhouse for 6 weeks. After the first 2 weeks of days after planting, the plant height, number of leaves, and plant biomass of the chili plants were observed everyweek.

## RESULTS AND DISCUSSION

### Proximate content of coffee pulp

The proximate levels of coffee pulp analysis were conducted to determine the amount of sugar and fiber content in the coffee pulp. The data from the proximate analysis of the coffee pulp are presented in Table 1.

**Table 1**. The results of the coffee pulp proximate analysis.

| No | Parameter | Result | unit | Methods |
|---|---|---|---|---|
| 1 | Total sugar | 0,12 | % | Luff Schroll |
| 2 | Raw fiber | 31,9 | % | Gravimetric |
| 3 | Total lipid | 0, 24 | % | Gravimetric |

Based on the data above, it is known that the crude fiber content is 31.9% which indicates that fiber is the predominant composition of the coffee pulp. Klingel et al. (2020) stated that the coffee husks are composed of skin, pulp, and parchment. The coffee husk contains 8%–11% of protein, 0.5%–3% of lipids, 3%–7% of minerals, and 58%–85% of total carbohydrates. Meanwhile, the fiber amount of coffee husk contains 24.5% of cellulose, 29.7% of hemicellulose, and 23.7% of lignin. The composition of fiber i.e. cellulose, hemicellulose, and lignin are polysaccharide sugar groups that have β (1-4) glycosidic bonds which tend to be stable and are difficult to hydrolyze.

Therefore, to describe the composition of these compounds, it is necessary to have a source of β (1-4) glycosidase or hydrolytic enzymes that can hydrolyze these polysaccharide compounds. These enzymes are obtained from microorganisms distributed in both prokaryotic and eukaryotic domains including bacteria, fungi, and actinomycetes (Ahmed et al. 2017). Actinomycetes are attractive microbial group for the production of lignocellulose degrading enzymes. They are known as Gram-positive group bacteria that are capable to produce cellulase enzymes and have been applied in several composting processes include coffee pulp. Therefore,the actinomycetes have been always predominantly found in the compost. The addition of

organic compost increased the number of actinomycetes compared to the untreated soil and also provides lower incidence of phytopatogen (Javoreková et al. 2019). Hence, the hydrolysis of coffee pulps in this study was conducted using several strains of actinomycetes.

## The hydrolytic activity of actinomycetes in hydrolyzing coffee pulp *in vitro*

The *in vitro* analysis results showed that 7 isolates of actinomycetes have hydrolytic activity with the formation of a clear zone around the bacterial colony (Figure 1). Meanwhile, the hydrolysis index is various among actinomycetes isolates. The highest hydrolysis activity was shown by ASR 45 isolate with a hydrolytic index of 3.85, while the lowest hydrolysis activity was shown by ASR 49 isolate with a hydrolytic index of 1.7 (Table 2).

**Table 2**. The hydrolytic index of actinomycetes isolates on the coffee pulp substrate using the plug agar method.

| No | Isolate Code | Hydrolytic Index (HI) |
|---|---|---|
| 1 | ASR 45 | 3.81 |
| 2 | ASR 47 | 3.04 |
| 3 | ASR 49 | 1.79 |
| 4 | ASR 58 | 2.00 |
| 5 | ASR 67 | 3.04 |
| 6 | ASR 69 | 2.71 |
| 7 | ASR 71 | 2.13 |

According to the data above, the 7 isolates of cellulolytic actinomycetes can hydrolyze coffee pulp cellulose and can be used to degrade coffee pulp waste. The ability of actinomycetes isolates to degrade coffee pulp polysaccharides *in vitro* is indicated by the formation of a clear zone around the actinomycetes colony (Figure 1). The coffee pulp in growth medium is hydrolyzed by an extracellular hydrolytic enzyme which is produced by cellulolytic actinomycetes isolates. The type of hydrolytic enzymes produced by actinomycetes isolates is depending on the type of polysaccharide compounds contained in coffee pulp waste. Some actinobacteria were reported

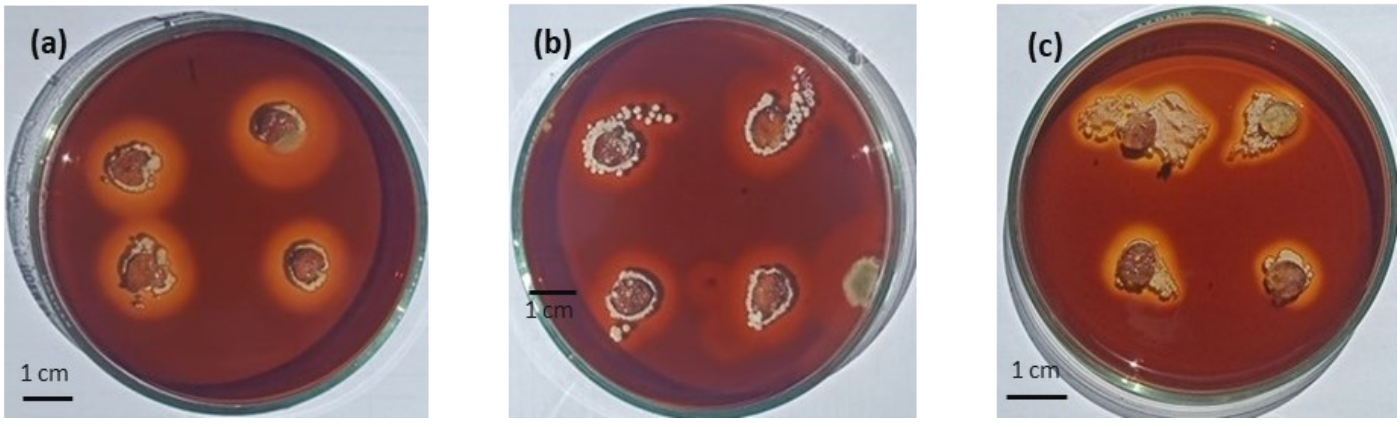


**Figure 1**. The clear zone around the actinomycetes colonies is an indicator of the activity of cellulolytic enzymes. (a) ASR 45 isolate; (b) ASR 67 isolate; (c) ASR 69 isolate.

can produce cellulase like *Streptomyces albogriseolus, Streptomyces lividans*, *Cellulomonas fimi, Microbispora bispora*, and *Thermobifida fusca* (Saini et al. 2015), as well as hemicellulase in *Streptomyces* spp. (Boroujeni et al. 2012).

## Physical and chemical parameters as a quality indicator of coffee pulp compost

The physical parameters of compost, including temperature, pH, smell, color, and texture were measured after 21 days of incubation (Table 3). The results showed that the temperature of compost decreased continuously after 7 days of incubation in all treatments. According to (Lucitawati et al. 2018) the decreasing of compost temperature at the end of the composting period is due to the reduction of nutrient availability. (Wahyono 2011) also mentioned that increasing of compost temperature is caused by a high level of microbial activity during the composting process. The temperature range of all composting treatments was 29°C to 47°C which is appropriate with optimum temperature for composting process i.e. 27°C - 60°C (Chinakwe et al. 2019). The compost pH tends to increase until 21 days of incubation with a pH value of 8.3 to 8.7. From the research conducted by (Huang et al. 2004) the increase of compost pH occurred due to the production of ammonia through the ammonification process and mineralization of organic nitrogen by microorganisms. The odor parameters showed that the compost product from EM4 treatment (P1), cellulolytic actinomycetes treatment (P2), and zeolite treatment (P3) have more intense smell than compost product from the combination strarter of cellulolytic actinomyecetes and zeolite treatment (P4). Meanwhile, the color of compost in all treatments were darker along the incubation period. These change indicate the high temperature during the composting (Ilvo et al. 2020). The compost texture also tends to be more delicate during 21-days of incubation, except the compost resulted from EM4 treatment (P1) which becomes rougher at 21 days of incubation. The darker color of compost indicates high humus content that is beneficial to reduce the absorption of high solar energy and temperature fluctuations of soil if applied to the plant (Setyorini 2006).

The organic nutrient (N, P, K, C) and moisture content of compost during incubation are displayed in Table 4. According to the nutrient analysis results, nitrogen (N) content in compost from cellulolytic actinomycetes composted (P2) and EM4 composted (P1) treatments tend to increase up to 25 mg/kg and 24.6 mg/kg respectively on the fourth week. The increase of nitrogen levels in P1 and P2 treatments was due to an excretion process by microorganisms were in P1 and P2 inoculums, there were microbes which were capable of converting ammonia to nitrate by nitrifying bacteria at the end of the fermentation process. In addition, the presence of microorganisms also contributed to some single-cell proteins obtained during fermentation, after the decomposition process, nitrogen was released back as one of the components contained in fertilizers (Sundari et al. 2014). On the other hand, the addition of zeolite 5% (w/w) (P3) and the combination of

**Table 3**. Physical parameters of compost after 21 days of the incubation period.

| No | Treatment | Incubation Time | | | |
|---|---|---|---|---|---|
| | | 0 day | 7 days | 14 days | 21 days |
| | | Temperature (°C) | | | |
| 1 | P1 | 31 | 47 | 39.5 | 35 |
| 2 | P2 | 29 | 41 | 35 | 35.5 |
| 3 | P3 | 29 | 43.5 | 38.5 | 34 |
| 4 | P4 | 29 | 46 | 35.5 | 36 |
| | | pH | | | |
| 1 | P1 | 5.3 | 6.4 | 7.45 | 8.32 |
| 2 | P2 | 6.9 | 8.4 | 8.35 | 8.38 |
| 3 | P3 | 6.5 | 7.5 | 8.45 | 8.52 |
| 4 | P4 | 6.9 | 7.8 | 8.65 | 8.71 |
| | | **odor** | **odor** | **odor** | **odor** |
| 1 | P1 | + | ++ | ++ | + |
| 2 | P2 | + | ++++ | ++++ | ++++ |
| 3 | P3 | + | +++ | +++ | +++ |
| 4 | P4 | + | + | + | ++ |
| | | **color** | **color** | **color** | **color** |
| 1 | P1 | * | * | * | *** |
| 2 | P2 | * | ** | ** | ** |
| 3 | P3 | * | **** | **** | **** |
| 4 | P4 | * | *** | *** | ** |
| | | **Texture** | **Texture** | **Texture** | **Texture** |
| 1 | P1 | + | ++ | + | + |
| 2 | P2 | + | ++++ | ++++ | ++++ |
| 3 | P3 | + | + | ++++ | ++++ |
| 4 | P4 | + | +++ | +++ | +++ |

**Notes:**

P1 = EM4 5% (v/w)

P2 = Cellulolytic actinomycetes (*Streptomyces* spp.) 5% (v/w)

P3 = Zeolit 5% (w/w)

P4 = Cellulolytic actinomycetes 2.5% (v/w) +Zeolit 2.5% (w/w)

**Odor**

\+ = no odor

++ = smelly

+++ = strong smell

++++ = very strong smell

**Color**

* = light brown

** = brown

*** = dark brown

**** = black

**Texture**

\+ = rough

++ = rather rough

+++ = tender

++++ = delicate

cellulolytic actinomycetes 2.5 % (v/w) and zeolite 2.5% (w/w) (P4) treatments show that the nitrogen content was reduced during the 4-week incubation period. This may have occurred in the evaporation of nitrogen into the atmosphere, in addition to the presence of zeolite molecules that absorb ammonium ions to the surface of the zeolite and are tightly bound, then slowly released into the environment and to the plants (Soudejani et al. 2019). The organic carbon of compost sample was decreased during the 4 week incubation period in P1 and P2. The decrease of organic carbon was caused by the reduction of available carbon sources to the synthesis reaction

**Table 4**. Results of the analysis of the parameters of organic N, P, K, C, and moisture content of coffee pulp compost.

| No | Treatment | Chemical Parameters | | | | | |
|---|---|---|---|---|---|---|---|
| | | N (mg/kg) | P (mg/kg) | K (mg/kg) | C organic (mg/kg) | Water content (%) | C/N ratio |
| 1 | P1M1 | 16.62 | 5.10 | 25.70 | 438.65 | 0.122 | 26.39 |
| 2 | P1M2 | 17.87 | 1.90 | 14.52 | 403.79 | 0.122 | 22.59 |
| 3 | P1M3 | 19.37 | 4.77 | 23.77 | 401.80 | 0.009 | 20.74 |
| 4 | P1M4 | 24.60 | 7.05 | 32.66 | 370.50 | 0.059 | 15.41 |
| 5 | P2M1 | 14.09 | 4.67 | 17.37 | 438.65 | 2.41 | 31.13 |
| 6 | P2M2 | 26.09 | 4.93 | 27.52 | 400.75 | 2.09 | 15.36 |
| 7 | P2M3 | 21.39 | 8.90 | 27.25 | 397.52 | 3.03 | 18.58 |
| 8 | P2M4 | 25.00 | 7.05 | 33.00 | 387.70 | 1.31 | 15.50 |
| 9 | P3M1 | 22.00 | 5.50 | 22.48 | 283.66 | 2.61 | 12.89 |
| 10 | P3M2 | 21.14 | 5.46 | 20.06 | 305.73 | 2.36 | 14.46 |
| 11 | P3M3 | 14.75 | 5.49 | 18.68 | 297.60 | 0.77 | 20.17 |
| 12 | P3M4 | 4.42 | 5.91 | 15.93 | 307.58 | 1.03 | 69.58 |
| 13 | P4M1 | 29.46 | 3.76 | 12.30 | 325.54 | 2.52 | 11.05 |
| 14 | P4M2 | 25.36 | 7.80 | 18.92 | 326.47 | 2.13 | 12.87 |
| 15 | P4M3 | 22.06 | 6.67 | 20.25 | 314.88 | 1.26 | 14.27 |
| 16 | P4M4 | 10.26 | 6.74 | 33.56 | 363.17 | 1.02 | 35.39 |

**Note:**

P1 = EM4 5% (v/w)

P2 = Cellulolytic actinomycetes (*Streptomyces* spp.) 5% (v/w)

P3 = Zeolit 5%

P4 = Cellulolytic actinomycetes (*Streptomyces* spp.) 5% (v/w) + Zeolit 5%

M1 = time incubation 1st week

M2 = time incubation 2nd week

M3 = time incubation 3rd week

M4 = time incubation 4th week

of the new complex and polymerized organic compounds (humification) during the maturation phase (Bernal et al. 2009). Other posibilities can also causethe loss of carbon in the form of $CO_2$ during the decomposition process (Getahun et al. 2012). While in the P3 and P4 treatments, the organic C content fluctuated. The coffee pulp which contains mostly of fiber is great carbon source for actinomycetes. This is corresponding to Khatoon et al. (2017) which stated that the availability of carbon sources in the decomposition process is very important to provide energy for microorganisms to grow optimally. The initial C/N ratio of coffee pulp composition in P1 and P2 treatment complied with the requirement of organic material to be composted according to the Indonesian National Standard (SNI 19-7030-2004) of mature compost i.e. 10-20. The initial C/N ratio will affect the rate of composting process by microorganisms including cellulolytic actinomycetes which inoculated in the coffee pulp. When the initial C/N ratio exceeds approximately 30, the composting process will occur longer. Otherwise, if the initial C/N ratio is too low, the nitrogen will volatilize along the composting process (Amalia 2016). Surtinah (2013) stated that the C/N ratio of compost indicated the maturity rate of compost, the higher

C/N ratio of compost showed that compost has not decomposed completely. In addition, the final C/N ratio of compost which is lower than 20 indicated that nutrient contained in organic waste has been degraded and mineralized into available nutrient for plant (Hanafiah 2005).

The phosphorus (P) content in compost from all treatments showed an increase on the fourth week of incubation. The highest increase in phosphorus and potassium (K) content after 4 weeks of incubation was seen in coffee pulp compost with the actinomycete inoculum (P2) which were 7.05 mg/kg and 33.0 mg/kg, respectively. In addition, the potassium (K) content in compost from P1and P4 treatments also increased. Meanwhile, the moisture content which indicates the quantity of water is contained in the compost decreased in all treatments. Generally, the phosphorus and potassium content as well as the water content in compost, also meet the Indonesian National Standard (SNI 19-7030-2004) of nutrient content in compost. The phosphorus and potassium elements in $P_2O_5$ and $K_2O$ are macronutrients in which their amount was determined by the compost material (Amalia 2016). Besides the final C/N ratio, the P and K content in compost also indicated that actinomycetes are able to transform coffee pulp-containing fiber into organic matter effectively due to their ability to produce cellulolytic enzymes (Saini et al. 2015).

## Effect of compost on the growth of chili plants in the greenhouses

Compost has an important role in plant growth and development due to its nutrient content and potency to increase the soil physicochemical properties. As an organic fertilizer, compost provides nutrient that can be used directly by the plant. To determine the effect of coffee pulp compost on chili plant, we cultivated the chili plant on modification growing media in the greenhouse. The growing media for chili plants consisted of soil, coffee pulp compost, and sand with a ratio 2: 1: 1, respectively. The growth parameters of chili plants such as the stem height, the number of leaves, and dry biomass were measured each week a long four weeks of cultivation.

The length of stem, the number of leaves, and biomass of chili plants treated with coffee pulp compost grew better than chili plants without coffee pulp compost. The highest stem height and number of leaves are generated from chili plants with coffee pulp composted by 5% zeolite treatment (Figure 2). The length of stem of chili plant treated with coffee pulp compost added with zeolite was higher than untreated plant. Zeolite is an aluminum silica crystal flake that is hollow in it and has metal ions, usually alkaline or alkaline earth, and can move freely (Kurniasih et al. 2017). Zeolite is widely used in agricultural land and functioned as a soil repairer to improve soil cation-excange capacity (CEC) and pH value in acidic soils as well as increase the soil's ability to absorb and to release water and nutrients slowly (Widyanto et al. 2013). Zeolites can also improve soil aggregation thereby increasing soil pores which stimulate plant root growth.

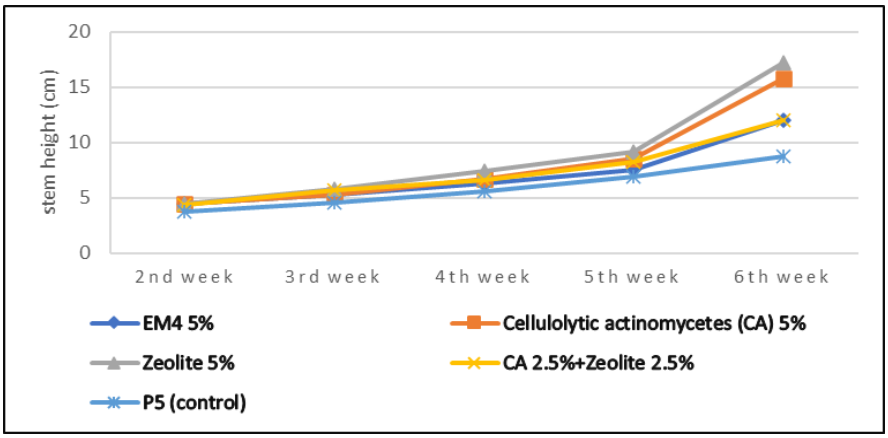


**Figure 2**. Stem height of chili plants treated with various coffee pulp compost.

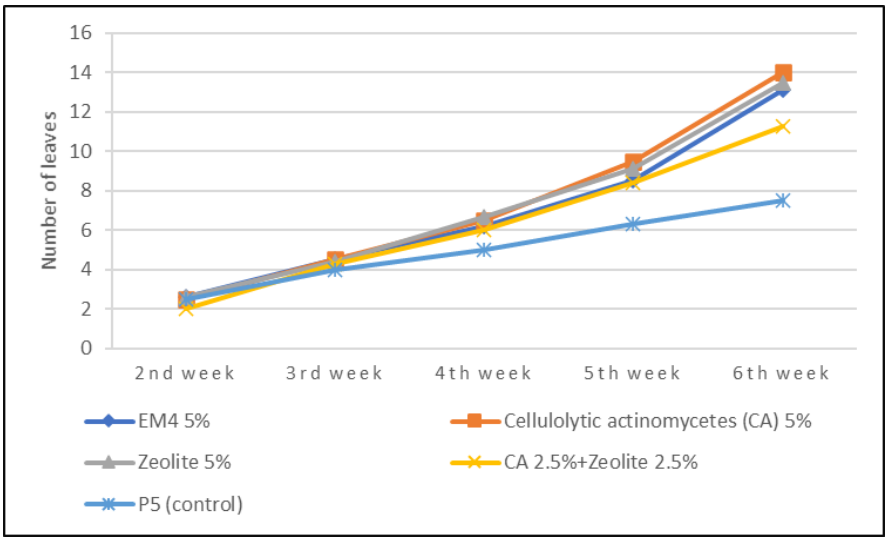


**Figure 3**. Number of leaves of chili plants treated with various coffee pulp compost.

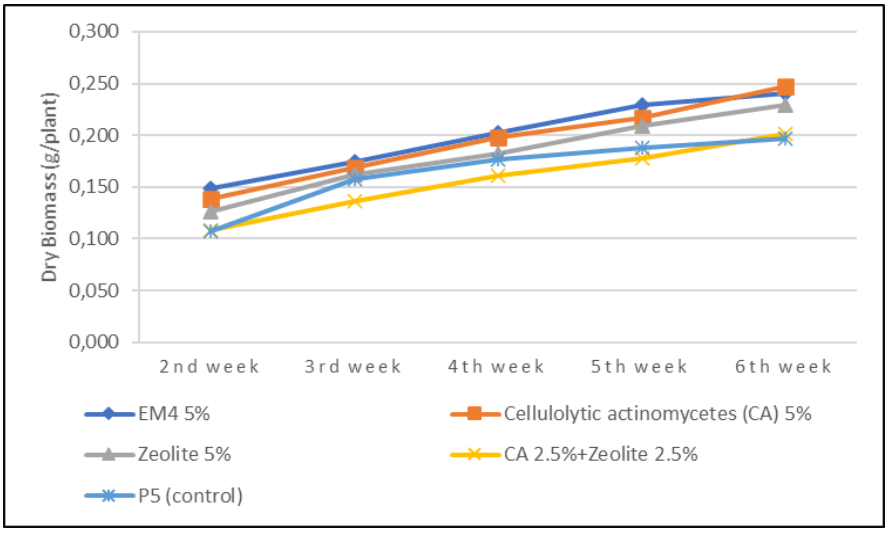


**Figure 4**. Biomass of chili plants treated with various coffee pulp compost.

The coffee pulp compost treatment also positively affects the number of chili leaves compared to the control treatment (Figure 3). The chili plant with coffee pulp composted by cellulolytic actinomycetes resulted the highest number of leaves i.e. 13.1 ± 2.8, increased by 96% compared to the control.

While the highest biomass was plant treated by amending compost by cellulolytic actinomycetes starter increased by 25% compared to the control (Figure 4). In this study, actinomycetes involved in the degradation of coffee pulp into compost which is rich in organic matter that can be used directly by the chili plant. In addition, the existence of cellulolytic actinomycetes in coffee pulp compost also increases the growth of the chili plant due to its role as plant growth promoter bacteria. As previously mentioned, cellulolytic actinomycetes isolate used in this research are capable to dissolve phosphate, providing nitrogen, and producing IAA- phytohormones (Fatmawati et al. 2019). Actinomycetes are Gram-positive bacteria that have a mycelium-shaped morphology that is commonly found in soil. The provision of actinomycetes culture to increase plant growth has been widely studied, including maize (Wahyudi et al. 2019); tomatoes (El-Tarabily 2008); wheat (Sadeghi et al. 2012), rice (Gopalakrishnan et al. 2013). Actinomycetes directly play an important role as plant growth-promoting rhizobacteria (PGPR) by producing phytohormones, nitrogen fixers, phosphate solvents, and siderophore production (Khamna et al. 2010).

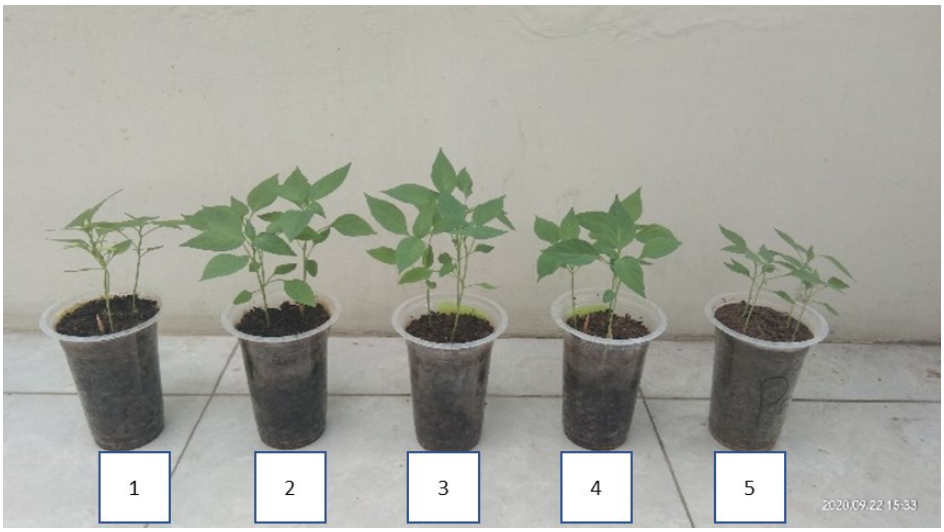


**Figure 5**. The growth of chili plants in the treatment of the planting medium with coffee pulp compost in a greenhouse. (1) EM4 5%; (2) 5% cellulolytic actinomycetes; (3) zeolite 5%; (4) 2.5% cellulolytic actinomycetes and 2.5% zeolite, and (5) soil without the addition of compost.

According to this study, the addition of coffee pulp compost that was decomposed using cellulolytic actinomycetes starter was proven to increase the growth of chili plants in the greenhouse (Figure 5). Furthermore, the coffee pulp compost is potentially applicable to other horticultural crops in the field and can substitute the use of chemical fertilizer in the future. Coffee pulp compost can be used as a soil conditioner to increase soil organic matter content, thereby increasing soil fertility. Good compost must meet the following requirements: contains slow-release nutrients and improves soil fertility. The existence of compost can alter the loose soil texture as a result of the decomposer microorganisms' activity as well as soil microbial myceli-

um which functions as soil particle adhesive. This is also related to the presence of actinomycetes in the compost where their mycelium can serve as soil particle adhesive and improve soil aggregates which create appropriate soil area for plants (Setyorini 2006). Application of compost to the soil will also increase the population of beneficial microbes in the soil, as well as suppress several types of soil pathogenic microbes (Bonanomi et al. 2020). The addition of organic compost can control soil borne pathogen directly by producing the fungitoxic compound (Blok et al. 2000) or indirectly enhancing the suppresive microbiome by the competition of space and nutrient (Hoitink & Boehm 1999), direct parasitism (Bonilla et al. 2012), and antibiosis (Raaijmakers & Mazzola 2012).

## CONCLUSION

According to this research, seven selected cellulolytic actinomycetes isolates have hydrolytic activity toward coffee pulp waste based on *in vitro* assay and proven to be effective in the coffee pulp composting process. This result indicated that the actinomycetes have the potency to be utilized as a composter agent of coffee processing waste to generate a good quality of coffee waste compost. Coffee pulp composted with cellulolytic actinomycetes showed the best result in nutrient content parameters especially in nitrogen content (25 mg/kg), P (7.05 mg/kg), K (33.0 mg.kg) as well as the delicate texture in the physical parameter during the four week incubation. The coffee pulp compost resulting from cellulolytic actinomycetes is also effective to increase the growth of chili plants in greenhouse experiments. The treatment of coffee pulp compost resulting from cellulolytic actinomycetes and zeolite showed the equivalent effect to the height of the chili plant. However, the highest number of chili leaves and the plant biomass were resulted from the coffee pulp compost resulting from cellulolytic actinomycetes Furthermore, it is necessary to prove the effect of coffee pulp compost on other horticultural crops in greenhouse and field experiments.

## AUTHORS CONTRIBUTION

UF was responsible for planning and conducting the research, collecting, analyzing the data, and writing the manuscript. DPS was responsible for conducting the research and interpreting the data. MI collected and interpreted the data. SS was responsible for providing the material and interpreting the data. H was responsible for conducting the research and interpreting the data. SMW was responsible for designing the methodology and writing the manuscript

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## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Research Article

# Cytotoxic Evaluation of *Eurycoma longifolia* Jack Root Extract on Chromosome Aberrations in Human Lymphocytes *In vitro*

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## ABSTRACT

This study aimed to investigate chromosomal aberrations of *Eurycoma longifolia* Jack (*EL*) root extract in human lymphocytes *in vitro*. Human whole blood was cultured in medium solution that treated with distilled water, 20% DMSO, extract of *EL* roots at the concentration of 2.5, 5, 10, 20, 40, 80 µg/mL (extracted with distilled water and ethanol), and nontreated (blank: only culture medium and whole blood). All experiments were cultured for 72 hours in the 37°C incubator .The effects of *EL* roots extract on cytotoxicity were compared with the control groups including the blank, distilled water, and 20% DMSO. This study found that *EL* root extract significantly decreased metaphase cell number and increased chromosome aberrations dose dependent manner ($p<0.01$). The 7 types of chromosome aberration that were observed consisted of dicentric chromosome, single chromatid breaks, isochromatid break, isochromatid gap, single chromatid gap, fragmentation, and deletion. The dicentric chromosome was the most common chromosomal aberrations type that was treated with *EL* root extract both distilled water and ethanol. Moreover, the ethanolic extract of *EL* root was more effective to stimulate chromosome aberrations compared to the water extract of *EL* root (the deletion and fragmentation were not found in the water extract of *EL* root). This study demonstrated that the phytochemicals of *EL* root extract had cytotoxicity effect (decreased metaphase cells and increase cells death) and genotoxic effect (increased chromosomal aberrations. The use of *EL* root crude extract with distilled water is therefore safer for cells. However, when *EL* is used at high levels, it may lead to the inhibition of cell division process and cause side effects (toxicity). *EL* extracts consist of various phytochemicals with different properties and dosages, thus more studies should be conducted on the effect of those substances on cytotoxicity, especially their effects on genotoxicity humans.



## INTRODUCTION

Amongst the tropical herbal plants in Southeast Asia, *Eurycoma longifolia* Jack

(*EL*) is a well-known and widely used medicinal herb. It is a native flowering and a long life of the family Simaroubaceae. There are different names for each area, such as Pasak Bumi or Bedara Pahit (Indonesia), Cay ba binh (Vietnam), Tongkat Ali, Ali's Umbrella or Malaysia ginseng (Malaysia), tho nan (Laotian), and Ian-don (Thailand) (Rehman et al. 2016). It is an evergreen, slender, tall, slow-growing shrub plant with maximum height of 15-18 m and commonly found in tropical forests. It is also popular as medicinal plant. The local people claim that the tea made from this plant can improve sexual abilities and virility (Kuo et al. 2003). It has now become a protected plant species to avoid extinction due to high demand for herbal medicines and health supplements production (Kuo et al. 2003). The *EL* root is commercially available in powdered form, formulated crude extract, capsule, tablet and is commonly used as food additives in beverages such as coffee and tea in healthy food markets in Southeast Asia. The well-known commercial trademarks of *EL* in markets are Maxidus®, LIbidus®, Passion Rx®, Tribulus®, and LJ100® (Bhat & Karim 2010). Among consumers, *EL* in the capsules with raw crude root powder or standardized *EL* extract is the most common form-of using. This standardized *EL* extract includes the processes of extracting the active ingredients, adjusting the preparation to a defining content of a constituent, and concentrating it to a standard level (Mohd et al. 2012) There is a high demand of *EL* production for herbal medicines and supplement with more than 54,000 kg per year for commercial product development and 200 *EL* root products registered with the National Phamaceutical Control Bureau of Malaysia (NPCB) (Rehman et al. 2016).

*EL* is considered as one of the King of herb. Its popularity is due to high amount of various bioactive compounds. The phytochemicals of *EL* root extracted and isolated by different solvents consisted of eurycomalactone, eurycomanol, triterpenes-type tirucallane, canthine-6-one alkaloid, 9-hydroxycanthine-6-one, 14,15 *β*-hydroxyklaineanone, phenolic components, eurycomanone, quanissoids, 5-hydroxymethyl-9-methoxycanthin-6-one, 1-hydroxy-9-methoxycanthin-6-one, *n*-pentyl *β*-carboline-1-propionate, and tannins (Choo & Chan 2002; Kuo et al. 2004). It has been reported since the 1980s that they were widely applied for the treatment of chronic diseases and wellbeing promotion. Because of its distinctive medicinal value, *EL* root extracts have been intensively investigated for pharmacological purposes. Several studies have performed both *in vitro* and *in vivo* focusing on the aphrodisiac, anticancer, antimalarial, antiosteoporosis, and antidiabetic properties using different assays (Segaran et al. 2021). It is also used as an anti-stress (decrease cortisol) (Talbott et al. 2013), anti-microbial (Khanam et al. 2015), appetite stimulant, health supplement (Rehman et al. 2016), immunomodulation to improve immunity (George et al. 2016), and anti-inflammatory (Han et al. 2016). The *EL* root extract was verified increasing the sexual performance in male rats. It also developed prostate and seminal vesicle's growth (Ang et al. 2000; Ang & Cheang 2001). In 2019, Hien et al. studied the effects of alkaloids from EL roots extract and found significant anti-inflammatory effect *in*

*vitro* and *in vivo*. They suggested that alkaloid exhibits the anti-inflammatory activity via suppression of pro-inflammatory mediators (nitric oxide (NO), inducible nitric *oxide* (iNOS), cyclooxygenase 2 (COX-2). These results demonstrated that *EL* dose-dependently protected mice from lipopolysaccharide (LPS-induced mortality) and inhibited LPS-induced NO production (decreasing NO) as well as the protein iNOS and COX-2 expressions. Therefore, the impacts of *EL* root extract are interesting due to its ability to improve immune system and to prevent diseases in humans. However, most importantly, *EL* is highly recommended to use with precaution due to its toxicity and side effects if used in excessive doses.

Studies in *EL* safety have shown that the composition of *EL* extracted with n-butanol, ethanol, and water is different. The *EL* extract with water is considered the safest among other, as its $LD_{50}$ is comparatively higher (>3000 mg/Kg) than ethanol and n-butanol extracts (most toxic). High doses of *EL* (Tongkat Ai Plus®) (1000 mg/Kg) reduced the percentage of pregnancy rates in rats (Li et al. 2013; Aida et al. 2016). The *EL* extract showed an acute toxicity in mice. Alcoholic extract of *EL* has oral lethal dose 50 ($LD_{50}$) between 1500-2000 mg/kg, whereas the oral $LD_{50}$ of the water extract of *EL* was more than 3000 mg/kg (Rehman et al. 2016). The *EL* root extract was indicated to cause hepatotoxicity in subacute toxicity via daily oral gavages for 28 days, in male rats (Shuid et al. 2011). Toxicity levels and safe doses of a major compound of *EL* eurycomanone) had been *studied* in catfish (*Clarias magur*). The 96 h $LD_{50}$ of eurycomanone was found to be 0.3917 µg/g while the subacute toxicity level and safe dosage of eurycomanone were 0.274 µg/g (70% $LD_{50}$) and 0.137 µg/g (35% $LD_{50}$). Furthermore, there had been a significant in micronuclei, DNA damage, and biochemical parameters in the subacute toxicity groups compared to the safe dosage (35% $LD_{50}$) and control groups. The transcript levels of apoptotic genes were significantly higher in the subacute toxicity groups than those in the optimal dosage and control groups. According to this study, it is concluded that cytotoxic effect of eurycomanone or major compound of *EL* extract depends on level of an administration and extracting solvents (Bhat et al. 2017). Chromosomal aberration assay is a sensitive and reliable test for genotoxic experiments *in vitro*. Nonetheless, chromosome aberrations could rise from secondary mechanisms involved in cytotoxicity. Therefore, these compounds that did not react with DNA were not genetically toxic in vivo, and some positive results *in vitro* aberration assay did not exhibit cytotoxic in human (Galloway 2000). In the past, the *EL* cytotoxic effect was investigated as anti-proliferative, mutagenic effect, and chromosome aberration in animals, virus, bacteria, normal cell, as well as cancer cell. In addition to that, it is also applied as antimicrobials, anti-parasite, anticancer, and antimalaria in humans. The various *concentrations* of *EL* powdered root in the range of 1.25-5.0 mg/mL significantly affected on the cell viability (Li et al. 2013). *EL* was revealed to contain mutagenic and genotoxic substance (alkaloids β-carboline), some components of *EL* could be carry mutagenic effects in low concentration

(Razak & Aidoo 2011). Cytotoxicity effect of *EL* is greatly crucial for further studies in terms of its pharmacological activities. In the meantime, there is no study reported in human cells. This study aimed to investigate further on the cytotoxicity effect and the safety of *EL* root extracts with distilled water and ethanol. Typically, *EL* roots are boiled or pickled in liquor and were previously used as a traditional herbal remedy.

## MATERIALS AND METHODS

### *EL* root sample and extractions

*EL* root was washed in water for 3-4 times and cut to thin sheet, then it was sun dried for 1-2 days. After that, *EL* root was incubated at 60 °C for 4 hours. Dry root of *EL* was grinded to 200 grade mesh and extracted using a ratio of 1 part of *EL* powder per 10 parts of solvent (distilled water or ethanol). In ethanol extraction method, the mixture of *EL* roots was agitated in a shaking water bath at 30 °C for 2 hours, while the sample from distilled water extraction method was shaken at 60 °C for 6 hours. *EL* extract from two solvents were filtered and then dried by vacuum evaporator for ethanol extraction of *EL* root and dried by freeze dehydration for distilled water extraction of *EL* root. All *EL* extracts were stored at 4 °C for further experiments.

### Experimental design

In treated groups, the water crude extract of *EL* roots was dissolved by distilled water while 20% DMSO was used for dissolving ethanol crude extract of *EL* roots. The final concentrations of both *EL* root extracts were 2.5, 5, 10, 20, 40, and 80 µg/mL. In a control group, whole blood cells were culture in a combination of cell culture medium (blank), distilled water, and 20% DMSO respectively without an addition of *EL* extract whilst in treatment groups, cell culture were treated with *EL* extracted with distilled water and ethanol respectively. All experiments in the treated groups and control groups consisted of 2 replications.

### Human whole blood cell culture

The *EL* herbal is commonly used especially among middle age men, but it has side effects and can be dangerous for pregnant and lactating women (Turck et al. 2021). In this study, blood sample was collected (15 mL) from a male volunteer at the aged of 49 years old by the permission of the Srinakarin hospital, the aseptic technique was used during the processes of the blood collection. This study was performed according to the international, national, and institutional rules considering human experiments. The clinical studies received the permission from an ethical committee, Khon Kaen University, Faculty of Science (ID U1-04498-2559). The sample was kept on ice in 5 mL vacuum test tubes coated with heparin. Each T-25 flask for each condition comprised 0.5 mL of whole blood that was collected in 8 mL RPMI 1640 medium. In treatment groups, the sample was supplemented with 100 µL of each concentration of *EL* root extract, whereas the control groups were

filled with distilled water and 20% DMSO (blank did not added with any substance) and incubated at 37 °C with flows of 5% $CO_2$ for 72 hours (Freshney 2005). After incubation, colchicine was introduced and mixed for 30 minutes before a further incubation. After colchicine incubation, the blood mixture was centrifuged 1.200 rpm at room temperature for 10 minutes. After that, the supernatant was discarded by sucking it off carefully with a glass pipette. The cells were treated with hypotonic solution (0.075 M KCl) and kept at room temperature for 30 minutes. They were centrifuged and the supernatant was discarded. Fresh cool Canoy's fixative was used to fix the cells by gradually added up to 8 mL (Sangpakdee 2016). Later, to make the supernatant clear, the fixation would be repeatedly conducted (twice). The cells were finally resuspension in 1 mL fixative, cells suspensions were dripped with 2 drops (20 µL) by micropipette on a clean and cooled glass slide and followed by air drying. Finally, chromosomes were stained using the Giemsa staining. The dried slide was stained with 20% Giemsa's solution for 30 minutes (Rooney 2001).

### Chromosome checking and statistical analysis

The sample in control and treated groups (6 slides/treatment) were prepared for cytotoxic evaluation. The cell counting and recording were performed in mitotic metaphase and abnormal chromosomes under the light microscope at 1,000 times magnification. Distinctly observable chromosome plates with well-spread cells were chosen and pictures were taken by digital cameras. All chromosome parameters such as metaphase index and chromosomal aberrations were analysed by one-way ANOVA as a completely randomized design (CRD) using the general linear models (GLM) procedure of SAS software (SAS® Institute, 2005). All statistical tests in this study were operated at p-value less than 0.05 ($p < 0.05$).

## RESULTS AND DISCUSSION

### Effect of *EL* root extract on mitotic division

Mitogen in medium culture was a stimulated factor for mitotic division of lymphocyte in human whole blood. The results of mitotic division showed that no significant difference among the control groups (blank, distilled water, and 20% DMSO) ($p > 0.05$). The mitotic division of the treated groups tended to decrease dose dependent manner and no mitotic division (no metaphase cell) was observed at the concentration of 80 µg/mL-distilled water and ethanol *EL* root extracts (Figure 1). In all treated groups with *EL* root extract, the number of metaphase cell was decreased and chromosomal aberrations were significantly increased different with the control groups ($p < 0.01$).

Effect of distilled water extract of *EL* root at the concentration of 2.5, 5, 10 and 20 µg/mL showed that the metaphase cell was significantly different with that of at the concentration of 40 µg/mL ($p < 0.01$). As well as, the result of ethanol extract of *EL* root exhibited that the number of metaphase cell and chromosomal aberration were significantly different between the concentration

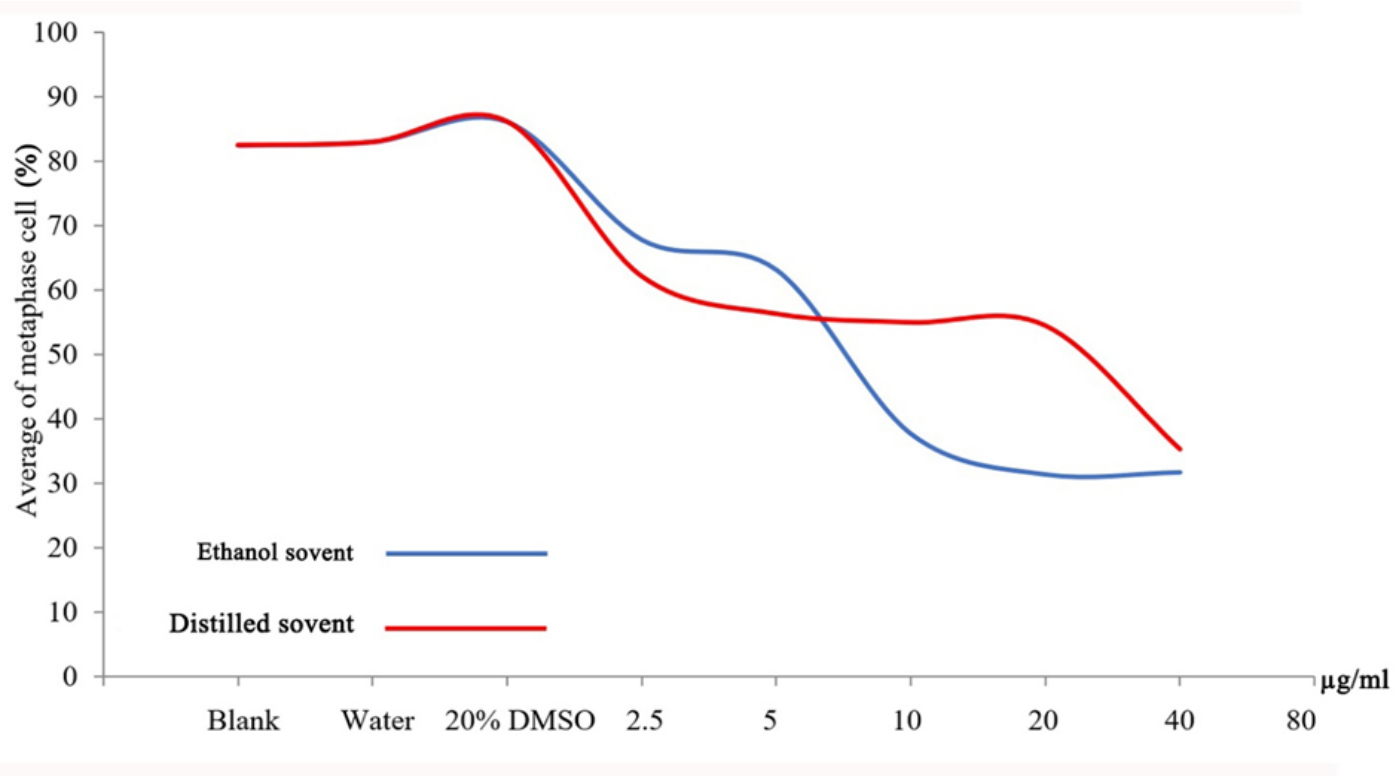


**Figure 1.** Decreasing of mitotic division of male human lymphocytes affected by increased concentration of distilled water and ethanol extract of *EL* roots between 2.5 - 80 µg/mL.

group of 2.5 and 5 µg/mL as well as with the concentration group of 10, 20 and 40 µg/mL (Table 1).

### Effect of *EL* root extract on chromosomal aberration

Human chromosome contains 46 with 44 autosomes and 2 sex chromosomes (Figure 2). During a process of cell division, chromosomal abnormalities rarely occur thanks to the mechanism of cellular repair in a cell cycle. In this study, an extract of *EL* roots induced seven types of chromosomal aberration including

**Table** 1. Effects of *EL* roots extract on average and percentage of metaphase cells compared with control groups.

| Control groups[1] and treated groups[2] | Water extract | | Ethanol extract | |
|---|---|---|---|---|
| | Average of meta-phase cell (cell/20 µL) | % metaphase cell | Average of meta-phase cell (cell/20µL) | % metaphase cell |
| Blank | 82.50 ± 1.17[a] | | 82.50 ± 1.17[a] | |
| Distilled water | 83.00 ± 0.47[a] | | 83.00 ± 0.47[a] | |
| 20% DMSO | 86.17 ± 4.01[a] | 100.00 | 86.17 ± 4.01[a] | 100.00 |
| 2.5 µg/mL | 62.17 ± 5.42[b] | 72.14 ±14.85 | 67.83 ± 7.78[b] | 78.72 ± 23.33 |
| 5 µg/mL | 56.33 ± 1.89[b] | 65.37 ±15.56 | 63.17 ± 4.01[b] | 73.30 ± 12.02 |
| 10 µg/mL | 55.00 ± 2.36[b] | 63.83 ±7.07 | 37.67 ± 8.01[c] | 43.71 ± 24.04 |
| 20 µg/mL | 54.50 ± 2.12[b] | 63.25±12.02 | 31.33 ± 4.72[c] | 36.36 ± 14.04 |
| 40 µg/mL | 35.33 ± 8.49[c] | 41.00 ±1131 | 31.17 ± 7.78[c] | 36.17 ± 23.33 |
| 80 µg/mL | 0.00 | 0.00 | 0.00 | 0.00 |

Remarks: Values in the same column with different letters are significantly different ($p < 0.01$)

[1]Control groups: Blank, Distilled water, 20% DMSO

[2]Treated groups: Concentrations of *EL* root extract at 2.5, 5, 10, 20, 40, and 80 µg/mL

isochromatid break (ISCB), single chromatid gap (SCG), deletion (D), fragmentation (F), single chromatid break (SCB), isochromatid gap (ISCG), and dicentric chromosome (DC) (Figure 3). The total aberration points of all type of chromosomal aberrations in the control groups (blank, distilled water, and 20% DMSO) were 4, 8, and 5 points respectively. Whereas, the total aberration points of all type of chromosomal aberrations in the distilled water extract of *EL* roots groups at the concentration of 2.5, 5, 10, 20, and 40 μg/mL were 29, 43, 53, 55, and 53 points, respectively. Surprisingly, cell division and metaphase cells were not detected when treated with *EL* extract at the concentration of 80 μg/mL, therefore, the number of metaphase cells and chromosomal aberrations cannot be assessed. Dicentric chromosome (DC) was the most common chromosomal aberration type in human lymphocyte that treated with distilled water extract of *EL* root. The percentage of cell with chromosomal aberrations of all treated groups were significantly different compared with the control groups. The extract of *EL* roots had significantly different effects on chromosomal aberrations dose dependent manner. The extract of *EL* roots at the concentration of 40 μg/mL displayed the highest chromosomal aberration and there were significantly different compared to the *EL* roots extract at the concentration of 2.5, 5, 10, and 20 μg/mL ($p < 0.01$) (Table 2).

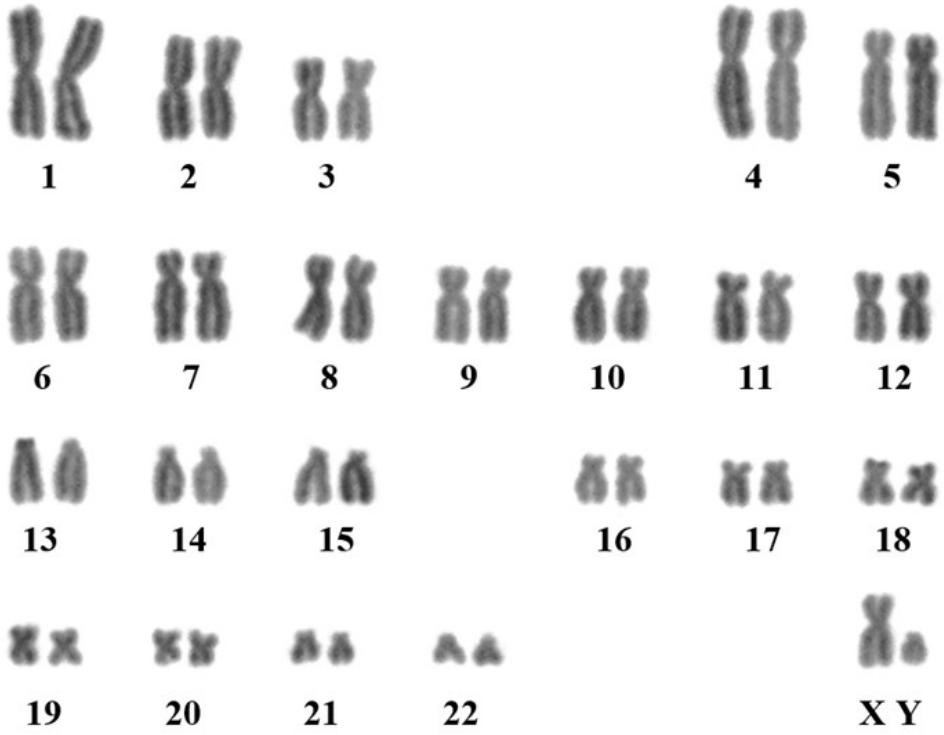


**Figure 2.** Human normal karyotype from normal metaphase cell of this study (control group).

When the cell treated with ethanol extract of *EL* root, the total points of chromosomal aberration types in test groups, including the control group (blank, distilled water, 20% DMSO), at the concentration of 2.5, 5, 10, 20, 40, and 80 μg/mL were 4, 9, 5, 25, 24, 33, 53, 77 and no cell division, respectively. Total 84 dicentric chromosomes (DC) which were observed were the most common ab-

erration type in ethanol extract of *EL* root groups. The number of chromosome aberration of treated groups was significantly different when compared to the control groups ($p < 0.01$). In addition, there were significantly different chromosome aberrations in various concentration groups of ethanol extract of *EL* root ($p < 0.01$) (Table 3).

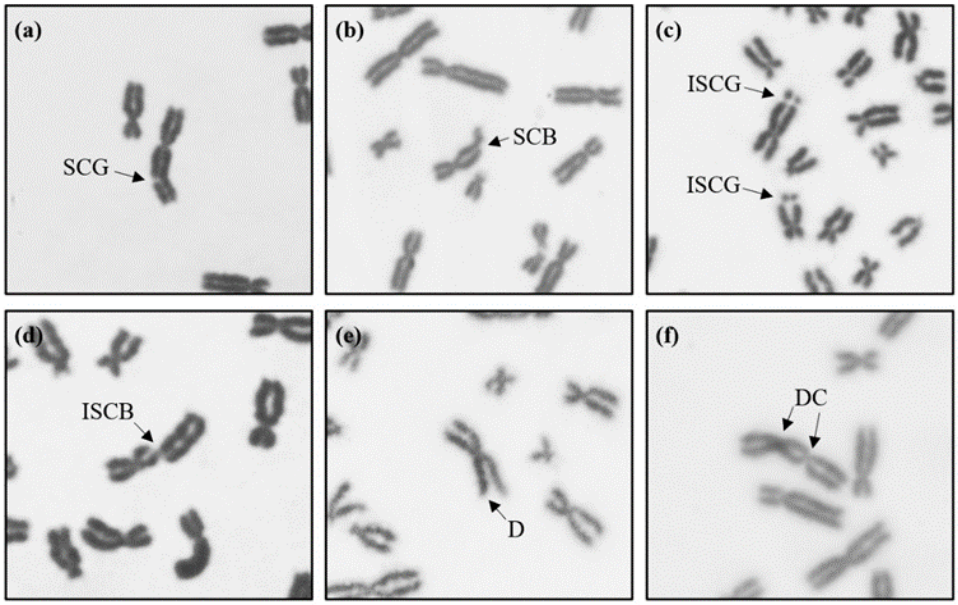


**Figure 3.** Six types of metaphase chromosome abnormalities in male human lymphocytes *in vitro* affected by *EL* root extract, such as single chromatid gap (SCG), single chromatid break (SCB), isochromatid gap (ISCG), isochromatid break (ISCB), deletion (D), and dicentric chromosome (DC).

**Table 2.** Effects of distilled water extract of *EL* roots on number and percentage of chromosomal aberrations in human lymphocytes *in vitro*.

| Control group[1] and treated group[2] | Number of abnormality points | | | | | | | Total of abnormal points | Total of abnormal cells | The percentage of cell number with aberrations (mean ± SD) |
|---|---|---|---|---|---|---|---|---|---|---|
| | SCG | ISCG | SCB | ISCB | D | F | DC | | | |
| Blank | 2 | 0 | 1 | 1 | 0 | 0 | 0 | 4 | 4 | 0.81 ± 0.01[a] |
| Distilled water | 4 | 2 | 1 | 1 | 0 | 0 | 0 | 8 | 7 | 1.41 ± 0.29[a] |
| 20% DMSO | 3 | 0 | 1 | 1 | 0 | 0 | 0 | 5 | 5 | 0.96 ± 0.23[a] |
| 2.5 µg/mL | 6 | 5 | 4 | 3 | 0 | 0 | 11 | 29 | 29 | 7.89 ± 2.58[b] |
| 5 µg/mL | 8 | 10 | 3 | 2 | 0 | 0 | 20 | 43 | 41 | 12.16 ± 1.66[c] |
| 10 µg/mL | 16 | 11 | 1 | 3 | 0 | 0 | 22 | 53 | 51 | 15.44 ± 0.62[c] |
| 20 µg/mL | 14 | 13 | 5 | 4 | 0 | 0 | 19 | 55 | 52 | 15.96 ± 3.22[c] |
| 40 µg/mL | 19 | 12 | 4 | 3 | 0 | 0 | 15 | 53 | 49 | 23.22 ± 0.91[d] |
| 80 µg/mL | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.00 |
| Total | 72 | 53 | 20 | 18 | 0 | 0 | 87 | 250 | 238 | |

Remarks: SCG= single chromatid gap, ISCG= isochromatid gap, SCB= single chromatid break, ISCB=isochromatid break, D= deletion, F= fragmentation and DC= dicentric chromosome.

Values in the same column with different letters are significant different ($p < 0.01$)

[1]Control groups: Blank, Distilled water, 20% DMSO,

[2]Treated groups: *EL* root extract at the concentration of 2.5, 5, 10, 20, 40, and 80 µg/mL

**Table 3.** Effects of the ethanol extract of *EL* root on number and percentage of chromosomal aberrations in human lymphocy.

| Control group1 and treated group2 | Number of abnormality points | | | | | | | Total of abnormal points | Total of abnormal cells | The percentage of cell number with aberrations (mean ± SD) |
|---|---|---|---|---|---|---|---|---|---|---|
| | SCG | ISCG | SCB | ISCB | D | F | DC | | | |
| Blank | 2 | 0 | 1 | 1 | 0 | 0 | 0 | 4 | 4 | 0.81 ± 0.01[a] |
| Distilled water | 4 | 2 | 2 | 0 | 1 | 0 | 0 | 9 | 8 | 1.41 ± 0.29[a] |
| 20% DMSO | 3 | 0 | 1 | 1 | 0 | 0 | 0 | 5 | 5 | 0.96 ± 0.23[a] |
| 2.5 µg/mL | 9 | 4 | 2 | 2 | 1 | 0 | 7 | 25 | 25 | 6.20 ± 1.06[b] |
| 5 µg/mL | 4 | 2 | 1 | 1 | 3 | 2 | 11 | 24 | 23 | 6.21 ± 4.05[b] |
| 10 µg/mL | 7 | 6 | 4 | 1 | 2 | 0 | 13 | 33 | 33 | 14.60 ± 0.03[c] |
| 20 µg/mL | 14 | 8 | 6 | 2 | 2 | 2 | 19 | 53 | 49 | 26.76 ± 9.29[d] |
| 40 µg/mL | 15 | 15 | 4 | 5 | 3 | 1 | 34 | 77 | 70 | 36.30 ± 9.09[e] |
| 80 µg/mL | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.00 |
| Total | 58 | 37 | 21 | 13 | 12 | 5 | 84 | 230 | 217 | |

Remarks: SCG= single chromatid gap, ISCG= isochromatid gap, SCB= single chromatid break, ISCB= isochromatid break, D= deletion, F= fragmentation and DC= dicentric chromosome

Values in the same column with different letters are significant different ($p < 0.01$)

[1]Control groups: Blank, Distilled water, 20% DMSO,

[2]Treated groups: *EL* root extract at the concentration of 2.5, 5, 10, 20, 40, and 80 µg/mL

This study showed that various compounds of ethanol extract of *El* root carried higher level of cytotoxicity than those distilled water extracted. The ethanol extract of *EL* roots and distilled water presented cytotoxic activities, the half maximum inhibitory concentration ($IC_{50}$) on cell division were between the range of 5-10 and 20-40 µg/mL, respectively (Table 1). Some of these results differ from previous studies with cells microorganisms. Kavitha et al. (2010), showed that all fraction from *EL* root possessed a moderate cytotoxicity activity against Vero cell, the 50% cytotoxicity concentration ($CC_{50}$) value was 5-23.50 µg/mL. In the study of Ming et al. (2014), it was indicated that aqueous extract of *EL* roots at dose <5 µg/plate did not show any cytotoxic effect on *Salmonella* spp. *in vitro*. The results showed that the ethanol extract of *EL* roots was more effective to chromosomal aberrations than the distilled water extract of *EL* roots. The effect of *EL* toxicity on the mitotic index (metaphase cell number) was statistically significant ($p<0.01$) in both groups of the ethanol extract of *EL* roots and distilled water. Hence, the decreases of cells during metaphase in the groups treated with *EL* root extracts may be strongly suggested that components in *EL* extracts act as cytotoxic agents. In accordance with *in vitro* studies, the compounds found in the extracts are also associated with cytotoxic effects in humans. Furthermore, the outcome of chromosomal aberration in each test group which affected from *El* roots extracted with distilled water exhibited the significant difference (Table 2) which was in an agreement with the results from the group treated with the ethanol extracts (Table 3). Significant decrease of cell division at higher concentrations (40-80 µg/mL) is due to the suppression of DNA synthesis at S phase of cell cycle as well as-RNA and DNA biosynthesis were impaired and thus formation of nucleic acid and protein was de-

clined (Qari & El-Assouli 2019). It is possible that compounds at high concentrations in crude extracts lead to irreversible DNA damage in lymphocytes (genotoxic effect) and cell death (cytotoxic effect) by inhibiting mitotic event (Askin & Asanturk 2018).

Dicentric chromosome was the most common aberration type that treated with distilled water and ethanol extract of *EL* root groups. In the distilled water extracted groups were discovered five aberration types such as SCG, ISCG, SCB, ISCB, and DC while in the ethanol extract groups were found seven aberration types (SCG, ISCG, SCB, ISCB, D, F, DC) of abnormal chromosome were determined. Deletion and fragmentation were found only in cells treated with ethanolic extract-of *EL* roots which completely cause the breakage chromosome. The completely breakage chromosome was the non-appearing of a little fragment on main centric chromosome (deleted and fragment), but the non-completely breakage chromosome was the appearing of a little fragment on main centric chromosome (gab, break, and dicentric). In the distilled water extract of *EL* root groups cellular samples were only aberration in the non-completely breakage chromosome, but in the ethanolic extract groups, both completely and non-completely breakage chromosomes were observed in the samples. These results may indicate that the presence of one or more components of *EL* root extract is able to break the DNA strands in DNA in a certain way, that may lead to chromatin gap due to deletion or absence of one or more nuclei and couldn't be repaired by the repair mechanism (Qari & El-Assouli 2019). The result from the study in the particle of *EL* root isolated suggested that several compounds such as eurycomanone, eurycomalactone and alkaloids β-carboline may cause acute cytotoxic, genotoxic and mutation. Several investigations reported that *EL* extracts induce side effects on human lymphocyte as well as in animals such as mice, rats, and catfish (Razak & Aidoo et al. 2011; Rehman et al. 2016; Bhat et al. 2017). When consumed by pregnant women, substances in *EL* have direct effects on foetal development by inducing chromosomal abnormalities due to its cytotoxicity. It is also known as a major cause pregnancy loss in humans with more than 90% of early abortions were contributed by foetal aneuploidy (Low et al. 2013).

Currently, prolonged consumption of *EL* root extract has associated with a range of side effects, including sleep apnea, facial flushing, pressure in the testicles, and hyper-aggressiveness, but there is no scientific evidence to support the mechanism of action. In clinical practice, specific dosages of *EL* (water extract) in humans have been suggested and supplementation of the extract for 2-8 weeks at doses of 100-600 mg/day had a pharmacological effect in men (Tambi 2005). Besides that, Bhat & Karim (2010) recommend a safe daily dose of *EL* root extract between 270–350 mg/kg with no cytotoxic effects. Shuid et al. (2011) suggested that oral administration of *EL* extracted with water and ethanol at 300 and 200 mg/Kg/day, respectively did show any sign of toxicity. It should be noted, that we do not recommend to using the *EL* root extracted with ethanol (pickled in liquor) regarding the safety issue (cytotoxicity). An intake of *EL* roots powder including capsules, bolus,

coffee, and tea (water extraction) is better way to prevent cytotoxic effect in consumers. Most importantly, *EL* extract should be used at appropriate level and thereby bioactive components can be excreted from the body without being accumulated in a large amount that may induce health problems in long term consumption.

## CONCLUSION

This study revealed that the herbal extraction method had an effect on various types and quantities of phytochemicals in the crude extract. The phytochemicals of *EL* extracts one or several compounds have a cytotoxic effect and affected the process of cell division or mitotic cell index. The ethanol extract of *EL* roots showed more severe cytotoxic effects than those distilled water extracts. High concentrations of *EL* root extracts contributed an effect (cytotoxicity) on a cell reduction during metaphase and increased chromosomal aberrations (genotoxicity) in human lymphocytes in vitro. Hence, the decrease of the MI values obtained in the blood lymphocytes indicated that *EL* root extract may be suggested as cytotoxic agents. Aqueous extracts are safer than ethanol extracts and the higher concentrations, the susceptible to cytotoxic effect it is. This information is valuable to consumers for a safe selection on *EL* root extract as well as for a production of *EL* extract as medicines and nutritional supplements.

## AUTHORS CONTRIBUTION

SC, IP designed the study. SC, SP performed laboratory work. SC, IP, SP analyzed the data. SP, CS, AT wrote the manuscript. All authors read and approved the final version of the manuscript.

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## CONFLICT OF INTEREST

The authors declared that there is no conflict of interest.

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Research Article

# Carbon Stock Potential of Gara Gola Natural Vegetation in East Hararghe Zone, Eastern Ethiopia

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## ABSTRACT
This study was conducted at Gara Gola, in the Oromia regional state of Ethiopia, to examine the carbon sequestration potentials under three altitudinal gradients [i.e., Lower altitude (LA: 1500–1800 m.a.s.l.); Middle altitude (MA: 1801–2000 m.a.s.l.) and upper altitude (UA: 2001–2300 m.a.s.l.)]. A total of 60 quadrats of 20m x 20m, 5m x 5m, and 1m x 1m with six horizontal transect lines were employed to gather data on the tree, shrub, herbaceous, and soil, respectively. To estimate organic carbon percentage, soil parameters were collected from three soil profiles (i.e., 0 to 10 cm, 10 to 20 cm, and 20 to 30 cm). The mean total carbon stock of the study area was 641.18 Mg ha-1. MA had relatively higher TC than the other gradients. But the LA had the lowest TC stock, due to a high amount of human and animal interference. The results showed that the UA had significantly higher above-ground (AGC) and below-ground carbon (BGC) stocks with 147.3±39.4 Mg ha-1 and 18.37±7.8 Mg ha-1, respectively, compared to other gradients. However, LA had the lowest AGC (66.8±8.7 Mg ha-1) and BGC (12.06±2.6 Mg ha-1). Lower altitude exhibited a significantly higher SOC value than the other two altitudinal gradients followed by MA. The UA had the lowest SOC value. SOC across the three soil profiles follows a reduction trend from topsoil depth to lower soil depth with significant variation. In conclusion, LA should embrace better ecological, policy, and socioeconomic considerations than the other gradients.



## INTRODUCTION
Carbon dioxide (CO2) and other Green House Gases (GHGs) concentrations in the atmosphere are now universally acknowledged as the primary source of global warming. Carbon accumulates at a rate of 3.5 pentagrams per year in the atmosphere. The majority of it comes from fossil fuel combustion and the conversion of the tropical forest into agricultural fields (Paustian et al. 2016). Increased atmospheric concentrations of so-called greenhouse gases are assumed to be the primary cause of the increase in temperature of the earth near-surface, air, and oceans in recent decades (GHGs). CO2 is also a major greenhouse gas (GHG) that contributes to global warming. Thus, Climate change has resulted in a lot of influence on the micro and macroclimate of the world, including losses of biodiversity, degradation of

natural vegetation and soil, and loss of important natural ecosystems with their services and indigenous knowledge (Alemu 2014).

Natural vegetation plays an essential part in mitigating climate change, according to the 1997 Kyoto Protocol, which is the first major international agreement on climate change, by naturally collecting carbon from the atmosphere and therefore lessening the impact of CO2 emissions (IPCC 2007a; Perschel et al. 2007). Natural vegetation potentially stores more than 80% of all above-ground carbon on the planet and 70% of all organic carbon in soil (Fauzi et al. 2017). On the other side, deforestation and forest degradation account for 12 to 20% of annual greenhouse gas emissions, more than the total amount of emissions from all forms of transportation combined (Saatchi et al. 2011). According to a recent assessment, carbon storage in forest biomass dropped by an estimated 0.5 Gt per year between 2005 and 2010, attributable primarily to a loss in worldwide forest acreage (Gebrewahid et al. 2018).

As mountain regions cover about 27.2 % of the global land area and there have been rapid climate changes in mountain regions during the past few decades (IPCC 2007a), understanding the shifts in forest carbon storage and allocation along altitudinal gradients in mountain regions will help us better predict the response of regional and global carbon balance to future climate change (Poudel et al. 2020). The above and below ground carbon stocks were increased with increasing altitude while the total carbon stock decreased with increasing altitudinal gradient (Kumar et al. 2021). The analysis of carbon stock variation of different carbon pools along the altitude of the forest showed a significant variation, whereas the above and below-ground carbon stock variation with slope, a gradient was also significant except soil organic carbon and liter carbon (Kumar et al. 2021).

Climate change consequences are also posing several issues in Ethiopia due to the country inadequate adaptive capacity. The country is vulnerable to climate change due to its remoteness and complexity, low income, and reliance on climate-sensitive economic sectors such as agriculture and pastoralism, it also has a limited adaptive ability (FAO 2016a). Rainfall is becoming more unpredictable as the temperature rises, and the resulting decrease in precipitation is frequently harmful to Ethiopian agriculture (Fonta et al. 2011). Droughts, which are frequently followed by soil erosion, are also becoming further common (Edwards 2010), due to increased deforestation and deterioration of land resources. Population growth has resulted in extensive forest loss for agricultural use, grazing, and exploitation of existing forest for fuelwood, feed, and construction materials. Ethiopia government launched the Climate-Resilient Green Economy (CRGE) in 2011 in response to the effects of climate change, such as rising average temperatures and erratic rainfall patterns. The objective of the CRGE is to protect and restore forests for economic, environmental, and carbon-storage purposes. As an accountable member of the international public, Ethiopia understands the importance of natural vegetation and forests in mitigating global climate change (Eshetu

& Hailu 2020a).

One of the four pillars of Ethiopia Climate Resilient Green Economy (CRGE) Strategy is Reducing Emissions from Deforestation and Forest Degradation (REDD+), to avoid emissions from the forest sector while absorbing greenhouse gases from other sectors to achieve a carbon-neutral economy by 2030 (Gonzalo et al. 2017). Furthermore, REDD+ has the greatest possible for mitigating climate change in a poor tropical country (FAO 2016b). As a result, natural forest management and enhancing their carbon stock potential are critical for large-scale carbon absorption and generating carbon credits to meet the CRGE strategy by increasing carbon sequestration potential and biodiversity conservation while also improving local community livelihoods (Eshetu & Hailu 2020a).

In this context, numerous researches in natural vegetation areas, including Ethiopian rangelands (Alemu 2012; Bikila et al. 2017; Tessema et al. 2017) were undertaken in various parts of the nation, concentrating on carbon stock potential. However, these investigations could not provide complete data on the country carbon stock potential. In east Hararghe, there are no detailed studies on carbon stock potential. Even some of the earlier studies aimed to estimate the potential for biodiversity and carbon storage in vegetation did not take into account ecological gradients such as elevation, slope, and aspect; others were only aimed at contrasting natural forests with different land uses such as community grazing (Chinasho et al. 2015). Ecological parameters such as elevation and slope, on the other hand, have a considerable impact on carbon stock potential (Acharya et al. 2011). More importantly, elevation is an important environmental factor since it influences other nonliving and living factors such as soil, temperature, landscape, and flora (Simegn & Soromessa 2015). Therefore, there is a significant demand for information on natural forest carbon store potential in height, aspect, and slope gradients. As a result, such baseline data will aid in good land use development for huge watershed areas, taking elevation into account.

This study will give baseline information for policymakers, local experts, community members, and researchers on the vegetation ability to mitigate climate change and its impact if the current land use is changed to another. Because the surrounding area is regarded as one of the country industrial pools, this change could happen soon. This modification will add to global GHG emissions and local climate change impacts. This study tested the hypothesis that soil organic carbon (SOC) and carbon stock would increase within creasing altitudes and soil organic carbon stock would increase with increasing natural vegetation and decreasing soil depth (Bargali & Bargali 2020). The primary goal of this study was to evaluate the carbon stock capability of Gara Gola natural vegetation across altitudinal gradients in eastern Ethiopia.

## MATERIALS AND METHODS

### Area Location

The research was administered within the Gara Gola Natural Vegetation within the Goro Gutu district of the Oromia regional state eastern Hararghe Zone. The district covers 531 km², accounting for about 2.35 % of the zone total area. Karamile, the capital, is 108 kilometers west of Harar. The agro-climatic zones of Goro-Gutu district are Dega (2,000-2,657 m.a.s.l), Woinadega (1,500-2,000 m.a.s.l), and Kola (1,500 m.a.s.l), which cover roughly 11, 52 and 37 % of the district total area, respectively (Figure 1). The climate is defined by the district agro-climatic zones. Agriculture (including crop and livestock production) is the people primary source of income and employment. Within the district, the average landholding per household is 0.37 hectares. Agriculture is practiced, with the most common crops being sorghum, maize, and wheat.

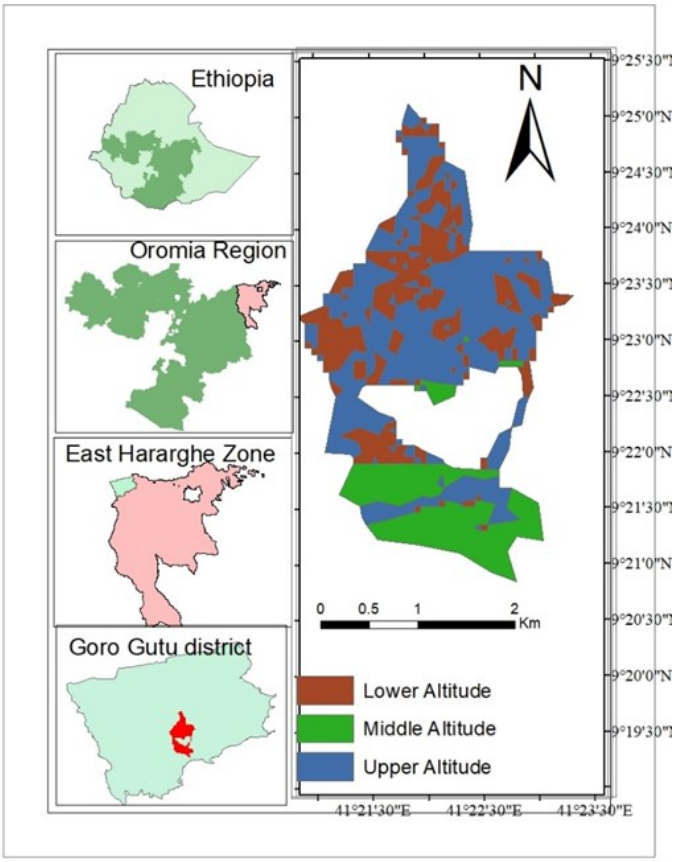


**Figure 1.** Gara Gola Natural Vegetation Map.

### Climatic

The region is classified as a semi-arid tropical belt in eastern Ethiopia, with a sub-humid climate. The area means annual rainfall (2000-2020) is roughly 924.7 mm, according to data from a meteorological station. A bimodal rainfall distribution pattern characterizes the studied area. The local season of short rain, called Badheessa, lasts from March to May, while the main/long rainy season, known as Ganna, lasts from late June to September (Zeleke et al. 2021). The mean monthly low temperature ranges between 4.1°C in De-

cember and 16.4°C in January, though the maximum temperature ranges amid 24.5°C in December and 28.3°C in May, respectively.

## The Study Area Stratification

The study area boundaries were created so that forest carbon stocks could be accurately measured and accounted for. The study area border was delineated using the Global Positioning System (GPS). Lower altitude (LA: 1500–1800 m.a.s.l), Middle altitude (MA: 1801–2000 m.a.s.l), and Upper altitude (UA: 2001–2300 m.a.s.l) were used to classify the study location. Systematic sampling of transect by altitude sections was conducted to establish relatively homogeneous units and obtain accurate data from the fieldwork. The study area had an elevational variation that helped determine the variants in elevation as a predictor variable to relate with woodland carbon stocks—sampling and measurement design.

### Woody Species Sampling

Two parallel transect lines were laid at 200m intervals in each site, parallel to the stand gradient and 50m far from the edge to prevent edge outcome. In each transect line, a quadrat sized 20mx20m was established systematically for data collection of trees, 5mx5m for shrubs, and 1m x1m for Herbs, Grasses, and Litter (GHL) and samples of the soil (Yayneshet 2011; Hasen-Yusuf et al. 2013; Bazezew et al. 2015). A total of 60 quadrats along six transects lines were obtained at a distance of 100 meters (20 quadrats with three in each gradient). GPS was also used to record the latitude and longitude of each research quadrat. Nested plots were established for sampling and gathering separate size classes of growth form. The methods included laying out 40 $m^2$ (20 mx20 m) nested plots for trees and shrubs.

A 1m x 1m plot was erected at the four corners and central locations of each main 20m x 20m quadrat to sample herbaceous vegetation and litter (figure 2). Pieces of $1m^2$ quadrat made of thin wood timber were used to sample GHL and soil made by the local carpenter. All herbaceous vegetation in every quadrat, which includes litters, were clipped at the ground level, weighed, and a 100g composite pattern was once introduced to the laboratory, where moisture content, dry biomass, and oven-dry mass had been determined the use of suitable laboratory method (Roshetko et al. 2002; Jina et al. 2008) to estimate the amount of carbon stocked using GHL. Litter is defined as all non-living biomass larger than the soil organic matter limit (recommended 2mm) that is dead and in numerous stages of decay above organic soil (IPCC 2006). Scientific nomenclature was carried out using published volumes "Flora of Ethiopia and Eritrea" (Sebsebe 1997), Useful Trees and Shrubs of Ethiopia (Bekele-Tesemma & Tengnäs 2007) and Natural Database for Africa (NDA) Version 2 (Ermias 2011). For some species that were unable to identify directly in the field, plant specimens were collected, pressed, dried, and identified in the herbarium.

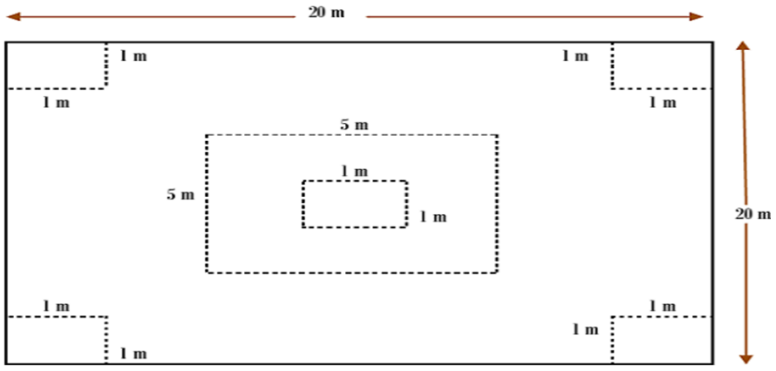


**Figure 2.** Position of the quadrat in the diagram. (1m x 1m for herbaceous, litter, and soil sampling; 5m x 5m for shrub sampling; and 20m x 20m for tree sampling).

A 1m x 1m plots were employed at all corners and middle places of all main quadrat for soil sampling. For SOC determination, soil samples were obtained in five 1m2 quadrats, with GHLs samples taken with a graded 30cm soil auger. A calibrated soil auger was used to collect soil samples up to 30cm in depth (between 0 up to 10, 10 up to 20, and 20 up to 30 cm) (IPCC 2006). A soil composite pattern was once created by mixing soil from 5 sub-quadrats of every primary quadrat to quantify organic carbon. Equal weights of every sample from all the major quadrants (400m$^2$) alongside a single transect had been mixed and blended collectively in accordance to their depth, air -dried and processed via a 2mm sieve to separate particles and gravel to form one soil pattern for every soil depth alongside a transect. As a result, a total of 24 composite soil samples (3 altitude x 2 transects x 3 soil depth) were produced from a total of 60 sample quadrats.

The bulk density was determined using the core technique (Blake 1965). Soil bulk density determination has been taken in the center of every transect relying on their soil profile, lead-off from the floor soil with a 5 cm depth and 2.5cm diameter core sampler gently pushed into the soil to keep away from compaction (Roshetko et al. 2002). All of the samples were tagged with the quadrat to which they belonged and taken to the lab for bulk density examination.

## Carbon Stocks Estimation

### Carbon stock and above-ground biomass

Allometric equations developed by Chave et al. (2014) were employed to provide a valid estimate of forest carbon reserves for AGB. The model was found to remain true across a wide range of tropical vegetation kinds, with no discernible consequence from geography or ecological conditions (Chave et al. 2014; Victor 2015). Equations that combine more than one tree measurement, according to Henry (2010), increase the accuracy of forest biomass calculation. As a result, many research employed the model of Chave et al. (2014), which used to be the satisfactory model for carbon inventory evaluation in Africa (Victor 2015) based totally on climatic conditions, DBH of trees, and wooded area kind of the study region to decide biomass of tree species with a diameter of much less than 5cm.

According to Chave et al. (2014), incorporating country-specific wood density into the equation improves biomass calculation substantially. As a result, Ethiopia conducted a thorough investigation to identify the most acceptable wood density estimate for the country, and data on the basic wood density of 421 indigenous and exotic tree species found in Ethiopia were gathered. The species' overall average wood density is 0.612 g/cm3. This is similar to the global average and the value for tropical Africa. (Chave et al. 2009; Reyes et al. 1992; IPCC 2006). Accordingly, in this study, I have used data on wood density from the database.

$$ABG = 0.0673 \times (WD \times DBH^2 \times H)^{0.967} \quad \text{eq.1}$$

Whereas;
AGB= Aboveground Biomass (in kg dry matter)
H= height of the tree (in m)
DBH = Breast Height Diameter (in cm)
WD = Density of wood (g/cm3)

(Brown 1997; Inventory 2004) allometric equations were used for trees/ shrubs with a DBH of less than 5 cm and shrubs. For assessing woody carbon stocks, (Inventory 2004) established equations for all woody species in Ethiopia.

$$AGB = (1.4277 \times DSH + 0.0088 \times DSH^{3.0}) \quad \text{eq.2}$$

Whereas;
AGB (Kg) = Above-ground biomass and
DSH (cm) = Diameter at stump height (1.3m)

50% of total tree and shrub biomass used to be assumed to be the carbon inventory when converting above-ground dry biomass to carbon. As a result, the carbon store of above-ground biomass in trees and shrubs was calculated as follows (Brown 2002):

$$AGTCS = AGTSDBM \times 0.5 \quad \text{eq. 3}$$

Whereas;
AGTSCS = Above-ground trees and shrubs carbon stocks
AGTSDBM=Aboveground trees and dry shrub biomass

## Calculation of dead woods carbon stock

For fallen deadwood, De Vries formula (de Vries 1986) has been applied, estimating log volume in m3 $ha^{-1}$. This formula requires the length of the transect (L) and the log diameter (d) at the point of intersection.

$$V = \frac{\pi^2 \Sigma d^2}{8L} \quad \text{eq. 4}$$

Whereas;
V = volume per hectare of deadwood

d = log diameter at the point of intersection of the transect perpendicular to the axis of the log,

L = length of the transect.

There are two decomposition classes recorded for deadwood particles: sound and rotten. If the decomposition class was missing in the data, it was assumed that the deadwood piece was sound. Because a rotten wood contains less biomass than a sound wood, the wood density of deadwood is scaled down using lower wood densities than for standing trees, as follows:

Sound deadwood biomass = volume x 90% x default WD eq. 5

Rotten deadwoon biomass = volume x 50% x default WD eq. 6

The default wood density for the species is 0.612 g/cm3, similarly as for trees, since the overall average wood density for the species is 0.612 g/cm3.

### Estimation of carbon stock and below-ground biomass of woody

To estimate the BGB carbon pool default values proposed by IPCC (2006) have been applied. For biomes, a root-to-shoot ratio of 27% is applied as suggested for tropical mountain sysstems (Singh et al. 1994).

BGB = AGB x 0.27 eq. 7

Whereas;

BGB = below-ground biomass.

AGB = above ground biomass

### Carbon stocks estimation in grasses, herbs, and dead litter

Samples have been gathered from the specified sub-quadrats of every important quadrat to evaluate litter, herbs, and grasses (GHLs). Fresh samples have been weighed with a 0.1g accuracy withinside the field. A hundred g sub -samples from every important quadrat were labeled inside the box and introduced to the laboratory. The sub-samples have been utilized to calculate an oven-dry-to-wet mass ratio, which was then used to convert the complete moist mass to oven-dry mass (Pearson et al. 2013).

$$\text{GHL's} = \frac{W\,field}{A} \times \frac{\text{W sub, fresh} - \text{sample, dry}}{2!} \times \frac{1}{10000} \quad \text{eq. 8}$$

Whereas;

GHL's (t. ha-1) = Biomass of grass, herbs, and leaf litter

Wfield A (Kg),= Weight of a freshly sampled destructively sparkling field sample of leaf litter, herbs, and grasses inside a place of measurement

A(ha) = The dimension of the collection place for leaf litter, herbs, and grasses

Wsub-sample, dry (g) = weight of an oven-dried sub-sample of leaf litter, grasses, and herbs delivered to the lab to decide moisture content and

Wsub-sample, Fresh (g)= Weight of a sparkling sub-sample of leaf litter, grasses and herbs that used to be taken to the lab to take a look at moisture content.

The following method was used to calculate carbon inventory in the litter and herb layer (Lasco et al. 2006):

C stored (Mg $ha^{-1}$) = total dry weight x C content eq. 9

The carbon stock (C content) of the dry biomass of herbs and litters accounted for 47% of the quadrat total dry biomass (IPCC 2007b).

### Estimation of organic carbon reserves in the soil

Field damp soil becomes dried in a laboratory oven at 105°C for 12 hours to evaluate SOC, then re-weighted to measure dry bulk density and moisture content material. I applied the WB method for SOC measurement (Walkley & Black 1934). The mechanism of this method is to oxidize the organic carbon in the samples to $CO_2$ by excessive strong oxidant $K_2Cr_2O_7$ (using $Ag_2SO_4$ as a catalyst), $FeSO_4$ is then used to titrated the remnant $Cr_2O_7^{2-}$, and the organic carbon content is estimated by the $Cr_2O_7^{2-}$ volume consumed during the reaction (Visconti & de Paz 2021). A calibration coefficient of 1.10 was used for oxidation efficiency. 0.1–0.5 g soil sample is treated with 5 mL 0.8 M 1/6 $K_2Cr_2O_7$ standard solution, and then mixed with 5 ml concentrated $H_2SO_4$ (Chen et al. 2015). The mixture is heated at 170 –180°C for 5 minutes with an oil bath furnace and cooled at room temperature. The solution is transferred into a 250 ml Erlenm eyer flask to keep at 60 –80 ml, and unreacted $K_2Cr_2O_7$ is determined d by titrating with 0.2 M $FeSO_4$. Soil organic C (SOCMWB) content is calculated from the difference in $FeSO_4$ used between a blank and a soil solution (Chen et al. 2015).

The volume and bulk density of the soil had been used to compute the carbon inventory density of soil organic carbon (Pearson 2007).

$V = H \times \pi r^2$ eq. 10

Whereas:

V(cm3) = the volume of soil withinside the core sampler.

H(cm)= core sampler height which is five.

r (cm) =radius of the core sampler, which is 2.5.

A soil sample bulk density can also be estimated as follows:

$$BD = \frac{Wav, dry}{V}$$ eq. 11

Whereas;

BD = soil sample bulk density per quadrat

Wav, dry = average air-dry weight per quadrat of the soil sample,

V ($cm^3$) = soil sample volume in the core sampler

The carbon stock in soil was determined as follows:

$$SOC = BD \times d \times \%C \quad \text{eq. 12}$$

Whereas:

SOC (Mg $ha^{-1}$) = Soil Organic Carbon Stock per unit area

BD (g.cm-3), = Bulk Density of Soil

d = The depth to which the sample will be taken in total (30cm) and

%C = Carbon concentration (percentage) measured in the lab

### Total Carbon Stocks Estimation

Finally, by including the biomass and carbon inventory of the different pools, the total woody biomass carbon inventory accumulation in all altitudinal gradients per quadrat and then per hectare used to be computed. As a result, the total dry biomass was calculated by using summing all biomass swimming pools for each quadrat and the converting the average of all quadrats to hectares the usage of the formula below:

$$\text{Total biomass (Mg } ha^{-1}) = AGTSDBM + BGTSDBM + HLDBM \quad \text{eq. 13}$$

Whereas;

AGTSDBM (Mg $ha^{-1}$) = Dry biomass of above-ground trees and shrubs

BGTSDBM (Mg $ha^{-1}$) = Dry biomass from below-ground trees and shrubs

GHLDBM (Mg $ha^{-1}$) = Dry biomass of grasses, herbs, and litters

Using the same formulae as for whole biomass, the complete carbon stock per quadrat and per hectare had been calculated.

$$\text{TCS (MG } ha^{-1}) = (TAGC + TDWC + TBGC + C(GHL's) + SOC \quad \text{eq. 14}$$

Whereas;

TCS (Mg $ha^{-1}$) = total carbon stock in total dry biomass

TAGC (Mg $ha^{-1}$) = Aboveground Tree Biomass Carbon Stock

TDWC (Mg $ha^{-1}$) = Dead Woods Carbon Stock

TBGC (Mg $ha^{-1}$) = Total Belowground Carbon Stock

C(GHL's) (Mg $ha^{-1}$) = Carbon Stock in Biomass of Grass, Herb and Litter

SOC (Mg $ha^{-1}$) = Soil Organic Carbon

## Statistical Analyses

All data was once organized as fixed factors (altitude gradients) and random

variables (sample plots) for each sampling site. The collected data of tree DBH, tree height, fresh and dry weight of litter, and soil sample were recorded, organized, and compiled in excel sheets. The carbon stock of tree vegetation, litter, and soil were calculated. The influence of altitude variation on carbon stock was tested using a one-way analysis of variance (ANOVA) at a 95% confidence interval, since each sample is taken from a normally distributed population, each sample has been drawn independently of the other samples and the variance of data in the different altitude is the same. The least significant difference test was performed to separate means by using a one-way analysis of variance (ANOVA) when the result showed the presence of significant differences along an altitude gradient on biomass carbon and SOC stock. Tables and Figures have been used to present the result of descriptive and inferential statistics of the find out about.

## RESULTS AND DISCUSSION

### Vegetation characteristics

A total of 52 vegetation species belonging to 33 genera and 24 families were collected in Gola natural vegetation were measured for the estimation of aboveground and belowground biomass carbon. The present study showed different tree populations among the stratum with a mean density of 114±21.54, 279±109.25, and 430.66±205.36 trees per hectare in LA UA, and MA respectively. In all altitudinal gradients, the study revealed that the top three dominant tree species such as *Acacia tortilis, Acacia bussei, and Grewia schweinfurthii* were the leading dominant tree species in all altitudinal gradients by their relative dominance.

This study found a high percentage of woody species in lower frequency groups and a low percentage of species in higher frequency classes. This indicates that generally, the study sites had heterogeneous species composition. MA had more species percent with higher frequency class which are 11.33% as compared to UA and LA that had only 5.5% and 4% of species respectively. While in the lower frequency class UA had species percent (58.33%) than LA (52%) and MA (50%). As a result, the study confirms that each altitudinal gradient has a significant degree of floristic heterogeneity. Among the total tree species, 8, 7, and 6% of species were observed only in MA, UA, and LA respectively, while 30% of tree species were observed in all altitudinal gradients (Figure 3).

Mean of species richness of species decreased non significantly from the MA site through the UA to the LA gradient shows an average number of species per sampling unit was also higher in the MA than in the UA and LA gradient in Gola natural vegetation. Several tree species with large DBH class were measured in MA than LA and UA gradient. However, large numbers of trees with lower DBH class (<5) were recorded in LA than MA and UA (Figure 4).

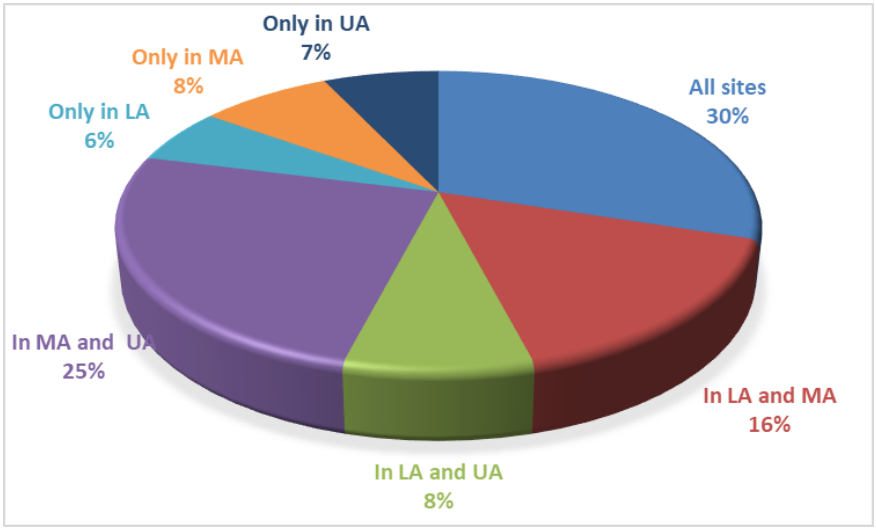


**Figure 3.** Percentages of species distribution across three altitudinal gradients (LA=lower altitude, MA=middle altitude land, and UA=upper altitude).

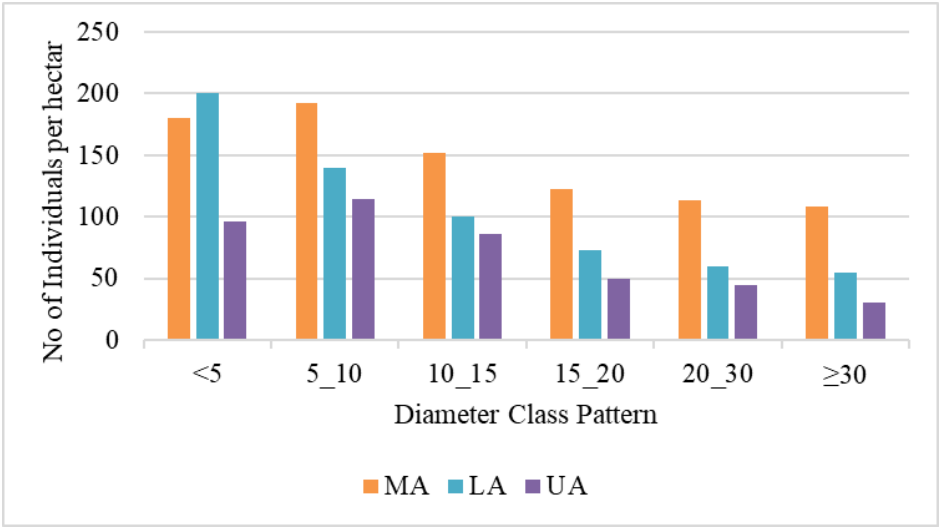


**Figure 4.** Diameter distribution of species in three altitudinal gradients (LA=lower altitude, MA=middle altitude land, and UA=upper altitude).

## Carbon Stock Estimation

### Above-ground and Below-ground Carbon Stocks

The result indicated that there is a significant difference in the amount of AGC in the three altitudinal gradients (Table 1). The maximum AGC was found in the MA gradient (85.7±19.2 Mg $ha^{-1}$). However, LA had s significantly lower AGC with 66.8 ±8.7. This finding demonstrated that the altitudinal gradient strongly influences AGC, which may be stated as a decrease in AGC as the altitude rises. The result is also in accordance with expectations (Gebrewahid et al. 2018). The presence of large trees increases in the MA, and their manipulation by legal and illicit cutting and grazing is rather modest, which is the basis for this trend. So, such large trees in MA resulted in the accumulationof larger AGC in UA. On the other hand, because few large individuals can account for the massive amount of carbon above and below ground, the presence of species characterized by giant individuals, and possibly due to favorable conditions for tree growth in middle altitudes. The ex-

planation for the disparity in similar studies is likely to be the diverse vegetation in MA and biodiversity status, and other physical or climatical factors (temperature, soil, humidity, topography, and so on), biological component variations also contributed to the wide range of studies. This concept was also backed up by (Bikila et al. 2017).

The three altitudinal gradients had a significant difference in BGC. The BGC of UA was much higher than that of MA and LA.LA had also significantly lower BGC than MA and UA. However, there was no significant difference between MA and LA of BGC (Table 1). The reason for such differences was The disturbance and diameter class distribution of vegetation LA Local people have a considerable influence on the study area in LA, which is likely the source of the lower biomass at lower elevations through cultivable land expansion and the acquisition of critical forest products (Gebrewahid et al. 2018). The present study of AGC and BGC stock results was agreed with the earlier study of (Teshager et al. 2018).

**Table 1.** (Mean±SD) of AGC, BGC, DWC, and GHL in three altitudinal gradients.

| Gradients | Carbon pool(Mg ha$^{-1}$) | | | |
|---|---|---|---|---|
| | AGC | BGC | DWC | GHL |
| LA | 66.8$^{b}$ ±8.7 | 12.06$^{b}$ ±2.6 | 0.00 $^{b}$ ±0.00 | 1.01$^{a}$ ±0.45 |
| MA | 85.7$^{b}$ ±19.2 | 14.93$^{b}$ ±5.2 | 0.97$^{a}$ ±0.25 | 1.22$^{a}$ ±0.48 |
| UA | 71.3$^{a}$ ±39.4 | 18.37$^{a}$ ±7.8 | 1.26$^{a}$ ±0.40 | 1.45$^{a}$ ±0.34 |
| P | 0.042* | 0.038* | 0.06 | 0.15 |

AGC=above ground carbon, BGC= below ground carbon, DWC=deadwood carbon, GHL=grass herbs litter, LA=lower altitude, MA=middle altitude land, UA=upper altitude

## Carbon Stock of Dead Wood

In the LA forest stratum, no standing and fallen dead woods were estimated, and more stumps were measured as compared with the MA and UA forest stratum. The absence of dead woods and stumps measured in LA gradients indicated the existence of human interference in LA is more widespread than the other two gradients, with local men and women collecting firewood. This enables the collecting of dead plants at LA more quickly. In MA and UA, dead woody plants were only found in 15 quadrants. The total mean carbon stock from the deadwood in this study was found at 0.74±0.54-Mg ha$^{-1}$. The mean of deadwood carbon was 0.97±0.25, 1.26±0.40Mg/ha$^{-1}$ and 0 for MA, UA and LA respectively. In general, deadwood carbon differs insignificantly crosswise between the two altitudinal gradients as compared to other carbon pools (Table 1). The dead carbon stock variation among the three strata was happened due to the high human intervention in the LA gradient and densely populated tree species were surveyed in higher altitude gradient (Simegn & Soromessa 2015). A similar trend was observed and reported in Yegof mountain natural vegetation in North East, Ethiopia (Eshetu & Hailu 2020b).

## Carbon Stocks in Herbs and Grass, Little

The mean GHL carbon stock The average GHL carbon stock of Gara Gola natural vegetation was about 1.21±0.45Mg ha$^{-1}$(Figure 3). LA, MA, and UA had mean GHL carbon stocks of 1.01±0.45, 1.22±0.48, and 1.40±0.34-Mg ha-1, respectively (Table 1). According to this result, UA had insignificantly higher GHL carbon than the other two locations. LA had a lower GHL carbon concentration than the other two, with MA as an intermediary between them (Table 1). The explanation for this variance could be due to unlawful grazing and grass cutting for cultural and religious holidays and the gathering of little for fuel in a lower altitudinal gradient than the others, causing it to have the lowest GHL carbon value (Eshetu & Hailu 2020). While there was little intervention in UA, it had a greater GHL carbon content than the others. According to (Zhang et al. 2008) the lack of a discernible pattern in litter carbon density in this study could be attributed to a decrease in litterfall amount and breakdown as altitude increases (Eshetu & Hailu 2020). According to (Bazezew et al. 2015; Chinasho et al. 2015; Simegn & Soromessa 2015; Abere et al. 2017) altitude variation have different carbon stock in GHL. Accordingly in this study, the GHL carbon inventory of the three altitudinal gradients did not change significantly (Figure 5).

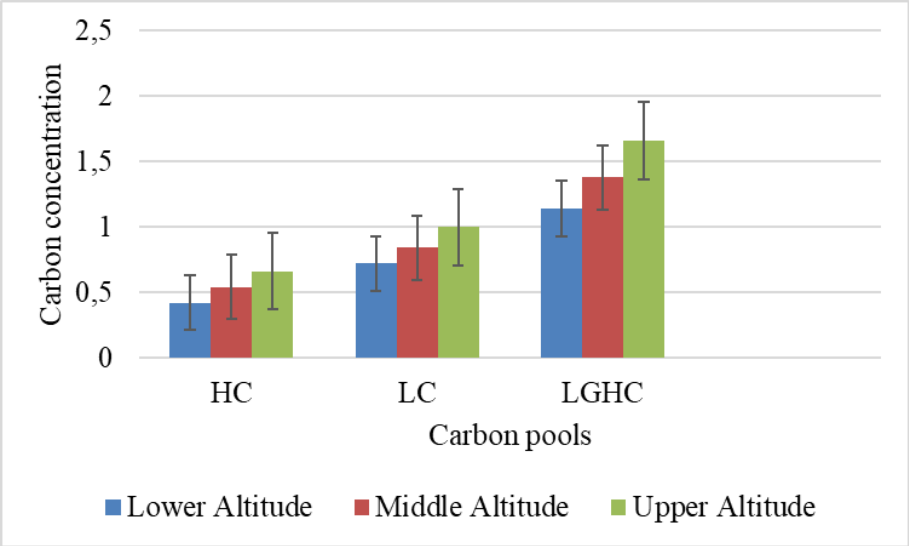


**Figure 5.** Herb and litter imply carbon stock distribution (HC=herbaceous plant carbon, LC=litter carbon, LGHC= little and herb carbon (Mg ha$^{-1}$).

## Soil Organic Carbon

In the Gara Gola, the average bulk density of soil was calculated to be 0.94±0.8g.cm$^{-3}$ (Table 2 ). Bulk density of soil across altitudinal gradient indicates that there is a significant mean difference between LA and UA, however, MA has a lower mean bulk density than LA and higher than UA insignificantly (Table 2). The carbon concentration of the soil in the research region significantly decreases with the increments of altitudes. As a result, in this investigation, the LA has significantly higher carbon concentration than MA and UA (Table 2). The SOC of the three altitudinal gradients differed signifi-

**Table 2.** Means (±SD) of soil bulk density. %Carbon and SOC across altitudinal gradients.

| Soil parameter | Altitudinal class | | | Grant mean | P-value |
|---|---|---|---|---|---|
| | LA | MA | UA | | |
| BD (g.cm$^{-3}$) | 1.31$^{a}$ ±0.9 | 0.82$^{ab}$ ±0.4 | 0.73$^{b}$±0.2 | 0.94±0.8 | 0.013* |
| %C | 3.23$^{a}$ ±1.0 | 2.66$^{b}$±0.7 | 2.05$^{b}$±0.8 | 2.61±0.9 | 0.02* |
| SOC (Mg ha$^{-1}$) | 140.4$^{a}$ ±47.1 | 109.2$^{b}$ ±30.1 | 97.3$^{b}$ ±43.5 | 118.9±46.4 | 0.00*** |

BD=bulk density, %C= carbon percentage, LA=lower altitude, MA=middle altitude land, SOC=soil organic carbon, and UA=upper altitude.

cantly. SOC followed an inversely proportionate trend to the increasing altitude. As the altitude rises, SOC steadily decreases from lower to upper altitude. As a result, the LA had significantly the greatest SOC value followed by MA. However, the UA had a significantly lower amount of SOC than the (Table 2). Similarly, (Eshetu 2014; Kassahun et al. 2015; Tefera 2016) observed a decreasing pattern of SOC with increasing altitude. The cause for this decrease in SOC with altitude could be due to greater temperatures in LA facilities breakdown in lower altitude soils (Zhu et al. 2010). Organic matter generation on the soil may be aided by LA relatively intense sunlight radiation, which is less dominated by huge trees with a closed canopy. On the other hand, human and animal intervention is high in LA, which may lead to the accumulation of manure and other organic substances, resulting in rapid litter decomposition. According to (Chinasho et al. 2015; Kassahun et al. 2015; Tefera 2016), soil type, siltation as a result of soil erosion and topography, as well as leaching and runoff, may additionally produce a circumstance for such a trend, with high SOC at the decrease gradient and reducing as altitude increases. This could be due to changes in vegetation structure and diversity throughout the elevation gradient, resulting in different amounts of organic matter being accumulated due to high inputs from root biomass and above-ground biomass (Gebrewahid et al. 2018).

The mean soil bulk density of the study area increased with the depth of the soil. The mean value of bulk density from the top (0-10cm), middle (10-20cm), and lower (20- 30cm) soil profile was increased insignificantly (Table 3). On the other hand, the percentage of organic carbon significantly decreased with depth increment. The mean % organic carbon Upper profile has significantly higher than the subsequent depth. (Table 3). Similarly, SOC across the three soil profiles follows a reduction trend from top to lower soil with significant variation. The top (0-10cm depth) had the highest SOC and followed by the middle (10-20 cm depth) profile. While the lower soil profile (20-30 cm depth) had the lowest SOC value (Table 3). This means that a high amount of SOC tends to accumulate in the top layer of the soil. This trend is caused by higher organic matter accumulation and decomposition activity in topsoil as it highly interacts with the surrounding plant roots and environmental elements. (Bazezew et al. 2015; Bikila et al. 2017; Tessema et al. 2017) also confirmed such a trend in the top three soil profiles.

**Table 3.** Means (±SD) of BD, %C, and SOC across soil depth.

| Depth (cm) | Parameter | | |
|---|---|---|---|
| | BD (g.cm$^{-3}$) | %C | SOC (Mg ha$^{-1}$) |
| 0-10cm | 0.83±1.3 $^{a}$ | 3.24$^{a}$ ±1.0 | 131.2±44.6 $^{a}$ |
| 10-20cm | 0.92±0.3 $^{a}$ | 2.39$^{b}$ ±0.8 | 122.5±40 $^{ab}$ |
| 20-30cm | 1.08.4±0.5 $^{a}$ | 2.21$^{b}$ ±0.5 | 103 ±47.3 $^{b}$ |
| p-value | 0.509 | 0.01* | 0.04* |
| Grant mean | 0.94±0.8 | 2.61±0.9 | 118.9±46.4 |

BD=bulk density, %C= carbon percentage, and SOC=soil organic carbon.

## Total carbon stock

The total carbon density of the forest ecosystem was calculated by summing each carbon pool estimated in the study area. As a result, the present study revealed that the total mean carbon density of 621.94 Mgha$^{-1}$ in the whole forest ecosystem. Biomass carbon and SOC estimation of the study area showed variation in carbon storage in different carbon pools. The highest carbon stock was estimated in SOC with 55.7% of the total forest ecosystem, whereas the lower carbon stock density was revealed in AGC, BGC, LHG, and DWC carbon pools with 35.90%, 7.2%, 0.59, and 0.35% respectively. In general, the belowground part contains a total of 64.3% and the above-ground share takes 35.9% (table 4). According to (Chinasho et al. 2015), more percentage of the total carbon store of tropical forest is located in the soil. Soils, on average, are the greatest carbon sinks in global terrestrial ecosystems, as they can hold three times as much carbon as vegetation (Schlesinger 1990). Most research suggests that soil organic carbon exceeds above-ground carbon (carbon in vegetation). This finding is also aligned with the investigation of (Girmay et al. 2008; Chinasho et al. 2015; Assaye & Asrat 2016; Bikila et al. 2017) also suggested that more than 90% of the total carbon stock was contributed from soil organic carbon in the wooded grassland.

The MA has the highest total carbon biomass, followed by the LA (Table 4). The lowest TC biomass was found at UA. This meant that the en-

**Table 4.** Mean summary of five carbon pools and total carbon in three altitude gradient.

| Gradients | Carbon pool(Mg ha$^{-1}$) | | | | | |
|---|---|---|---|---|---|---|
| | AGC | BGC | DWC | GHL | SOC | TC |
| LA | 66.8b±8.7 | 12.06b ±2.6 | 0.00 b±0.00 | 1.01 $^{a}$ ±0.45 | 130.4 $^{a}$ ±47.1 | 209.8 |
| MA | 85.7 a±19.2 | 14.9 b ±5.2 | 0.97a ±0.25 | 1.22 $^{a}$ ±0.48 | 109.2 $^{b}$ ±30.1 | 211.4 |
| UA | 71.3b ±39.4 | 18.37a ±7.8 | 1.26a ±0.40 | 1.45 $^{a}$ ±0.34 | 97.3 $^{b}$ ±43.5 | 189.71 |
| P | 0.042* | 0.038* | 0.06 | 0.15 | 0.0* | |

AGC=above ground carbon, BGC= below ground carbon, DWC=deadwood carbon, GHL=grass herbs litter, LA=lower altitude, MA=middle altitude land, SOC=soil organic carbon, TC=total carbon, and UA=upper altitude.

tire carbon stock pattern was humped, with the altitudinal gradient indicating the peak carbon stock at the intermediate altitude. As a result, in the majority of the carbon pools, the MA gradient behaved magnificently (de la Cruz-Amo et al. 2020). This may be due to the gradient high species diversity, favorable environmental circumstances, and soil characteristics. The reason for such variation may be due to the variation of vegetation structure in different mountain vegetation. Shrub species had a higher carbon proportion than large trees in some areas, especially in the MA class. This makes the variation in TC higher in MA than LA and UA class, which is relatively dominated by large trees, making the variation in TC much smaller than the other altitudinal gradients. Similarly, most findings in Ethiopia and overseas, such as (Muluken 2020; Matewos 2021; Tesema & Abera 2021) have previously reported a similar pattern (Abere et al. 2017).

## CONCLUSION

The research was carried out in the natural vegetation of Gara Gola in Eastern Ethiopia. Gara Gola natural vegetation supplied about 578 Mg/ha of TC. The ABC, BGC, and SOC of the three altitudinal gradients, on the other hand, varied significantly. The UA gradient had the greatest AGC value, whereas the LA gradient had the lowest. This implies that AGC rises as altitude rises. A similar result was also got for BGC as it is derived from AGC indirectly. In contrast to AGC, the connection between SOC and altitude is inversely proportional. As a result, LA had the highest SOC, followed by MA. However, no significant differences were detected in the three altitudinal gradients for DWC and GHL carbon. In terms of DWC, only the two top altitudinal gradients were sampled. SOC contributed the most to the area's total carbon stock, followed by AGC. GHL and DWC, on the other hand, made a negligible contribution to total carbon. In most cases, the stock potential MA outperformed the others because it had greater total carbon stock values. This suggests that stronger law enforcement and management are needed in other areas, particularly in LA, heavily impacted by unlawful human and animal activities.

## AUTHORS CONTRIBUTION

AH was the sole author for data curation; formal analysis; writing of original draft; writing review and editing, conceptualization; methodology; investigation, and authors read and approved the final manuscript.

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## CONFLICT OF INTEREST

The author declares that he has no conflict of interests. The content is new and has not been published in any journal.

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Research Article

# Biostimulant Activity of *Sargassum* sp. Extracts on Early Growth of *Zea mays* L. and the Phytohormones Content Analysis

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## ABSTRACT

Seaweed has been gaining global interest in agriculture for the development of marine-based plant biostimulants. This research aimed to study the effect of three different liquid extracts of *Sargassum* sp., acidic, alkaline, and water extract, on the germination and early growth of maize and to evaluate the phytohormones content responsible for the growth. Phytohormones content including Indole-3-acetic acid (IAA), gibberellins (GA), kinetin and zeatin were analyzed using high-performance liquid chromatography (HPLC) and bioassay was performed twice on maize. Parameters observed on the bioassay were germination percentage, number of roots, shoot length, shoot weight and root weight under 4 different concentrations with 0.5; 1.5; 3.5; and 5% in the first bioassay and 3.5% concentration in the second bioassay. Both bioassays following randomized complete design and the data were analyzed using one-way ANOVA using post hoc test of Duncan Multiple Range Test (DMRT) at error probability of 5% in Genestat software. Phytohormones content in the seaweed extract indicated that alkaline extract was rich in IAA, gibberellin, and zeatin content, while water extract showed the highest kinetin content. The first bioassay indicated that lower concentration of the seaweed extracts gave better growth in all extracts, therefore a 3.5% concentration was chosen for the second bioassay with higher replication for each treatment. The second bioassay confirmed alkaline extract resulted in the highest germination while the highest seedling height, number of roots, shoot and root weight were resulted from acidic extract treatment. In conclusion, *Sargassum* sp. extracts obtained from acidic, alkaline, and water-based extraction methods, were able to improve the shoot and root growth of maize plants. The acidic extract showed the highest growth promotion among other extracts with the lowest phytohormones content.



## INTRODUCTION

Seaweed has been gaining global interest for over the last decades, commonly due to its potential for natural products (Stengel & Connan 2015). Numerous algae-based products have been developed for Agri-horticultural, food, pharmaceutical and nutraceutical products. A recent review reported approximately 3200 seaweed-based products, covering 13% of natural compounds reported in marine organisms (Stengel & Connan 2015).

Seaweed was usually categorized into three phyla based on the pigmentation, including green algae (Chlorophyta), red algae (Rhodophyta), and brown algae (Ochrophyta) (Sangha et al. 2014). Among marine algae, the extracts of brown algae were reported to possess higher bioactive compounds, such as betaines, brassinosterols, jasmonates, isoprenoid cytokinin, polyamines, salicylates, and signal peptides (Craigie 2011). Thus, they have been widely used continuously in agriculture and horticulture as natural plant growth stimulants or bio-fertilizers (Craigie 2011). The current commercial agricultural products mainly were produced from brown seaweeds of *A. nodosum*, *Laminaria* spp., *Ecklonia maxima*, *Sargassum* spp., and *Durvillaea* spp. (Craigie 2011). One of the brown algae, *Sargassum* sp. (Sargassaceae) has been attracted great interest for the higher phytohormones, macro- and micronutrients contents than other species in different phyla (Hernández-Herrera et al. 2014).

Seaweed extract generally consists of useful bioactive compounds, including polyphenols, terpenoids, polysaccharides, polypeptides and free amino acids, pigments, vitamins, phytohormones, macro- and micro-nutrients (Mahmoud et al. 2019). Seaweed extracts were considered as plant biostimulants that act as metabolic enhancers to increase the effectiveness of conventional mineral fertilizers (Craigie 2011). Recent study by Sunarpi et al. (2021) evaluated bioactive and phytohormones roles of *Sargassum* extract as plant biostimulants in rice plant. The result suggested that *Sargassum* is a potential raw material for development of organic fertilizers which increased growth and yield of rice plants

Seaweed extracts have shown accelerated germination, vegetative growth, flowering and yield of many crops. Seaweed extracts of *Ascophyllum nodosum* at 0.5 g/L concentration improved total anthocyanin, yield and fruit size of strawberry (Roussos et al. 2009). Combination of *Sargassum wightii* and *Caulerpa chemnitzia* aqueous extract at low concentration (20% from 1 kg fresh seaweed in 1 L aquadest) reported to promote germination, vegetative growth and flowering of *Vigna sinensis* (Sivasankari et al. 2006). Enhancement of leaf chlorophyll content by various brown seaweed extracts application has also been reported in cowpea, red-radish and oil palm (Sivasankari et al 2006; Santoso et al 2011; Mahmoud et al. 2019). Improved vegetative and generative development and increase in crop growth treated by foliar spray of *Sargassum* extract of 10 ppm by 3 times application led to an increase in yield of harvested soybean (Sari et al. 2019). Foliar spray of *Sargassum* sp. extract at 2% concentration also reported to improve yield, vegetative and generative growth of rice and corn (Santoso et al. 2011). Vegetative growth of plantation crops, including oil palm and sugarcane were also improved after treatments of *Sargassum* sp. extract (Santoso et al. 2011; Putra et al. 2017). It was suggested that the stimulatory effect of seaweed extract may be controlled by phytohormones, such as auxins, cytokinins, or gibberelins, were probably in control for the improved growth and yield of the plants (Jannin et al. 2013).

This research was part of a grand research in the production of humic fertilizer supplemented with seaweed extract. Extraction of seaweed was developed to discover the suitable method with the highest biostimulant activity, which is suitable with humic acid extraction from lignite. Humic acid act as soil ameliorant which contains minerals to physically and chemically restore disturbed soil (Van Oosten et al. 2017). Application of humic acid to the crops directly improved abiotic stress tolerance in plants, for example application of humic acids to common bean under high salinity showed better adaptation to saline environments (Aydin et al. 2012). Supplementation of humic acid with seaweed extract was superior showed by increment of leaf hydration under dry soil conditions as well as improved vegetative growth and yield (Van Oosten et al. 2017). This study was conducted to evaluate the effect of *Sargassum* sp. liquid extracts under three extraction condition, which are acidic, alkaline, and water, on the germination and early growth of maize and evaluate the phytohormones content responsible for the growth.

## MATERIALS AND METHODS

### Materials

The seaweed *Sargassum* sp. was obtained from farmers in Gunungkidul coastal area, Yogyakarta, Indonesia, washed with fresh water to remove sand, stones and other debris and then dried under sunlight inside a plastic house for two days. The water contents were measured by weighing seaweed samples before and after drying. Commercial hybrid maize seeds of Bonanza F1 (Cap Panah Merah) was used for bioassay in this study. The production of seaweed extracts and bioassay was performed at the Laboratory of Biochemistry, Indonesian Research Institute for Biotechnology and Bioindustry, Bogor, Indonesia. The phytohormones analysis was performed at Agrochemical Material Residues Laboratory-The Agricultural Environment Research Institute (Balingtan), Bogor Indonesia. The research was performed from November 2020 until March 2021.

### Seaweed extraction

The *Sargassum* sp. extraction protocol was modified from Sharma et al. (2013). The dried material was mechanically milled with 40 mesh filter. *Sargassum* sp. meal (2 g) was mixed with 200 mL of aquadest and vortexed for water extract (WE). Alkaline and acidic extract were produced by mixing 2 g of *Sargassum* sp. meal with 200 mL aquadest pH 9.0 and pH 3.0, respectively. pH was adjusted by adding 2 M sulfuric acid or 2 N potassium hydroxide into aquadest to reach the designated pH. pH was adjusted before mixing with *Sargassum* sp. meal and measured by pH meter (Sartorius; PB-10 basic pH meter). The mixtures were incubated at 85°C in water bath for 2 hours and let them cool down at room temperature. The extracts were filtered with cheese cloth and then centrifuged at 6000 rpm for 5 minutes. The crude extracts were stored in a refrigerator for further analysis.

## Phytohormones analysis

*Sargassum* sp. extracts were adjusted to pH 5.8 as the final product of seaweed extract and then sent to Balingtan Bogor, Indonesia for phytohormones analysis. Phytohormones analysis was performed using in-house protocol from Barendse (1987) in HPLC and the separation of compounds, hypersil gold C18 column was used. Standard solution (0.1%) of kinetin, GAs, zeatin and kinetin, were prepared. The mobile phase used was methanol/water (65:35, v/v). The flow was 0.5 mL.min$^{-1}$ and 5-10 $\mu$L of sample was loaded onto the column. The temperature of the column was maintained at 40°C. Detection was carried out at the wavelength of $\lambda$= 254 nm. Samples prior to analysis were mixed with methanol 65% and centrifuged at 4000 rpm for 30 min. The supernatants were filtered with a syringe filter (0.45 µm) and the filtrate was ready to inject onto the column. Phytohormones content was calculated by comparing between chromatogram peak of sample and standard.

## Bioassay in maize

Three different *Sargassum* sp. extracts were tested for germination and early growth activity of maize in two bioassays. The first bioassay, maize seeds were germinated in water, acidic, and alkaline *Sargassum* extracts following the experiment design of randomized complete design with 4 concentrations (0.5; 1.5; 3.5; and 5%) in 3 replicates and aquadest was used as negative control. Maize seeds were soaked according to the treatment for 1 hour, followed by germination in Petri dish (10 seeds per Petri dish) covered with wet tissue paper. Germination was observed when negative control was germinated at 30-50%. Observation of germination was performed twice (during negative control germinated 30-50%) and the significant result between treatment was chosen. The germination rate was calculated by the percentage of number of germinated seeds (when negative control is 30-50% germinated) divided by total number of germinated seed at the end of germination period. All germinated seeds were grown for the next 7 days to observe the shoot length, number of roots, shoot and root weight. The optimum concentration was chosen for the next bioassay with higher replications number.

The second bioassay was performed under the same procedure but only one concentration of *Sargassum* sp. extract was used. Maize seeds were germinated in Petri dish soaked with treatment solutions, aquadest as negative control, and treatment solution including water, acidic, and alkaline extract in six replicates (15 seeds per Petri dish). Germination was observed following the first bioassay condition. The growth parameter was observed 4 days after germination including shoot length, number of roots, shoot and root weight.

## Data analysis

Quantitative data including germination and early growth parameters from first and second bioassay were statistically analyzed using Genstat software in

one-way ANOVA and Duncan's multiple range test was performed at $\alpha$ = 0.05.

## RESULTS AND DISCUSSION

### Seaweed extract production

Seaweed extraction was performed using aquadest as the solvent with three different pH, namely acidic, neutral and alkaline. The seaweed used in this study is *Sargassum* sp. obtained from farmers in Gunungkidul coastal area (including Sepanjang beach, Krakal beach, Drini beach, Nglolang beach, Pok tunggal beach, Sanglen beach, Porok beach etc.; Personal communication with the supplier), Yogyakarta (Indonesia) which was harvested from the nature (Figure 1A). The seaweed was then dried until water content was around 5-10% (Figure 1B). The dried seaweed was milled mechanically with 40 mesh filter chosen (Figure 1C). Under this milling condition, it was able to obtain seaweed meal with particle size of < 2 mm. Sharma et al. (2013) reported that for agricultural purposes, seaweed was preferred in a coarse size grade (1–4 mm) for extraction, while the feedstock industry utilizes the finer particle for supplement.

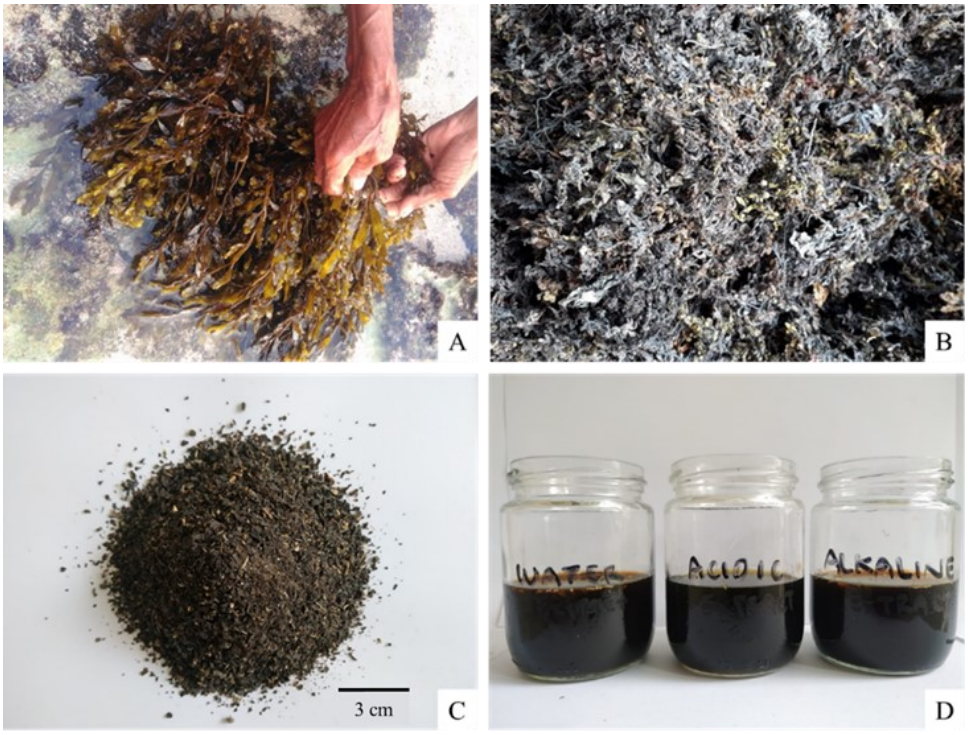


**Figure 1.** Seaweed used in this study: (A) Intertidal seaweed *Sargassum* sp. harvested from the nature, (B) Dried *Sargassum* sp., (C) *Sargassum* sp. meal, and (D) *Sargassum* sp. Extract.

*Sargassum* sp. which grows in intertidal zone may be exposed to unfavorable environmental conditions, including extreme variations in temperature, salinity, and light, which lead to the production of a wide range of secondary metabolites to survive in these environments (Shukla et al. 2016). Goñi et al. (2016) reported that different extraction protocols produce bi-

ostimulants with different bioactive compounds and activities. Various commercial plant biostimulant products have been utilizing the hydrolysis process of seaweed (Shukla et al. 2019).

## Phytohormones content in *Sargassum* sp. extract

Seaweed extraction protocol in this study resulted in three different solutions, they are acidic, alkali, and water extract, (Figure 1D) with dark brown color and the pH was adjusted to 5.8 before dilution with 2 N KOH or 1 M $H_2SO_4$. The phytohormones content in liquid extract of *Sargassum* sp. respectively as shown in Table 1.

**Table 1.** Phytohormones content of *Sargassum* sp. Extract.

| Seaweed extract | Concentration (mg.L$^{-1}$) | | | |
|---|---|---|---|---|
| | IAA | Gibberellins | Zeatin | Kinetin |
| Acidic extract | 10.64 | 18.89 | 34.92 | 4.09 |
| Water extract | 11.04 | 29.63 | 35.71 | 4.73 |
| Alkaline extract | 11.49 | 30.88 | 38.25 | 4.33 |

The data in Table 1 shows that even with slight difference from each extract, it was feasible that alkaline extract showed the highest IAA, gibberellins, and zeatin content. While kinetin content was almost the same in all extract and was the lowest phytohormone content among others. Gibberellins and zeatin content were considered high compared to IAA and kinetin in all extracts. Sunarpi et al. (2021) reported that different Sargassum extracts produced different phytohormones contents. Extract from *S. polycystum* produced the highest gibberellins and kinetin content of 8 mg. L$^{-1}$ and 10 mg. L$^{-1}$ respectively, compared to *S. cristaefolium* and *S. crassifolium* (Sunarpi et al. 2021). It was also reported that IAA is the most common growth hormone found in all *Sargassum* species (Sunarpi et al. 2021). Indole-3-Acetic Acid (IAA) has chemically poor solubility in water or aqueous buffers unless the pH of the solvent is alkaline (Yamakawa et al. 1979). This explained, IAA content of alkaline extract was slightly higher compared to other extracts. Previous research from Sumera & Cajipe (1981) reported that alkaline hydrolysis for *S. polycystum* extraction increases the auxin activity of algal extracts due to liberation of bound auxins or inactivation of co-existing inhibitory substances during hydrolysis.

## Bioassay in maize

The result of bioassay in maize was shown in Table 2, indicates that the application of seaweed extracts improved germination and early growth of maize significantly compared to negative control. The highest germination percentage was achieved by soaking maize seeds in 3.5% *Sargassum* alkaline extract. The highest number of roots and shoot height showed by treatment of 1.5% *Sargassum* alkaline extract, which was not significant with 1.5% acidic extract. Shoot weight and root weight showed the highest value under treatment of 0.5% acidic extract.

**Table 2.** The effect of liquid extracts of *Sargassum* sp. on germination and early growth of maize (first bioassay).

| Treatment | | Germination (%) | | Number of roots | | Shoot height (cm) | | Shoot weight (g) | | Root weight (g) | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Extract | Concen-tration | Mean | s.d. | Mean | s.d. | Mean | s.d. | Mean | s.d. | Mean | s.d. |
| Acidic extract | 0.50% | 62.50$^{abcd}$ | 14.6 | 6.97$^{b}$ | 1.0 | 14.47$^{abc}$ | 0.1 | 3.32$^{c}$ | 0.2 | 1.46$^{b}$ | 0.1 |
| | 1.50% | 62.96$^{abcd}$ | 8.6 | 7.23$^{b}$ | 0.8 | 15.61$^{c}$ | 1.0 | 3.06$^{bc}$ | 0.8 | 1.03$^{ab}$ | 0.2 |
| | 3.50% | 52.38 $^{abcd}$ | 8.0 | 6.70$^{b}$ | 0.0 | 12.91$^{abc}$ | 0.9 | 2.64$^{abc}$ | 0.4 | 0.93$^{ab}$ | 0.3 |
| | 5% | 61.64$^{abcd}$ | 16.7 | 4.97$^{a}$ | 1.4 | 10.77$^{a}$ | 2.4 | 1.83$^{ab}$ | 0.7 | 0.89$^{ab}$ | 0.4 |
| Alkali extract | 0.50% | 39.01$^{ab}$ | 15.1 | 6.40$^{ab}$ | 0.5 | 14.38 $^{abc}$ | 1.3 | 2.46$^{abc}$ | 0.7 | 1.06$^{ab}$ | 0.3 |
| | 1.50% | 74.60$^{cd}$ | 4.1 | 7.27$^{b}$ | 0.4 | 16.42$^{c}$ | 0.6 | 2.18$^{abc}$ | 0.2 | 0.88$^{ab}$ | 0.2 |
| | 3.50% | 81.06$^{d}$ | 3.5 | 7.03$^{b}$ | 0.8 | 15.39$^{bc}$ | 2.4 | 2.41$^{abc}$ | 0.9 | 1.10$^{ab}$ | 0.4 |
| | 5% | 66.67$^{bcd}$ | 14.7 | 6.87$^{b}$ | 0.7 | 14.53 $^{abc}$ | 0.8 | 2.81$^{abc}$ | 0.1 | 1.22$^{ab}$ | 0.1 |
| Water extract | 0.50% | 59.72$^{abcd}$ | 10.9 | 5.93$^{ab}$ | 0.1 | 13.86 $^{abc}$ | 1.4 | 2.33$^{abc}$ | 0.3 | 1.15$^{ab}$ | 0.3 |
| | 1.50% | 48.68$^{abc}$ | 4.3 | 6.07$^{ab}$ | 0.8 | 13.43 $^{abc}$ | 2.1 | 2.79$^{abc}$ | 0.6 | 1.00$^{ab}$ | 0.2 |
| | 3.50% | 55.73$^{abcd}$ | 9.1 | 6.43$^{ab}$ | 0.3 | 15.89$^{c}$ | 2.5 | 2.95$^{abc}$ | 0.4 | 0.99$^{ab}$ | 0.3 |
| | 5% | 76.67$^{cd}$ | 17.0 | 6.67$^{b}$ | 0.6 | 16.18$^{c}$ | 1.3 | 2.76$^{abc}$ | 0.4 | 1.02$^{ab}$ | 0.3 |
| Negative control | | 34.52$^{a}$ | 17.6 | 6.00$^{ab}$ | 0.3 | 11.50$^{ab}$ | 2.9 | 1.67$^{a}$ | 0.5 | 0.76$^{a}$ | 0.3 |

Based on Table 2, it was visible that different concentrations on the same extract were not significantly different towards germination and early growth of maize. However, based on the germination result which was the first activity observed during maize growth, it is showed that the highest concentration did not ally with high growth or germination. Lower concentration of the extracts gave higher germination and growth instead. Thus, to narrow down the effect of each extract towards maize growth in the second bioassay, lower concentration of 3.5% was chosen.

Several studies have reported that seaweed-based plant biostimulants were effective in low concentration. Khan et al. (2011) reported higher growth in roots and shoots of *Arabidopsis* following application of *Ascophyllum nodosum*-based biostimulants at 0.3% concentration. A similar trend was found on the evaluation of different concentrations (0.2; 0.4; 0.6; 0.8; and 1.0%) of seaweed extract (*S. tenerrimum*) on seed germination and growth of tomato plant (Sasikala et al. 2016). Full seed germination was only observed at 0.8% treatment together with a maximum number of leaves, root length, and total dry weight. While the maximum shoot length and total plant height were found at 0.6% concentration.

Figure 2 showed different growth of maize seedlings under different concentrations of acidic, alkali, and water extract compared to the negative control. The seedlings were grown for 7 days after germination. The non-significant difference in many treatments probably attributed to the over-grown seedling. Therefore, in the second bioassay, maize seedlings were grown for 4 days after germination in order to see more diverse effect of each extract towards early growth of maize seedling. Determination of concentration for the second bioassay was quite difficult due to no significant

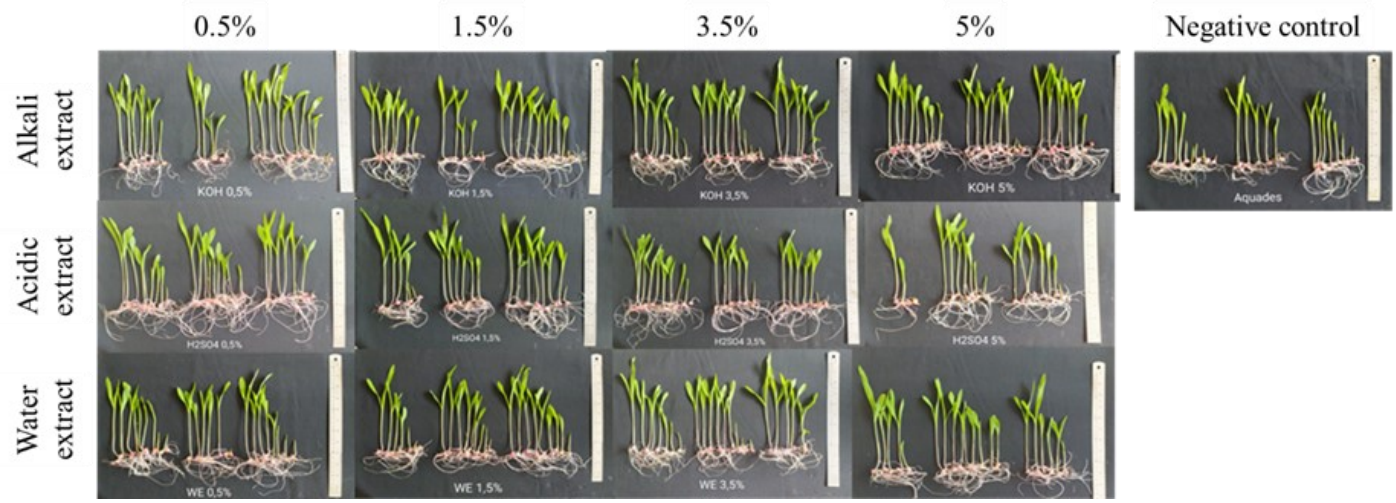


**Figure 2**. The growth of maize seedling under different treatments of *Sargassum* sp. Extracts.

different in all treatment, therefore, middle concentration given the highest germination was chosen, assumed that germination was the first stage influenced by the treatment.

The second bioassay was mainly performed to confirm the different actions of each extract in the germination and early growth of maize. A non-significant result from different treatments in the first bioassay making it difficult to determine the seaweed extraction method, therefore, the second bioassay was performed in higher replicates and minimum treatment. The result of the second bioassay is shown in Table 3 and Figure 3.

The second bioassay result showed improvement in germination and all growth parameters observed in this study for acidic, alkali, and water extract compared to negative control except for number of roots at water extract treatment. The highest germination occurred on alkali extract treatment but with no significant difference in all treatments. Acidic extract treatment occurred to give the maximum number of roots, shoot height, shoot weight, and root weight. The acidic extract was the only treatment with a significant difference in the number of roots compared to negative control than other treatments. Statistical analysis in the second bioassay helped in the determination of the optimum method to achieve high biostimulant activity from *Sargassum* sp. extract.

**Table 3.** The effect of liquid extracts of *Sargassum* sp. on germination and early growth of maize (second bioassay).

| Treatment | Germination (%) | | Number of roots | | Shoot height (cm) | | Shoot weight (g) | | Root weight (g) | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Mean | s.d. | Mean | s.d. | Mean | s.d. | Mean | s.d. | Mean | s.d. |
| Acidic extract | 52.22[a] | 9.4 | 4.87[b] | 0.5 | 4.94[c] | 1.9 | 4.94[c] | 0.7 | 4.94[c] | 0.3 |
| Alkali extract | 56.67[a] | 6.3 | 3.89[a] | 0.5 | 2.40[ab] | 0.3 | 2.40[ab] | 0.3 | 2.40[ab] | 0.1 |
| Water extract | 56.67[a] | 3.1 | 3.21[a] | 0.7 | 3.26[b] | 1.0 | 3.26[b] | 0.3 | 3.26[b] | 0.3 |
| Negative control | 44.44[a] | 11.3 | 3.62[a] | 0.3 | 1.78[a] | 0.4 | 1.78[a] | 0.5 | 1.78[a] | 0.3 |

* Concentration used was 3.5% for all extracts, negative control is aquadest

** Means in the same column followed by different letters are significantly different according to Duncan's multiple range test at $\alpha = 0.05$

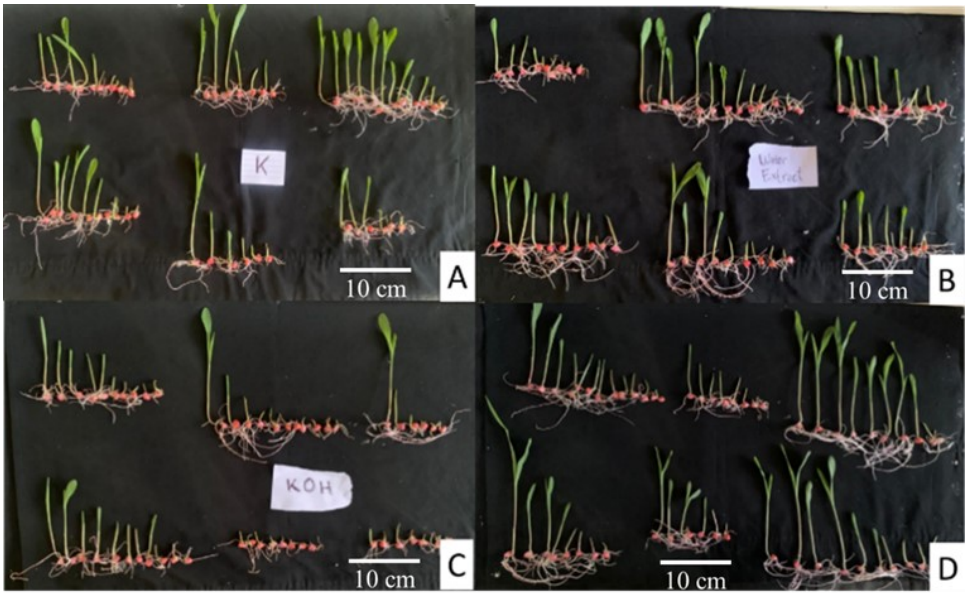


**Figure 3.** The growth of maize seedling under different treatments of *Sargassum* sp. extracts: (A) negative control (aquadest), (B) water extract (3.5%), (C) alkali extract (3.5%), and (D) acidic extract (3.5%).

Based on Table 1, it showed that acidic extract contained the lower phytohormones compared to alkali and water extract. However, it showed the highest biostimulant activity in the early growth of maize. Seaweed-based plant biostimulants were known to contain diverse compounds, ranged from single compounds such as amino acid and phytohormones, to complex matrices such as polysaccharides or polypeptides (Ugena et al. 2018). The activity of different groups of bioactive components in seaweed-based biostimulants has only been partly characterized. Understanding the mechanism of action requires multidisciplinary approach; not only based on phytohormones activity, but also from multiple interaction among different groups of bioactive compounds (El Boukhari et al. 2020). Therefore, further studies on the analysis of other bioactive compounds, such as bioactive peptides, amino acids, or polysaccharides, in *Sargassum* extract were highly recommended.

Previous study reported that acid hydrolysis of brown seaweed removed complex phenolic compounds and increased de-polymerization of polysaccharides (Flórez-Fernández et al. 2018). Generally, the acidic hydrolysis method was used for the extraction of fucose-containing sulfated polysaccharides, a class of bioactive compounds in seaweed extracts that promote plant growth (Shukla et al. 2016). Marais & Joseleau (2001) purified fucoidans from *Ascophyllum nodosum* by acid hydrolysis. AZAL5 is a commercially available biostimulant manufactured from *A. nodosum*, which is extracted through acid hydrolysis (Jannin et al. 2013).

The high germination rate under alkali extract treatment was linear to high gibberellins and zeatin content in the extract (30.88 and 38.25 mg. $L^{-1}$ respectively) compared to other extracts. Several hormones induce germination and break the dormancy including Gibberellins (GAs), brassinosteroids, ethylene, and cytokinin (Kucera et al. 2005). Rayorath et al. (2008) suggested

that the organic components of the *A. nodosum* extracts also play important role in promoting seed germination, by inducing α-amylase activity independently from gibberellic acid-3 (GA3), that might act, in concert with GA-dependent α-amylase production, resulting in enhanced germination and seedling vigour in barley plant.

Alkali hydrolysis extraction methods have been used in biostimulant production (Craigie 2011; Sharma et al. 2013; Flórez- Fernández et al. 2018). Alkali extraction method underwent degradation, re-arrangement, condensation, and catalysis reactions which also produces novel compounds that are not initially present in the brown seaweed (Craigie 2011). Several commercial biostimulants are manufactured from brown algae including Maxicrop (United States), Seasol (Australia), and Acadian (Canada) (Shukla et al. 2019). Alkali hydrolysis under relatively low temperatures 70 and 100 °C breaks down complex polysaccharides into oligomers with smaller sizes and lower molecular weight (Craigie 2011). Alkali treatments of brown seaweed biomass also act on polyphenols in the tissue to produce a complex array of reaction products, which are dependent on the hydroxylation pattern of the original polyphenol (Craigie 2011).

Several reports demonstrated that treatment of seaweed extracts improved root vigour, plant growth and development, shoot/root biomass and leaf numbers, which resulted in the higher yields of various crops under normal or stress conditions (Sangha et al. 2014). Mahmoud et al. (2019) utilized *S. vulgare* extract by soaking the seeds before planting and indicated that the treatment gave significantly higher values of plant length, number of leaves, root diameter, and leaves and root weights as well as leaves and root dry weights than water-soaked seeds.

Considering the high activity of acidic *Sargassum* sp. extract on early growth of maize and humic acid extraction process with alkaline condition, it was suggested that extraction of *Sargassum* and humic acid should be conducted separately and mixed at the end of the process to neutralized pH. However, *Sargassum* sp. extract concentration is supposed to be adjusted to the humic acid formula to achieve the highest biostimulant activity in the further study.

## CONCLUSION

In conclusion, *Sargassum* sp. extracts obtained from acidic, alkaline, and water -based extraction methods, were able to improve shoot and root growth of maize plants during the early stage of growth. The acidic extract showed the highest growth promotion among other extracts with the lowest phytohormones content.

## AUTHORS CONTRIBUTION

FF, P, and Si designed the study; MAA, SW, IML, and Su collected the research materials; FF performed data collection; FF and Si analysed the data;

FF wrote the draft paper; MAA, SW, HF, and P preformed review and paper editing.

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## CONFLICT OF INTEREST

The author declares that there is no conflict of interest in this study.

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**Research Article**

# A Study on Diversity and Distribution of Figs (*Ficus*, Moraceae) in Bogor City, West Java, Indonesia

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## ABSTRACT

*Ficus* (Moraceae) is a keystone resource species in the tropical region, and it contributes significantly to Bogor City's vegetation composition. *Ficus* spp. provide habitat for urban animals and contribute to providing environmental services for the community. Minimum data distribution and increasing land-use change possibly decrease *Ficus* diversity in Bogor City. This study aimed to analyse the diversity and distribution of *Ficus* spp. in Bogor City. The research was conducted by dividing Bogor City into 128 plots sized 1 x 1 km. Relative abundance and distribution analysis used QGIS version 3.10.2-A Coruña. A total of 37 species of *Ficus* spp. from six subgenera were found in Bogor. The highest distribution is mainly located around the Bogor Botanic Gardens and the Ahmad Yani City Forest in a tree and hemiepiphyte. *Ficus benjamina* and *Ficus septica* were the most common species found and spread throughout Bogor City. The significant land-use change in Bogor City has resulted in *Ficus* spp. generally spread in the northern and central parts. In contrast, in the southern part, they are relatively low. Some *Ficus* can also be bioindicators because they have growing habitats that tend to be specific and spread in certain areas. The presence of the Bogor Botanic Gardens has an important role in increasing the diversity of *Ficus* spp. in Bogor City.



## INTRODUCTION

Bogor City was one of the important cities during the Dutch colonial period. It was a cantilever city for Batavia, so schools based on agricultural cultivation (Khoiriyah & Nasution 2014), plant acclimatisation gardens, and various botanical research institutions (Ariati & Widyatmoko 2019) were built in this city. Cultural acculturation, architecture, and high plant species diversity attract domestic and foreign tourists to visit Bogor. The rapid growth development of Bogor city, especially in the 1997–2007 period, converted many land functions that were initially vegetated into settlements and trading centres (Nurwanda & Honjo 2018). To preserve existing nature and history, the local government issued Bogor Mayor Regulation No. 17 (2015) regarding the implementation of Bogor City as a Heritage City in the form of natural and

landscape heritage. Natural and landscape heritage consists of various areas that have the potential as Green Open Space, such as the Ciliwung river boundary, the Cisadane river boundary, forested areas, green belt corridors, and heritage trees. These heritage group trees commonly planted, such as java almond, mahogany, and several species of figs (*Ficus* spp.), are often found in the Bogor Botanic Gardens and Great Post Road (De Groote Postweg Lane) (Akbar & Nurhayati 2018). High adaptability and species distribution of *Ficus* spp. generate it to be commonly found in various areas in Bogor City until now.

The figs (*Ficus*) have various benefits to the environment, animals, and humans. *Ficus* is a genus of the Moraceae that can be a key resource in tropical forests (Kuaraksa et al. 2012). The presence of *Ficus* spp. in urban areas has been widely studied in various big cities (Lok et al. 2013; Ebika et al. 2015; Pradana et al. 2018), leading it to become an environmental indicator (Reyes et al. 2012) and restoration plants (Cottee-Jones et al. 2016). *Ficus* spp. in urban areas has various ecological benefits as a food source and habitat for urban animals (birds, bats, and small mammals), creating a microclimate, and maintaining water conservation (Siswo et al. 2019). Some species also have ethnobotanical potential (Shi et al. 2014).

The high level of landscape change in Bogor City allows for a decrease in plant species diversity, especially native plant species such as *Ficus*. Previous research on plant species diversity in Bogor City was limited to a few green belt corridors and urban forests (Soviyanti 2017; Kalam 2018), while *Ficus* spp. can grow in various habitats in the city. Limited information and data of *Ficus* diversity in Bogor City resulted in a lack of attention to the presence of these figs and the function of *Ficus* as an environmental bioindicator and ecological balancer has not been maximised. Recent studies on the presence and ecological function of *Ficus* in various ecosystems in Java have been published, including studies of diversity *Ficus* in the mountains (Hendrayana et al. 2019), *Ficus* at settlements around springs (Ridwan et al. 2015), and *Ficus* in Sentul City as urban area (Mulyani et al. 2021). The Bogor City, as a satellite town of the capital city, can be a liveable area with sufficient green open space and information on the diversity of *Ficus* in Bogor City can add a list of *Ficus* found on the Java, both native and introduced species. This research aimed to analyse the diversity and distribution of *Ficus* in Bogor City. This research is expected to provide important information in determining plants in green open space and settlements to increase plant diversity and the ecological function of Bogor City.

## MATERIALS AND METHODS

### Study Site

This research was conducted from August 2020 to May 2021. Observations of *Ficus* spp. were carried out in Bogor City excluding Bogor Botanic Garden, Presidential Palace, the office complex of Environmental and Forestry Instrument Standardisation Agency, and Dramaga-Situ Gede Research Forest.

The next step continued the herbarium identification at the Treub Laboratory and Herbarium Bogor Botanic Gardens (HBO). Bogor City has 11.850 hectares, divided into six sub-districts and 68 urban villages with an average height of 190 – 330 masl (The Central Bureau of Statistics of Bogor 2021). Monthly average temperature is 22° – 32°C, and humidity is 80% (Meteorological, Climatological and Geophysics Agency (BMKG) 2021). Observation of *Ficus* spp. were carried out throughout the Bogor City area, marked by the administrative boundary between Bogor City and Bogor Regency.

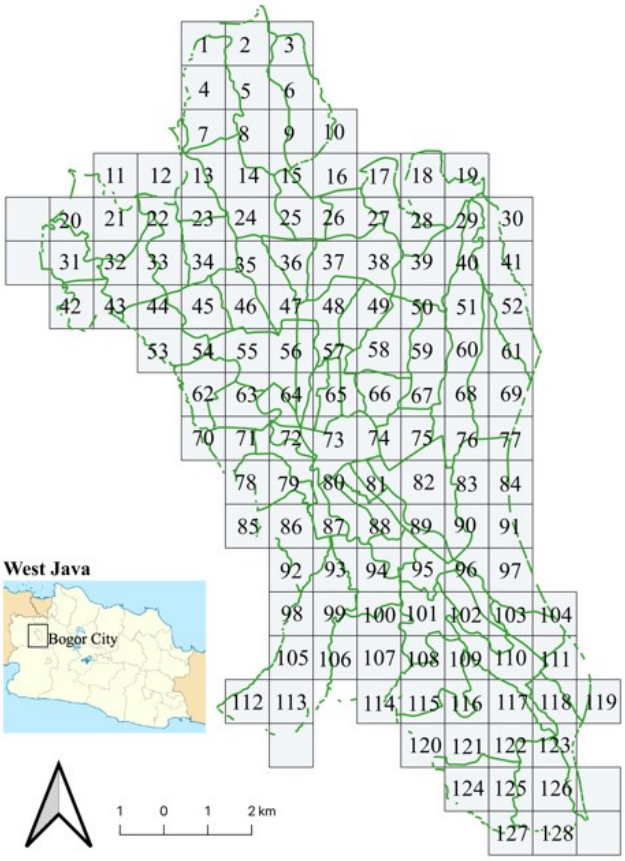


**Figure 1.** Map of the study area

## Material and Methods

Observations of *Ficus* spp. were done by forming observation plots sized 1x1 km (Kent et al. 1999; Serrato et al. 2004), thus there were 128 observation plots in Bogor City (Figure 1). The data collected were location point, species name, number of individuals, habitus based on Berg and Corner (2005) and description habitat. The specimens were documented by taking photograph for habitus, important morphological characters and making description of habitat. After that, specimens were preserved into the herbarium. The next stage was the identification of *Ficus* spp. found, identified host trees, and known distribution status by matching the specimen with the herbarium in HBO, and identification books based on Flora of Java vol 1-3 (Backer & Bakhuizen van den Brink 1963; 1965; 1968) and Flora Malesiana: *Ficus* (Berg

& Corner 2005). Scientific name validation and accepted name updating refers to the Plant of the World Online website (POWO 2021). Information about the sexual system of *Ficus* refers to various references that will mention in the results of this study.

### Data Analysis

Observation data were tabulated and analysed using Ms. Excel to classify *Ficus* to subgenus, sexual system, characteristic life form/habitus, and relative frequency of species (%F). The relative abundance of species shows to the percentage of individuals in a location by the following formula:

$$\%F = \frac{nA}{N} \times 100\%$$

Where %F is the relative abundance of species, *nA* is the number of individual species A found in Bogor City divided by *N*, the total individual of all species in Bogor City. The ecological studies were analysed based on distribution data of *Ficus* spp. using QGIS version 3.10.2-A Coruña. The base map for Bogor City is sourced from Ina-Geoportal (2021).

## RESULTS AND DISCUSSION

### Diversity *Ficus* spp. in Bogor City

Based on the research results in Bogor City, there were 37 species of *Ficus* from six subgenera (*Ficus*, *Pharmacosycea*, *Sycidium*, *Synoecia*, *Sycomorus*, and *Urostigma*) (Table 1, Figure 2). The diversity of *Ficus* species in Bogor City is high compared to other satellite towns such as Sentul city, which recorded 10 species (Mulyani et al. 2021). A total of 877 *Ficus* species have been recorded worldwide (POWO 2021), with 367 species of which can be found in the Malesia to Australasian regions, and 75 species can be found on the island of Java (Berg & Corner 2005). Based on the study, 25 species are native, and 12 species are alien. Native species were found in Bogor City, showing that one-third of the native *Ficus* species on the Java can be found here.

*Ficus* is divided into six subgenera and has distinctive morphological characteristics, including habitus and reproductive system diversity. A total of 1.726 individuals were observed in Bogor City. Most had a habitus as trees (72%), another habitus is shrubs (14%), hemiepiphytes (10%), and root climber (4%). A total of 25 *Ficus* species (73.76%) have a monoecious reproductive system that positively impacts species conservation, especially for key resource species that can bear fruit year-round. Monoecious figs such as *F. benjamina*, *F. virens*, and *F. racemosa* have more adaptive characteristics (Jim 2014; Krishnan & Borges 2018). The gynodioecious is a reproductive system in which each plant has female flowers and hermaphrodite flowers, perfect flowers (male-female) (Bawa 1980), but functionally the reproductive system of gynodioecious is similar to dioecious (Basso-Alves et al. 2014; Teixeira et al. 2018). There were 2 species of *Ficus* with a gynodioecious (14.25%) and 10 species of dioecious (12.01%).

**Table 1**. Diversity of *Ficus* spp. in Bogor City.

| Species | Sexual System | %F | Habitus |
|---|---|---|---|
| Subgenus *Ficus* | | | |
| *Ficus fulva* Reinw. ex Blume | Gyno[1] | 0.46 | T |
| Subgenus *Pharmacosycea* | | | |
| *Ficus callosa* Willd. | Mono[5] | 13.15 | T |
| Subgenus *Sycidium* | | | |
| *Ficus ampelos* Burm.f. | Mono[8] | 0.93 | Sh, T |
| *Ficus heteropleura* Blume | Dioe[9] | 0.06 | Sh, Hm |
| *Ficus montana* Burm.f. | Dioe[6] | 2.32 | Sh |
| *Ficus subulata* Blume | Dioe[7] | 0.06 | T |
| *Ficus tinctoria* subsp. *gibbosa* (Blume) Corner | Dioe[7] | 0.93 | T, Hm |
| Subgenus *Sycomorus* | | | |
| *Ficus auriculata* Lour* | Dioe[5] | 0.52 | T |
| *Ficus fistulosa* Reinw. ex Blume | Dioe[4] | 1.74 | T |
| *Ficus hispida* L.f. | Dioe[3] | 1.80 | Sh, T |
| *Ficus racemosa* L. | Mono[1] | 4.11 | T |
| *Ficus septica* Burm.f. | Gyno[1] | 13.79 | Sh, T |
| *Ficus variegata* Blume | Dioe[4] | 0.64 | T |
| Subgenus *Synoecia* | | | |
| *Ficus pumila* L.* | Dioe[3] | 3.24 | Rc |
| *Ficus trichocarpa* Blume | Dioe[10] | 0.70 | Rc |
| Subgenus *Urostigma* | | | |
| *Ficus altissima* 'variegata'* | Mono[4] | 3.19 | T |
| *Ficus annulata* Blume | Mono[7] | 0.12 | Hm |
| *Ficus benjamina* L. | Mono[4] | 24.68 | T, Hm |
| *Ficus benjamina* 'lanset'* | Mono[4] | 0.12 | T |
| *Ficus benjamina* 'variegata'* | Mono[4] | 0.81 | T |
| *Ficus callophylla* Blume | Mono[12] | 0.12 | T, Hm |
| *Ficus caulocarpa* (Miq.) Miq. | Mono[8] | 0.06 | T |
| *Ficus drupacea* Thunb. | Mono[4] | 0.41 | T, Hm |
| *Ficus elastica* Roxb. ex Hornem* | Mono[5] | 4.92 | T, Hm |
| *Ficus elastica* 'abidjan'* | Mono[5] | 2.26 | T |
| *Ficus elastica* 'tineke'* | Mono[5] | 0.23 | T |
| *Ficus kurzii* King | Mono[12] | 0.98 | T, Hm |
| *Ficus lyrata* Warb* | Mono[1] | 6.49 | T |
| *Ficus maclellandii* King* | Mono[5] | 2.14 | T |
| *Ficus microcarpa* L.f.* | Mono[1] | 3.94 | T, Hm |
| *Ficus microcarpa* 'variegata'* | Mono[1] | 0.06 | T |
| *Ficus natalensis* subsp. *leprieurii* (Miq.) C.C. Berg* | Mono[12] | 0.23 | T |
| *Ficus religiosa* L.* | Mono[1] | 0.23 | T |
| *Ficus retusa* L. | Mono[10] | 0.06 | T |
| *Ficus subcordata* Blume | Mono[11] | 0.06 | T |
| *Ficus superba* (Miq.) Miq. | Mono[5] | 0.06 | T, Hm |
| *Ficus virens* Aiton | Mono[2] | 4.40 | T, Hm |

%F is the relative abundance of species; * introduced species, and some naturalized; Gyno: gynoecious; Mono: monoecious; Dioe: dioecious; T: tree; Sh: shrub; Hm: hemiepiphyte; Rc: root climber; (1) Teixeira et al. (2018); (2) Fu et al. (2017); (3) Nazareno et al. (2013); (4) Corlett (2005); (5) Tarachai et al. (2011); (6) Parrish et al. (2003); (7) Zhang et al. (2020); (8) Huang et al. (2019); (9) Harrison & Yamamura (2003); (10) Jeevanandam & Corlett (2013); (11) Harrison (2008); (12) Berg and Corner (2005).

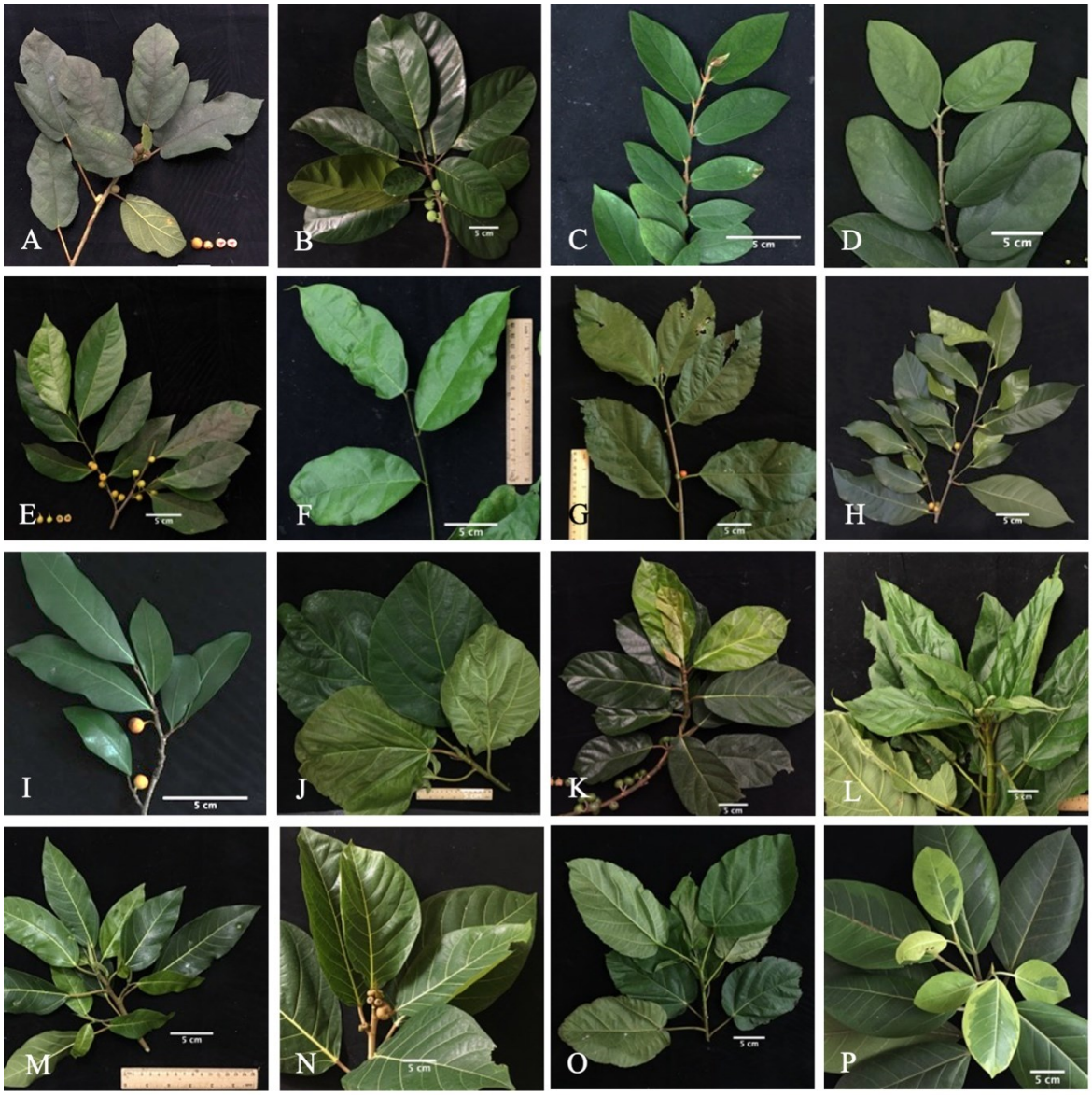


**Figure 2.** *Ficus* spp. found in Bogor City. A. *Ficus fulva*; B. *Ficus callosa*; C. *Ficus pumila;* D. *Ficus trichocarpa*; E. *Ficus ampelas*; F. *Ficus heteropleura*; G. *Ficus montana*; H. *Ficus subulata*; I *Ficus tinctoria* subsp. *gibbosa*; J. *Ficus auriculata*; K. *Ficus fistulosa*; L. *Ficus hispida*; M. *Ficus racemosa*; N. *Ficus septica*; O. *Ficus variegata*; P. *Ficus altissima* 'variegata'.

All species of *Urostigma* were monoecious trees and hemiepiphytes. *Urostigma* also has the highest number of species (22 species) with the highest relative abundance of 55.56%. Another subgenus with a high relative abundance is the *Sycomorus*, with 22.60% (6 species) with shrubs and tree habitus and generally dioecious. Subgenus *Pharmacosycea* has a high relative abundance of 13.15% (1 species), only *F. callosa* with tree habitus and monoecious. Three other subgenera in Bogor City have relative abundance below 10%. Subgenus *Sycidium* at 4.29% (5 species) with diverse habitus: trees, hemiepiphytes, or shrubs. Subgenus *Synoecia* at 3.94% (2 species) had habitus as root climber, and Subgenus *Ficus* at 0.46% (1 species) had tree habitus. These three subgenera generally have a dioecious.

Native *Ficus* are 25 species (75.64%), and 12 species (24.38%) are introduced or naturalized in Indonesia. Naturalized species such as *F. elastica* and *F. religiosa*, these two species have been naturalized for a long time and are

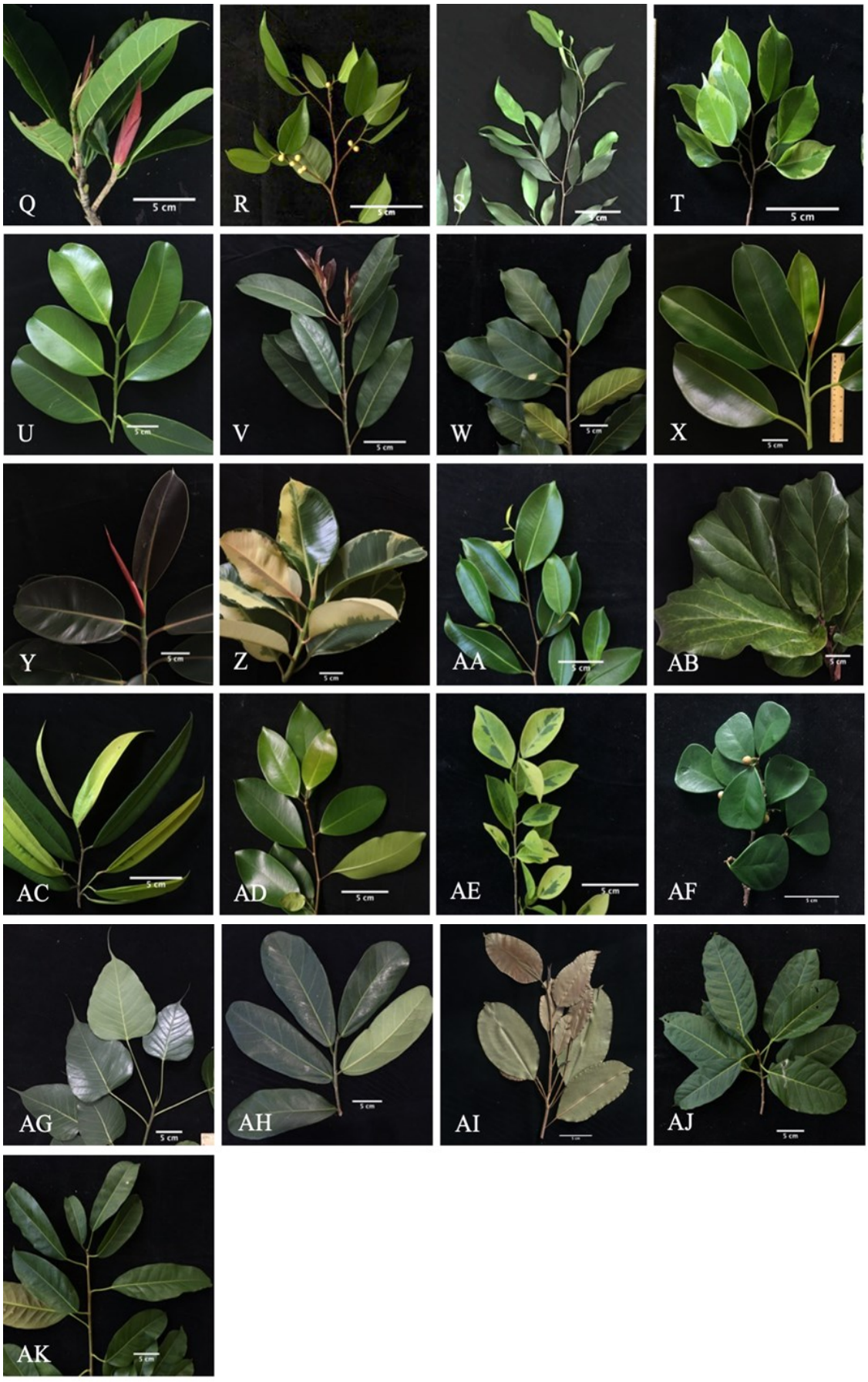


**Figure 2 (continued).** *Ficus* spp. found in Bogor City. Q. *Ficus annulata*; R. *Ficus benjamina*; S. *Ficus benjamina* 'lanset'; T. *Ficus benjamina* 'variegata'; U. *Ficus callophylla*; V. *Ficus caulocarpa*; W. *Ficus drupacea*; X. *Ficus elastica*; Y. *Ficus elastica* 'abidjan'; Z. *Ficus elastica* 'tineke'; AA. *Ficus kurzii*; AB. *Ficus lyrata*; AC. *Ficus maclellandii*; AD. *Ficus microcarpa*; AE. *Ficus microcarpa* 'variegata'; AF. *Ficus natalensis* subsp. *leprieurii*. AG. *Ficus religiosa*; AH. *Ficus retusa*; AI. *Ficus subcordata*; AJ. *Ficus superba*; AK. *Ficus virens.*

widely used by people in Indonesia. In the Lalitavistara relief at Borobudur temple, *F. elastica* and *F. religiosa* (bodhi tree) are found together with *F. benjamina*, *F. microcarpa*, and *F. racemosa* as species that have important values and have been known by the community as food, wood, and sacred (Metusala et al. 2020). Introduced *Ficus* are generally used as ornamental plants because they have unique plant parts, such as *F. elastica*, which has several variations in leaf color and stipules. In addition, the ability to absorb carbon and having a beautiful crown-like *F. lyrata* makes this species commonly planted in city parks or urban forests in Jakarta, Bekasi, and Bogor (Santoso et al. 2021). The high public interest in various species of introduced plants needs to be balanced with efforts to awareness about native *Ficus*, which also have beautiful crowns such as *F. annulata*, *F. drupaceae*, *F. virens*, or *F. superba*, so that planting in urban forests can be directed at native species.

## Distribution *Ficus* spp. in Bogor City

*Ficus* spreads throughout Bogor City. *Ficus* spp. found in Bogor City are generally species with a wide distribution in Indonesia, such as *F. benjamina*, *F. caulocarpa*, *F. microcarpa*, *F. subulata*, *F. variegata*, and *F. virens* (Sukmawati 2019). The abundance of *Ficus* spp. categorized as very high (45-140), was found around the Bogor Botanic Gardens, Sempur Field, Bogor City Square, Sudirman Street, and Ahmad Yani City Forest. The abundance of species in the high category (21-44) was also found in other green belt corridors around the Bogor Botanic Gardens, Dr. Semeru Street, and the largest residential area in Bogor City (Figure 3A). Areas with a relatively very high and high category of the abundance of *Ficus* are included in the Landscape Heritage Bogor City. Therefore, the vegetation in green belt corridors, river boundaries to settlements that are structuring the urban landscape gets attention and periodic maintenance by the local government. The maintenance activity carried out is routine pruning of trees alongside roads and rivers. In addition, planting tree species in Bogor City Green Open Space is still being carried out. *Ficus*, which has a habit of trees and hemiepiphyte, is maintained to create shady vegetation that provides coolness around this Landscape Heritage.

*Ficus* found around the Bogor Botanic Gardens are generally species planted and then spread and grow naturally as hemiepiphytes on green belt corridor and surrounding buildings. In addition, some species are also planted as ornamental plants in office areas and public facilities such as schools, hospitals, and city parks. *Ficus* found around the Bogor Botanic Gardens include *F. benjamina* and *F. virens* with habitus in the trees and hemiepiphytes.

Several *Ficus* spp. have limited distribution around the Bogor Botanic Gardens, including *F. annulata*, *F. callophylla*, *F. drupacea*, *F. superba*, and *F. tinctoria* subsp. *gibbosa*. These five species were probably dispersed with the help of animals and visitors to the Botanic Gardens. Generally, these *Ficus* species have a habitus as hemiepiphyte that grows on trees in the green belt around the Botanic Gardens, such as *Samanea saman* and *Canarium asperum*. Bogor Botanic Gardens in the middle of Bogor City provides many ecological bene-

fits to the surrounding area, such as for animals (birds, bats, insects, and small mammals). Botanic Gardens can be a habitat, a source of food, and a bridge connecting animals' movement space (Arifin & Nakagoshi 2011).

Alongside the Ciliwung River and Cisadane River, consistently *Ficus* abundance was moderate (7-20). Generally, the species found around rivers are *F. racemosa* and *F. variegata*. Both species of *Ficus* can be environmental bioindicators because their presence can indicate ecosystem function changes and provide animal feed (birds and small mammals) throughout the year. However, these two species may be slightly spread in residential areas due to fruit dispersal by animals (birds or bats). Another *Ficus* found in the riverbank, or edge water stream is *F. fulva*. It was commonly found in smaller streams/rivers around agricultural and open areas. Kuaraksa et al. (2012) explained that *F. variegata* and *F. fulva* are suitable species for habitat restoration due to their high adaptability and fast growth. The presence of tree species around the river is expected to preserve the habitat around the river and reduce erosion, landslides, and drought.

Open spaces in Bogor City such as agricultural land, plantations, and areas under development had a low species abundance (0-6) (Figure 3A). Agricultural and plantation activities require a wide area, with management activities including weeds control such as *Ficus* seedling weeds with trees and shrubs. Therefore *Ficus* spp. were found in small numbers in those areas and generally only grow alongside rivers and roads. The southern part of Bogor City currently has an extensive open area due to massive development in toll road construction to housing development, so land clearing is carried out. It has been predicted based on a study by Nurwanda & Honjo (2018), which analyses the land-use change in Bogor City that occurred very quickly, which increased the average air temperature. In 1990, air temperature in Bogor City was recorded at around 23,8°C, then increased to around 26,4°C in 2007, and around 25,7°C in 2017. The temperature increase would occur more often if this developed area does not provide sufficient vegetated area. *Ficus* species commonly found in open areas are *F. hispida* and *F. septica*, both of which can grow in open and dry areas (Parrish et al. 2003; Kuaraksa et al. 2012) because they have mechanisms to defend themselves from drought and maintain balance turgor (Hao et al. 2010). *Ficus* and other plant species in this open area benefit the soil from potential surface run-off and erosion.

*Ficus* spp. can grow in various habitats and sometimes have specific distribution. Subgenus *Synoecia* (*F. pumila* and *F. punctata*) has habitus as root climbers and is generally grown as ornamental plants that decorate buildings or fences. *Ficus variegata*, *F. racemosa*, and *F. fulva*, easy to grow around streams, have also been described. Subgenus *Sycidium* and *Sycomorus* also grow naturally and distribute in open areas or around settlements. There are shrubs and treelet. In contrast, *Ficus* as a tree is generally planted as ornamental plants or grows naturally around the parent tree.

The presence of several *Ficus* that dominates Bogor City certainly has a big part in the ecological function of the city. *Ficus benjamina* and *F. septica*

dominate in Bogor City and have relatively different habitat preferences, although they often grow together in open areas. *Ficus benjamina* generally grows on the street, while mostly *F. septica* grows around settlements. The presence of *F. benjamina* in urban areas can be used as biomonitoring because it has a very important role in the environment, as a recent study in Mexico showed that *F. benjamina* was able to absorb various metal pollutants (Pb, Zn, Cu) from the air (Castañeda-Miranda et al. 2020; Morton-Bermea et al. 2021). In addition, *F. benjamina* can create new niches for various birds in urban areas, such as Depok City, Sentul City, and Dramaga Campus (Pradana et al. 2018; Mardiastuti et al. 2021). The presence of *F. septica* around settlements also has a high ecological role because it can reduce the heavy metals content (phytoremediation) such as mercury (Hg) from the soil (Mariwy et al. 2020). *Ficus septica* also has much bioactive content that is antibacterial (Sudirga & Suprapta 2021). Ethnobiologically, the leaves, and roots of the *F. septica* are widely used as food, medicine, and natural fiber. The indigenous people of Kampung Pinolobu, Sabah, Malaysia, use the roots of *F. septica* as postpartum, stomach aches, and headaches (Awang-Kanak et al. 2021). Also, indigenous peoples in Lampung use these leaves as an eco-friendly leaf plate known as Tebakak (Martinus et al. 2021). Thus, the community can utilize the presence of *F. septica* around the settlements of Bogor City.

Another species that dominates Bogor City is *F. callosa*, which has a tree habitus. This species is common in open areas and settlements. Wijaya & Defiani (2021) in Gianyar, Bali, stated that *F. callosa* have distributed around the riverbank. *Ficus callosa* distribution is limited in the northern Bogor, towards the centre, and is no longer found in southern Bogor (Figure 3B). The main reason for the absence of *F. callosa* in the southern area of Bogor City is the high development and the wider agricultural area.

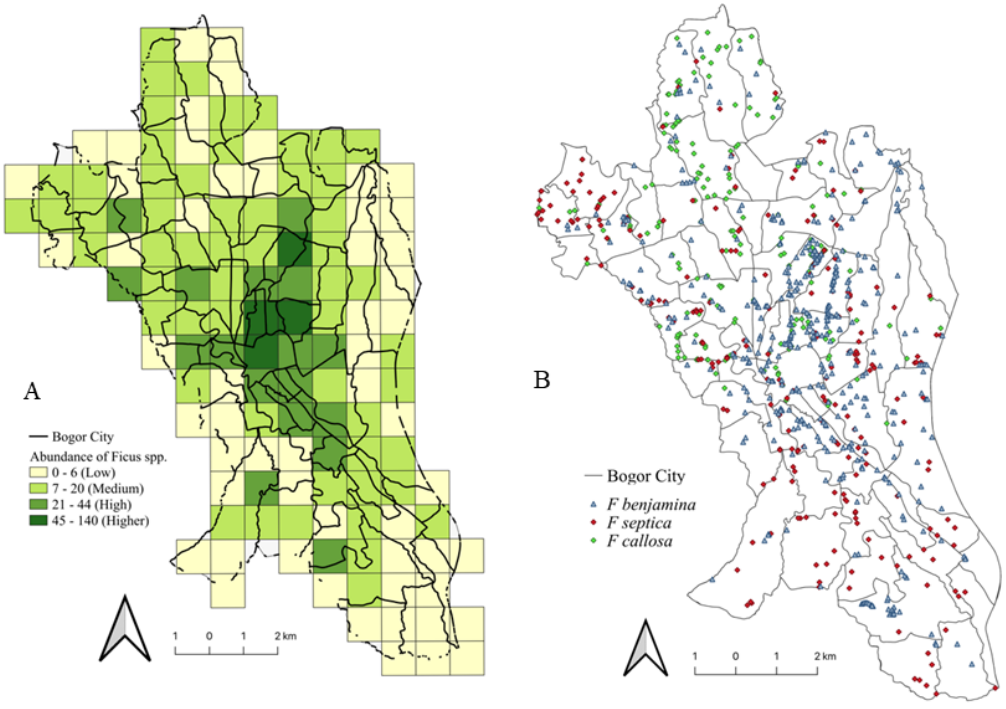


**Figure 3.** *Ficus* spp. distribution in Bogor City. (A) Abundance of *Ficus* spp.; (B) Distribution dominant *Ficus* spp.

Naturalized *Ficus* spp. in urban areas is common, especially as ornamental plants such as *F. lyrata*, *F. altissima* 'Variegata', *F. religiosa* to *F. pumila*. Thus, the abundance of naturalized *Ficus* spp. is getting higher and will gradually suppress the existence of the native *Ficus* spp., leading to a decrease in the number of these native *Ficus* spp. These introduced species are generally planted in the green belt corridors, green open space, and public facilities around settlements in relatively large numbers of individuals, even recently introduced species in Java, such as *F. auriculata*, *F. maclellandii*, and F. *natalensis* subsp. *lepreurii* has been widely planted in home gardens (Peniwidiyanti et al. 2021). Introduced species have a relatively high distribution and abundance of species potential (Table 1) and increased public demand. Planting introduced species in large numbers and areas, such as along roads in residential areas, certainly has a high potential for ecological disturbance in pest attacks, diseases, or decreased soil quality and fruit-eating animals in the vicinity (Krishnan & Borges 2018). The native *Ficus* species generally grows in open areas along roadsides and does not receive special attention from the local community, such as *F. retusa*, *F. subulata*, *F. heteropleura*, *F. caulocarpa*, and *F. subcordata*. Other species of *Ficus* spread with the help of animals such as *F. annulata*, *F. superba*, and *F. callophylla*, which do not receive regular maintenance. Several native *Ficus* recommended for planting activities in Green Open Spaces include species with beautiful crowns such as *F. annulata*, *F. retusa*, *F. subcordata*, and *F. virens*. Other native figs that can be recommended for planting rehabilitation include riverbanks such as *F. variegata*, *F. racemosa*, and *F. fulva*, while planting rehabilitation in open areas such as *F. hispida*, *F. fistulosa*, and *F. ampelos*.

## CONCLUSION

*Ficus* spp. in Bogor City identified 37 species. It is classified into six subgenera: *Ficus*, *Pharmacosycea*, *Sycidium*, *Sycomorus*, *Synoecia*, and *Urostigma*. Subgenus *Urostigma* has the highest species with 22 species, and all species were monoecious. The relative abundance of *Ficus* spp. most recorded around the Bogor Botanic Gardens and the Ahmad Yani City Forest. *Ficus benjamina* and *F. septica* had relatively high abundance and distribution in Bogor City. Another species with a relatively high abundance was *F. callosa*, which was distributed from the north to the centre of Bogor City. *Ficus racemosa*, *F. variegata*, and *F. fulva*, which generally grow around rivers, can be environmental indicators to show land-use change. Several *Ficus* spp. also only grows around the botanic gardens and can indicate that an ex-situ conservation area also significantly increases *Ficus* diversity in Bogor City.

## AUTHORS CONTRIBUTION

All authors (PNW, IQ, TC) had an equal contribution to the conceptualization of research, designing experiments. PNW collected, analyzed data, and wrote the manuscript. IQ and TC supervised all processes and wrote the manuscript.

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## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this article.

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Research Article

# Leaf Morphometric and Chlorophyll Content Study of Bisbul (*Diospyros discolor* Willd.) at the Bogor Botanical Garden

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## ABSTRACT
Bisbul (*Diospyros discolor* Willd.) is one of the collections from the Bogor Botanical (BBG). The wide distribution of this plant in Southeast Asia's tropical forests creates many morphometric variations of this species. The diversity of leaf morphometric variations of a plant species can be the basis for grouping various species. This study aimed to determine the morphometric diversity of Bisbul leaves from various locations of origin. The study took samples of bisbul leaves from Bogor Botanical Gardens from three accessions, namely the Philippines, West Java, and Papua. All the trees are over 30 years old. The leaves are taken based on a horizontal position, vertical crown, and leafage. The results showed that the origin of the location gave different multiplier values to calculate the area, namely the Philippines y = 0.733x + 0.034; West Java y = 0.765x-2,949; and Papua y = 0.758x-1.389. The length to leaf width ratio also has differences, namely, the Philippines, which is 2.64, West Java 2.65, and Papua 2.81. The chlorophyll content in young leaves increases in old leaves. The samples from Papua also have the highest chlorophyll content compared to the Philippines and West Java. This difference indicates morphometric variations between the three, even within one species. Some environmental conditions may affect shade areas and tree age. In addition, DNA research from accession *D. discolor* is also needed to determine the cause of the morphometric variation.



## INTRODUCTION
Bogor Botanical Gardens (BBG) is an ex-situ conservation area with more than 12,370 collected plants covering 3,555 species, 1,202 genera, and 191 families (Ariati et al. 2019). These collections are planted on a land area of 87 hectares in the center of Bogor City. BBG is located at an altitude of 260 m asl with a humidity level of 80-90% and an average rainfall of 3,000-4,000 mm per year (Statistics Bogor Regency 2014). This Botanical Garden focuses on conserving lowland humid and wet plant species. BBG collections originate from Indonesia and abroad. One of the plants that the BBG has successfully collected is the Ebenaceae family (Ariati et al. 2019).

The Ebenaceae family collected in the BBG has only one genus, namely Diospyros. Ebenacean diversity centers are Southeast Asia, Madagascar, Central Africa, and South America. The Ebenaceae family species has reached 1,698 species, and only 751 species have valid names (GBIF 2019; POWO 2019). The genus *Diospyros* has 500 - 600 species scattered in tropical forest areas. The genus *Diospyros* is known to have anti-diarrhea, antibacterial, anti-protozoal, anti-fungal, molluscicide, and anti-inflammatory properties (Howlader et al. 2012). This genus is an essential component of forest vegetation composition in Africa and Asia (Bakhuizen van den Brink 1933; White 1988). The number of these species decreases over time as deforestation increases. However, the Bogor Botanical Gardens have successfully conserved 198 specimens and 32 species of *Diospyros* spp. (Wanda et al. 2019). These collections come from exploration activities in almost all major islands in Indonesia and exchange seeds with botanical gardens from other countries.

*Diospyros discolor* is a species of the Ebenaceae family widespread in Southeast Asia. This plant is known as the Bisbul Tree, Butter fruit tree, and velvet apple tree. *D. discolor* is synonyms with *Cavanillea philippensis* Desr. These are scattered in Sumatra, Java, the Philippines, Taiwan, and the Malay Peninsula (Bakhuizen van den Brink 1936; GBIF 2019). Bisbul trees grow in primary and secondary forests with an altitude of up to 800 m above sea level. Besides producing edible fruit and becoming a food commodity, Bisbul also has potential as wood and an ornamental plant (Rauf et al. 2017).

Bisbul tree has tree habitus; up to 32 m high, free branches up to 10 m, diameter up to 100 cm, and conical. Elliptical leaves; varied in size between 8 -30 x 2.5-12 cm; rounded base; tapered leaf tips, hair with grey color on the underside. Male flowers; 3-7 in cymes; Solitary female flowers, tubular corollas, white. Globose-shaped fruit; 7-10 cm in diameter, brown to dark red, velvety; edible fruit (Bakhuizen van den Brink 1936; Knapp & Gilbert 2002). The seeds are dark brown and flattened. Some vegetative characters, such as leaves in this plant, vary in size, marked by the leaf's size range. Genetic and environmental factors can cause this difference. Ecological differences will cause differences in plant species diversity or plant plasticity (Koch et al. 2006).

In Angiosperm, the morphological characteristics of generative organs (flowers) are widely used as information on species identification and morphometric analysis. However, flowers are temporary, and not all plants can have flowers, resulting in identification using other plant parts, such as vegetative organs. Vegetative organs that can provide information about a plant include leaves, roots, and stems. Of the three organs, leaf organs have the most knowledge and can be the right solution to replace the identification process if no flower organs are found (Stuessy 2009). This condition can be seen from the identification process of *D. discolor,* which rarely uses generative characters because the organ is not always found in every season.

Meanwhile, other characters such as leaves are often found but are neglected because of their plasticity. Apart from leaf morphometrics, leaf chlo-

rophyll content can also be used as information for species identification. One of the causes of leaf color variation is the presence of chlorophyll pigment in leaf tissue. Chlorophyll is a green color-giving pigment in plants, which plays a role in plants' photosynthesis process. The intensity and levels of chlorophyll will be expressed in various green leaves (Buschmann et al. 2012; Ma et al. 2018).

Several studies using leaves for identification have been carried out. Afrinawaty (2007) found many morphological variations in the leaves of the Tabat Barito plant (*Ficus deltoidea*) in West Sumatra. Jawati (2006) also found morphological variations in Andalas (*Morus macroura*) plants in West Sumatra. Haq (2019) studied the Nepenthes pitcher in Central Kalimantan and West Kalimantan and found that morphometrics can be used to distinguish species in Nepenthes. Using a morphometric study, Utama et al. (2012) distinguished five Macaranga species in the Forest Biology Research and Education (HPPB) Padang. However, no studies use leaves to see the morphometric variations of bisbul (*D. discolor*). The use of morphometrics in taxonomy helps to improve scientific rigor in the description of crucial features of biodiversity's phenotypic dimension (Viscosi & Cardini 2011).

Leaf morphometrics can be used as one of the morphological characteristics describing the diversity of phenotypes. Genetic and environmental factors determine the phenotypic diversity of a living thing. Phenotypic properties can be observed in plain view in shape, color, and size (length and width). Leaf morphometric ratio and imaginary leaf area (intact) can determine leaf length and width, while the amount of chlorophyll can be associated with leaf color characters. This research will study the variation of leaf morphometric of *D. discolor*, which grows in the same environment (landscape) but has different origins or genotypes in leaf morphology. Leaf morphometric identification was chosen because it is easy to observe and vital for plant survival. Besides, morphometric studies and chlorophyll content of the Bisbul species have never been carried out.

## MATERIALS AND METHODS

### Materials

All samples are collections of Bogor Botanical Gardens. The samples used in this research were *Diospyros discolor* leaves from three accessions, namely the Philippines, West Java, and Papua. All the trees are over 30 years old.

### Methods

The study was conducted at Bogor Botanical Gardens-BRIN from January 2018 - to May 2020. The study method used a survey, observation, and purposive sampling. Microclimate data retrieval was done using several tools, including termohygro, Soil pH meter, altimeter, and camera.

### Morphometric Identification

Morphometric identification is made by taking leaf samples with the criteria

of young and old leaves at; 1) the four cardinal directions; 2) the position of the leaves on the tree (bottom, middle, top), and 3) the inside and outside of the canopy (Figure 1). Each leaf sample was taken in three replications so that the number of each individual was 48 leaf samples. Each *D. discolor* species from each accession (the Philippines, West Java, and Papua) used a sample from one tree. The parameters observed in this study include leaf length and width to determine the leaf morphometric ratio, imaginary leaf area (intact), and the amount of chlorophyll.

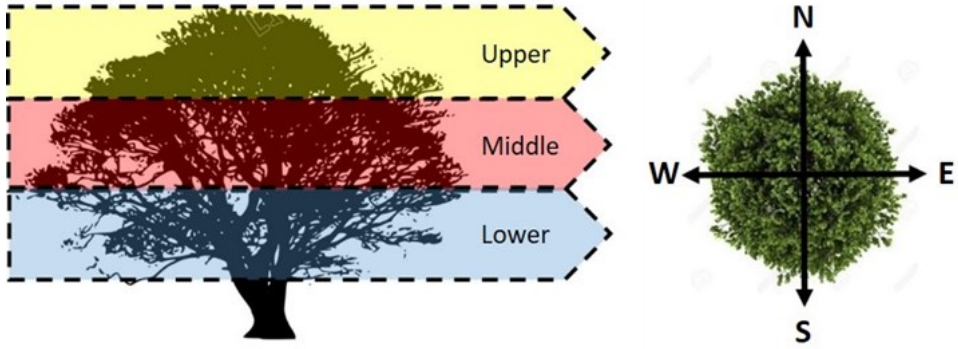


**Figure 1.** Leaf sampling.

Regression analysis was used to determine the relationship between length, width, and leaf area (Stowe 1995). Here is the formula:

$Y = ax + b$

Information:

Y: leaf area (cm2)

a: coefficient x

x: (length (cm) x wide (cm))

b: constant

## Morphometric measurements

Morphometric measurements were carried out by scanning the leaf area and measuring the leaves' size using the Epson L210 printer scanner with the Epson scan software. Image details use JPG format with a resolution of 300 dpi. Leaf scanning results were processed using ImageJ 1.46r software to determine the leaves' length, width, and area. The length and width of the scanned image area are 2481 x 3509 pixels. The calculation starts by calibrating the size to A4 paper size (21.0 x 29.7 cm). The calibration results obtained a scale of 118.14 pixels/cm. The image is then adjusted for color through the threshold technique and selected to calculate the leaf's length, width, and area.

## Chlorophyll content measurements

Chlorophyll content measurements were carried out on all leaves taken at each location of origin. The adaxial sides of the leaves were scanned using the Chlorophyll Meter SPAD-502 Plus tool.

### Data Analysis

The effect of *D. discolor* origin on the morphometric variation and analyzed using the Analysis of Variance (ANOVA) method. If a real impact is found, continue with the Duncan's Multiple Range Test (DMRT) 5% to determine the difference. Leaf morphological characteristics data on different landscapes were processed with Microsoft Office Professional Plus 2016 (Excel) and Minitab 16.1.1 software.

## RESULTS AND DISCUSSION

The results of *D. discolor* leaves' observations found that the three plants of *D. discolor* originating from different locations had different leaf morphometrics (Table 1). The statistical results showed that plant origin differences significantly affected the variables of leaf area, leaf length, and leaf width. From the recapitulation of the results of variance, it was found that the *D. discolor* leaves that had the most significant length, width, and leaf area were *D. discolor* leaves originating from West Java (length: 24.11 cm; width: 9.12 cm; area: 171.20 $cm^2$), and the smallest leaves are *D. discolor* from the Philippines (length: 19.02 cm; width: 7.25 cm; size: 107.40 $cm^2$). *D. discolor* from Papua is 22.34 cm long, 7.99 cm wide, and has a surface area of 137.99 $cm^2$. The results indicate that the difference in origin (parentage) of a plant species also means that the species have a different genotype characterized by different appearances of phenotypes.

The microclimate conditions around the sample are relatively the same, with 28-29 °C of air temperature, 67.6% of air humidity, 5.8 soil pH, and 85-100% of soil humidity. Phenotypic differences that can be seen from these leaf morphometric variations still appear even though these species grow and develop in relatively the same environment. However, there may be differences in the micro-environmental conditions where *D. discolor* grows, which causes differences in leaf phenotypes. Variations in plants due to environmental conditions indicate that a plant adapts. Some environmental conditions that may affect shading area and tree age include *D. discolor* from the Philippines in open areas, while *D. discolor* from West Java and *Diospyros* from Papua are grown in shading areas. In addition, the age of *D. discolor* from the Philippines is younger than the other two *Diospyros*. In response to shade,

**Table 1**. *Diospyros discolor* leaf morphometric variations based on the origin and position of leaves in plants.

| Category | Origin | Leaf area ($cm^2$) | Length (cm) | Width (cm) | Length: Width (cm) | Chlorophyll (SPAD) |
|---|---|---|---|---|---|---|
| Origin | Philippines | $107,40^{a} \pm 54,76$ | $19,02^{a} \pm 4,88$ | $7,25^{a} \pm 1,87$ | $2,64^{a} \pm 0,22$ | $41,95^{a} \pm 16,83$ |
| | West Java | $171,20^{c} \pm 59,57$ | $24,11^{c} \pm 4,81$ | $9,12^{c} \pm 1,77$ | $2,65^{a} \pm 0,20$ | $41,22^{a} \pm 23,70$ |
| | Papua | $137,99^{b} \pm 47,13$ | $22,34^{b} \pm 4,07$ | $7,99^{b} \pm 1,47$ | $2,81^{b} \pm 0,24$ | $45,29^{b} \pm 21,77$ |
| The position of the leaves on the tree | Lower | $139,07^{ab} \pm 57,86$ | $21,79 \pm 4,77$ | $8,20^{ab} \pm 1,86$ | $2,68 \pm 0,25$ | $41,03^{a} \pm 21,22$ |
| | Middle | $152,72^{b} \pm 63,02$ | $22,90 \pm 4,73$ | $8,54^{b} \pm 1,83$ | $2,69 \pm 0,19$ | $44,99^{b} \pm 21,19$ |
| | Upper | $124,79^{a} \pm 56,06$ | $20,79 \pm 5,45$ | $7,63^{a} \pm 1,83$ | $2,72 \pm 0,26$ | $42,44^{ab} \pm 20,50$ |

Note: The number in the same column, followed by a different letter, differs significantly from Duncan's Multiple Range Test (DMRT) 5%.

plants showed functional changes in physiological and morphological aspects. These functional changes can be seen in the plant *Enterolobium contortisiliquum* (Vell.) Morong (Souza et al. 2017). As a form of adaptation to natural conditions or environmental stresses, plants can experience phenotypic plasticity, which is the ability of an individual to modify some specific traits during their development period (Jones & Luchsinger 1987). Understanding genetic diversity and its origin distribution are fundamental to conservation and sustainable use.

Another result was obtained when the leaf morphometric variations were compared based on the leaves' position on the tree, namely the bottom, middle, and top. From the ANOVA statistical results, differences in leaf position significantly affected leaf area and leaf width variables. From the recapitulation of the effects of variance, it was found that the leaf area of the *D. discolor* leaves in the middle position (152.72 cm) was wider when compared to the bottom (139.07 cm) and upper (124.79 cm). This leaf area variation is supported by differences in leaf width, although the length and ratio of height to width are not significantly different. Physiological and environmental factors cause these variations, and tree age and shading area are suspected to be the causes of this condition. Leaves are one of the organs that develop pretty quickly and are classified as sensitive. Cox & Moore (1980) stated a correlation between climate and leaf character. Leaf size and leaf margins can explain plants' adaptation to average rainfall and temperature.

The leaf's length and width ratio describe the leaf's general shape. The leaf shape, formed from the rate of leaf length to width, also shows a significant difference from Papua (Figure 2). The most considerable leaf length and width ratio value was that of *D. discolor* from Papua (2.81). Then followed by *D. discolor* from West Java (2.65) and the Philippines (2.64). The length and width ratio did not significantly differ between the leaves at the top, middle, and bottom positions (Table 1). The value of the ratio of the length and width of the leaf affects the shape of a leaf. The L/W ratio has an intimate association with the geometrical features and the self-similarity of leaf shape (Shi et al. 2021). *Diospyros discolor* leaves from 3 locations, namely the Philippines, West Java, and Papua, have the same morphological shape. Based on the identification results, *D. discolor* from the 3 locations has elliptical leaves with a rounded base; symmetrical; the leaves' tips are tapered and hairy with grey color on the underside. Young leaves have a lighter green color than the fully developed leaves with darker colors. From the ANOVA statistical results, differences in plant origin and leaf position on trees significantly affected leaf chlorophyll content (Table 1). The leaf chlorophyll content is what causes color variations in *D. discolor* leaves. Colour variations in leaves are caused by chlorophyll, carotenoid, and anthocyanin pigments in the leaf tissue. The chlorophyll content in dark green leaves is greater than that of light green leaves (Pandey & Sinha 1979).

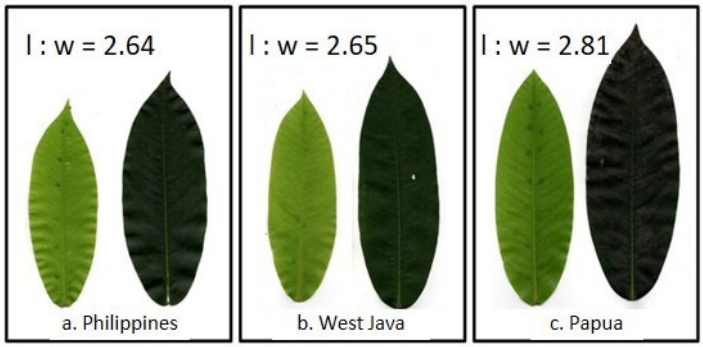


**Figure 2**. Length and width ratios of *Diospyros discolor* leaves from three locations. note: p = length; l = width; The color of the lower leaf surface is dark green, whereas the color of the top leaf surface is light green.

## Chlorophyll content

Internal and external factors of plants influence the difference in the content of chlorophyll pigment. Internal factors include genetics, while external factors include light, nutrients, water, temperature, and soil pH. Internal factors in different genes on the chromosome will regulate chlorophyll's biosynthesis process (Kurniawan et al. 2010). These external factors will later affect pigment synthesis (Mlodzinska 2009; Juneja et al. 2013) and enzyme activity involving chlorophyll synthesis (Raharjeng 2015).

The results showed that the highest chlorophyll content was *D. discolor* from Papua (45.29), and the lowest was *D. discolor* from West Java (41.22) (Table 2). Based on the leaf position, the middle part contained the highest chlorophyll (44.99), and the lowest was in the lower leg (41.03) (Figure 3). The tree's age is why *D. discolor* from Papua has the highest chlorophyll content. *D. discolor* from Papua has the youngest tree age compared to the other two *Diospyros*. Sunlight is the main factor in determining the amount of chlorophyll content in leaves and is directly proportional to the leaf's age. Early in leaf development, leaf meristem activity causes leaf elongation, and subsequent leaf elongation occurs due to intercalary meristem activity (Hidayat 2008). Chlorophyll biosynthetic ability is thought to be different between species and cultivars. According to Ai & Banyo (2011), pigment formation in plants is influenced by internal factors, namely plant genetics. Kurniawan et al. (2010) stated that different genes on chromosomes regulate chlorophyll biosynthesis.

**Table 2**. Leaf chlorophyll content of *D. discolor* from three locations based on leaf age.

| Leafage | SPAD value | | |
|---|---|---|---|
| | Phillippines | West Java | Papua |
| Young leaves | 26,54 | 18,19 | 25,04 |
| Old leaves | 57,37 | 64,24 | 65,53 |
| Average | 41,95 | 41,22 | 45,29 |

The chlorophyll content of young leaves and old leaves is other. This difference can be seen in the relatively different green colors of the leaves.

The darker green leaf color may indicate that they contain higher levels of chlorophyll. Also, chlorophyll in leaves is influenced by many things, especially genetics, light, oxygen, water, and temperature. The highest amount of chlorophyll in young leaves was found in *D. discolor* leaves from the Philippines (26.54), and the lowest in *D. discolor* leaves from West Java (18.19) (Table 2). In old leaves, the highest chlorophyll content was found in *D. discolor* leaves from Papua (65.53) and the lowest in leaves from the Philippines (57.37). This condition is influenced by genetic and environmental factors that provide phenotypic variations in the chlorophyll content of *D. discolor* leaves. Besides genetic factors, light, oxygen, water, and temperature, other factors that influence chlorophyll's formation include N, Mg, and Fe as builders and catalysts in chlorophyll synthesis. The chlorophyll content in plants is about 1% dry weight (Dwidjoseputro 1994). According to Sumenda et al. (2011), leaves' ability to photosynthesize increases until the leaves are fully developed and slowly decline. Old leaves that are almost dead become yellow and unable to photosynthesize because of chlorophyll breakdown and loss of chloroplast function.

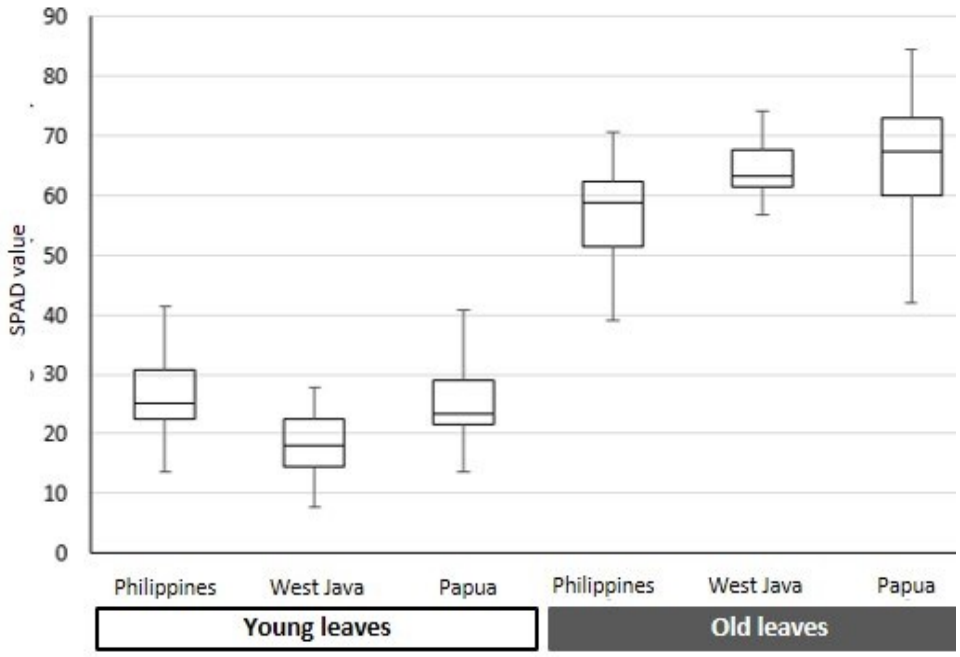


**Figure 3**. The chlorophyll content of *D. discolor* leaves from three different locations.

## Leaf Area Analysis

Figure 4 shows that *D. discolor* originating from West Java has a leaf area formula y = 0.765x - 2.949, Papua is y = 0.758x - 1.389 and the Philippines is y = 0.733x + 0.034. (y) is the actual leaf area value, while (x) is the length measurement value multiplied by the leaf width. The leaf area regression analysis showed that the leaves *D. discolor* originating from West Java had the highest coefficient compared to those from the Philippines and Papua

This coefficient difference value indicates that there are morphometrically based on length, width, and leaf area variations between the three, even

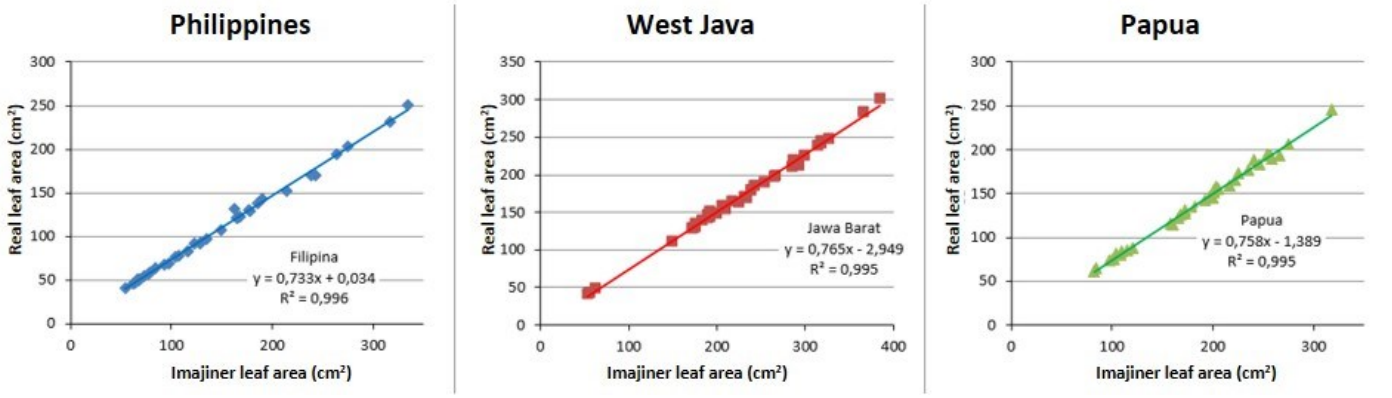


**Figure 4.** Relationship between imaginary leaf area and actual leaf area in *D. discolor* leaves.

though within one species. The value of the coefficient (a) in the leaf area formula (Y = ax + b) can be a reference to distinguish the difference in leaf area from three different locations. The greater the coefficient value, the higher the multiplier for the length and width. The coefficient will be directly proportional to the results of the leaf area calculation; the leaf area difference is thought to be because *D. discolor* underwent morphological adaptations to its environment. Changes in adaptation may cause genetic changes, so further research is needed regarding these changes. This coefficient can be used to reference further research for *D. discolor* living or originating from each location and research for other plant species. This difference is also thought to be caused by one factor, namely the condition of sun exposure. According to Sirait (2008), sunlight has an essential function in photosynthesis, affecting leaf growth.

Leaf morphometric differences can indicate canopy density, biomass, and a determinant of plant evapotranspiration. The similarity of the LAI pattern shown in Figure 4 also has implications for the health aspects of the three types of *D. discolor*, which are classified as the same. Further developments in determining the Leaf Area Index (LAI) can also estimate plant health and plant optimum productivity. LAI values have increased along with plant growth and development. The beginning of growth is marked by young leaves leading to adult development marked by aging leaves. Likewise, the leaf area in the crown and the area of protected land will also increase. Increasing the LAI value will increase the net assimilation result or what is known as the NAR (Net Assimilation Rate) (Zakariyya 2016).

## CONCLUSION

The difference in plant origin significantly affects the leaf morphometrics *of D. discolor*, namely the leaf area, leaf length, leaf length and width ratio, and chlorophyll in leaves. The highest chlorophyll content was in *D. discolor* leaves from Papua, and the lowest was *D. discolor* from West Java. Based on leaf area, there are morphometrically variations between the three, even though within one species. Some environmental conditions may affect shade areas and tree age. In addition, DNA research from accession *D. discolor* is also needed to determine the cause of the morphometric variation. Further inves-

tigations into the DNA from *D. discolor* accessions are expected to confirm the taxonomic status of this species.

## AUTHORS CONTRIBUTION

The author's contribution: IFW designed the research, collected and examined the data, and wrote the manuscript. ANR designed the research, collected and analyzed the data, and AAO improved the manuscript.

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## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this article.

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Research Article

# Bycatch of Amboina Box Turtle (*Cuora amboinensis*) by Fishermen in Rawa Aopa, Southeast Sulawesi

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## ABSTRACT
Rawa Aopa is a permanent swamp ecosystem that serves as one of the suitable habitats for amboina box turtles (*Cuora amboinensis*). Some of the area is part of a national park (Rawa Aopa Watumohai National Park) and is designated as a traditional zone for fishing activities, where local fishermen sometimes reported accidental catch *C. amboinensis* in their fishing gear. The aims of this study were to record the existence of *C. amboinensis* bycatch, size and age structure of bycatch, characteristic of fishing activity, and to discuss the conservation implication of bycatch. The number of bycatch was recorded by direct observation of 7 selected fishermen in 14 days. The *C. amboinensis* caught accidentally were measured and weighed. A total of 38 individuals of *C. amboinensis* were accidentally caught by fishermen during the study, having a size ranged of 7.4 to 18.5 cm (juveniles, young adults, and old adults; no hatchling), and weighed 248 to 996 g. Based on sex, there was no significant difference between morphological size of male and female, although bycatch for females (59%) tend to be slightly higher than males (41%). Most of fishermen lives in Pewutaa Village and used *bubu* traps to catch fishes. *C. amboinensis* are the most common bycatch compared to other species. In order to minimize the impact of bycatch of the *C. amboinensis* by fishermen, we need to ensure that the turtle that accidentally trapped in the fishermen's fishing gear would be released unharmedly to their habitat.



## INTRODUCTION
Inland fisheries in freshwater in Indonesia are considered small compared to the coastal and marine fisheries. Yet, artisanal fisheries of inland waters, especially in lakes, are important for the livelihood of the local communities, for instance in Tondano Lake, Sulawesi and Sentarum Lake, Kalimantan (Makmur et al. 2021). In the swamp of Rawa Aopa in Southeast Sulawesi, part of the Rawa Aopa Watumohai National Park, fishes are diverse and abundant (Wulandari et al. 2018; Muliani et al. 2021) which attract fishermen. In addition to fishes, the swamp is also a home to diverse birds and other wildlife species (Putri 2016; Ridha et al. 2021).

As a permanent swamp ecosystem, Rawa Aopa is considered as suitable habitats for Amboina box turtle *Cuora amboinensis* (Aini et al. 2019), a freshwater turtle with a wide distribution in Indonesia and Southeast Asia. The turtle has been harvested for food in many part of its range (Schoppe

2009; Fauzi et al. 2020), which eventually raised the conservation status from Vulnerable to Endangered in IUCN Red List 2020 (Cota et al. 2020).

To conserve a certain species, basic information on biology and ecology should be made available, including threats. Information is usually obtained from the local communities living around its habitat. This traditional ecological knowledge can be used to obtain relevant data and increase understanding to conserve certain species (Howard et al. 2011; Butler et al. 2012). Fishermen are one of the potential informants to gather information of certain species or to obtain information regarding bycatch (Thornton & Scheer 2012; Zappes et al. 2016; Lucchetti et al 2017).

Previous research about *C. amboinensis* in the Rawa Aopa Watumohai National Park mostly was conducted in temporary pools in the savanna ecosystem, focusing on population estimation and habitat suitability models to analyze its distribution (Widagti 2007; Schoppe 2009; Aini et al. 2019). Unlike the previous research, this research was focused on permanent swamp ecosystem, the Rawa Aopa (±30,000 ha; '*rawa*' means swamp), located in a traditional zone where local community utilizes natural various resources, including fishing (KLHK 2018).

Fishing activities by fishermen in the study area will be used as the source of information for the existence of *C. amboinensis*, as well as the possible threat for this species due to bycatch. Anecdotal report indicated that *C. amboinensis* were accidentally trapped in fishermen's traditional fishing gear (called *bubu*) during fishing (Aini et al. 2019) and thus considered as bycatch. Bycatch is an important issue in fisheries as it creates a problem due to unused and discarded catch of unwanted species (Nugroho et al. 2015). The study of bycatch is needed to understand the size and area of bycatch (Wallace et al. 2010). In order to mitigate the population decline of *C. amboinensis* due to human activities, it is important to collect information regarding the characteristics of captured *C. amboinesis* as bycatch. The aims of this study were to record the existence of *C. amboinensis* bycatch, the size and age structure of this bycatch, characteristic of fishing activities and to discuss the conservation implications of this bycatch.

## METHODS

### Study Area

The research was conducted in Rawa Aopa swamp, located in *Seksi Pengelolaan Taman Nasional (SPTN) I* of the Rawa Aopa Watumohai National Park, Southeast Sulawesi (Figure 1). Field data was collected from February to March 2020.

Rawa Aopa is a large peat swamp and the only major peat swamp in Sulawesi under administration of two regencies: South Konawe and Konawe. The water bodies of the swamp are covered with vegetation of more than 90% with substrates mostly peat. The topography is flat that gradually turns into hilly (Zwahlen 1992). Rawa Aopa has an important function as water regulator for the surrounding area. During rainy season, it serves as water

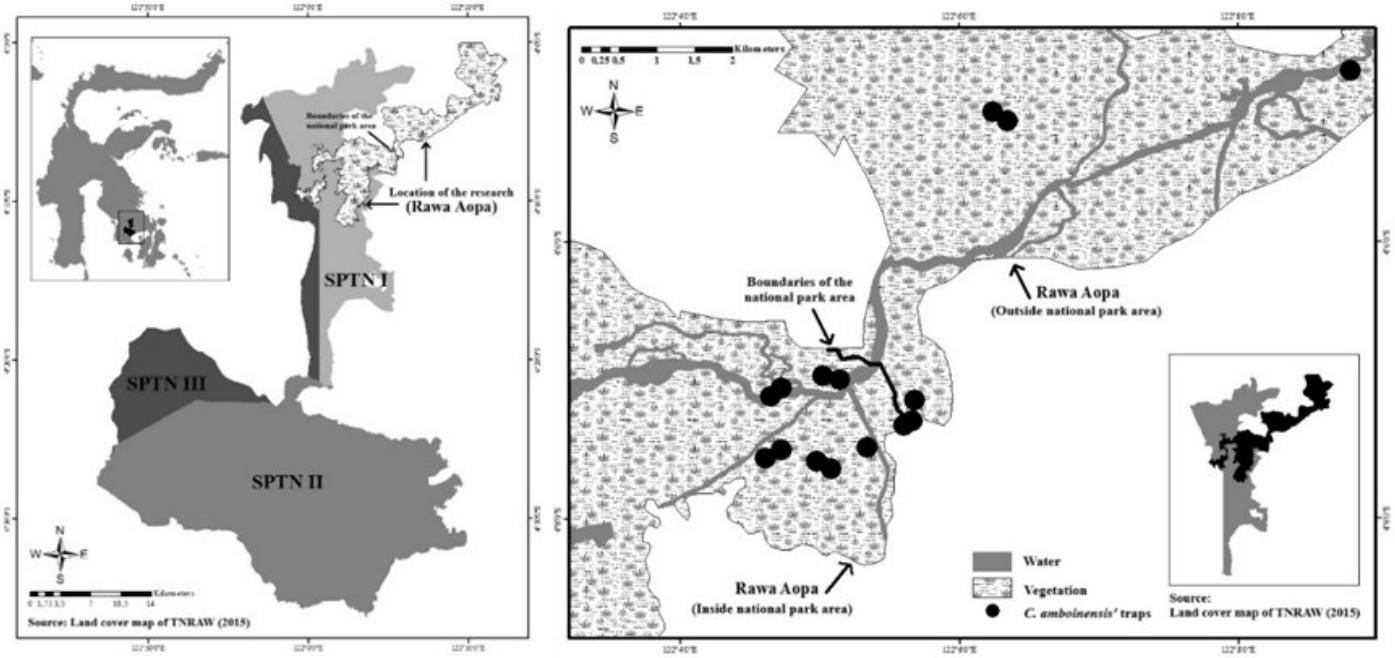


**Figure 1.** Map of Rawa Aopa, showing the placement of fish traps (right). Some of the swamp area is located inside the national park area (Management Section I), while some others is outside the national park.

catchment area and supplies the water to the local communities along the Sampara River Basin to Kendari during dry season. The water is also used by a regional drinking water company to fulfill water needs for the people of Kendari and its surrounding area (Sugiarto 2007).

Of the total ±30,000 ha of Rawa Aopa swamp, ±11,488 ha is managed by the national park authority (Sugiarto 2007; BTNRAW 2018). The boundary of the national park is marked by a bridge. The northeast area is outside the national park and the southwest area is inside the national park. Rawa Aopa within the national park is part of the traditional zone, where the local communities are allowed to utilize the natural resources, such as fishing and harvesting swamp plants (swamp pandanus or in the local languange called *totole*) for producing a mat (KLHK 2018). The community of Pewutaa Village located in the east of the Rawa Aopa has carried out traditional fishing from generations. The communities divided the year into three seasons: the rainy season (February to July), the dry season (August to January), and the transitional season (May to June).

## Data collection

Information regarding *C. amboinensis* was obtained through a 7-day face-to-face interview to 32 fishermen. Prior to the study, we obtained prior informed consent (PIC) by informing interviewees the purpose of the study commencing the survey. Interviews were only carried out after verbal consent were issued by the fishermen. We ensured that data collected were confidential and anonymity of fishermen was protected. After PIC were obtained, we began with showing a photo of *C. amboinensis* and its egg to ensure respondents recognized the turtle. We develop a set of open questions consisted of three categories: identity of respondent, frequency of *C. amboinensis*

captured (including number of captured, turtle size, condition of the turtle caught), and equipment (i.e., type of fishing gear, location of installation, species of harvested fish, treatment of caught turtle).

Based on the interview, we followed activities of 7 selected fishermen to gather more detailed information of *C. amboinensis* bycatch. Data were collected for 14 days in the morning (6-9 am) or evening (4-6 pm) by canoeing the swamp to find *C. amboinensis* trapped in fishermen's fishing gear or moving around the swamp.

We recorded sex and morphological characteristics of each individual of *C. amboinensis* obtained from the fishermen's trap. The morphological characteristic recorded included carapace length and width, plastron length and width, tail length, and body mass (Figure 2). To identify a possible of recapture, *C. amboinensis* then were marked on the marginal carapace using a marker with a unique code following Karraker et al. (2020). Individuals that have been measured were released to their original location of capture.

The fishing area as the turtle habitat (presence of plants, water depth) was recorded and described. In addition, the abiotic components were also measured, including air temperature, humidity, and water acidity.


**Figure 2.** Measurements of morphological characteristics of *C. amboinensis* (a) straight carapace length, (b) straight plastron length, (c) straight carapace width, (d) straight plastron width, (e) height, (f) curved carapace length, (g) curved carapace width, (h) curved plastron length, (i) curved plastron width, (j) tail length, (k) weight.

### Data analysis

Average and standard deviation were calculated to determine the range for each morphological variable of captured turtles, and presented in a box plot. An independent t-test was performed to obtain differences between sex, with a normality test carried out to ensure that the data distributed normally. The results of the calculations were displayed in tabular form for descriptive analysis. The age structure of *C. amboinenesis* was distinguished by the size of the straight carapace length and classified into four categories (Table 1) (Widagti 2007). Results of this study were compared to similar study in Southeast Asia.

**Table 1.** Age structure of *C. amboinensis* based on straight carapace length (SCL).

| Age Class | Straight Carapace Length (cm) | Age Structure |
|---|---|---|
| I | ≤ 5.0 | Hatchling |
| II | 5.1-11.5 | Juvenile |
| III | 11.6 -15.9 | Young adult |
| IV | ≥ 16.0 | Old adult |

## RESULTS AND DISCUSSION

### Morphological characteristic and age structure of *C. amboinensis*

During the research, 38 individuals of alive *C. amboinensis* were caught in fish traps, of which 4 individuals were recaptured. The *C. amboinensis* turtles were found in fish traps located inside (n = 21, 55%) and outside (n = 17; 45%) the national park. Most of turtles (n = 24; 63%) are normal, and unfortunately 14 turtles (37%; 4 juveniles and 10 adults) were found with certain abnormalities. The abnormality found in this research includes damage to the carapace and plastron, differences in number of marginal carapace, and defect in the legs and tail. There might be several factors that caused the abnormality, including fishermen handling when releasing turtle, predatory attack, and genetic disorders. As for nest finding, only one fisherman informed a finding of a nest near a dry grassy area underneath a *longgida* tree (*Nauclea orientalis*).

All *C. amboinensis* turtles were found inside traps (totalling 15 traps), except for one turtle which was found swimming near traps. All traps containing turtles were mostly traps located in areas with dense aquatic plants (Figure 3). Aquatic plants in Rawa Aopa consisted of lotus (*Nymphaea* sp.), *taboru-boru*, *doida* or swamp grass, *wolungatuleleo/apu-apu* or water lettuce (*Pistia stratiotes*), swamp pandanus (*Pandanus* sp.), water hyacinth (*Eichhornia crassipes*), *tolueda/lemidi* (*Stenochlaena palustris*), swamp fern, and *kurangkasoro* or water-moss (*Salvinia* sp.) (Figure 4).

Measurements of the abiotic components showed that the air temperature was between 25-33°C and the humidity was 94-99%. Meanwhile, the water color was blackish with acidity level (pH) 6. Water depth was between 2-10 m with peat substrate originated from rotting aquatic plants above it.

Based on the age structure (AS), the composition of bycatch *C. amboinensis* consisted of juveniles (AS II) and adults (AS III and AS IV) (Figure

5). No hatchling (AS I) was found. As for the sex ratio, of the 27 adult individuals that were able to be clearly distinguished the sex difference, the composition was 11 males (41%) and 16 females (59%).

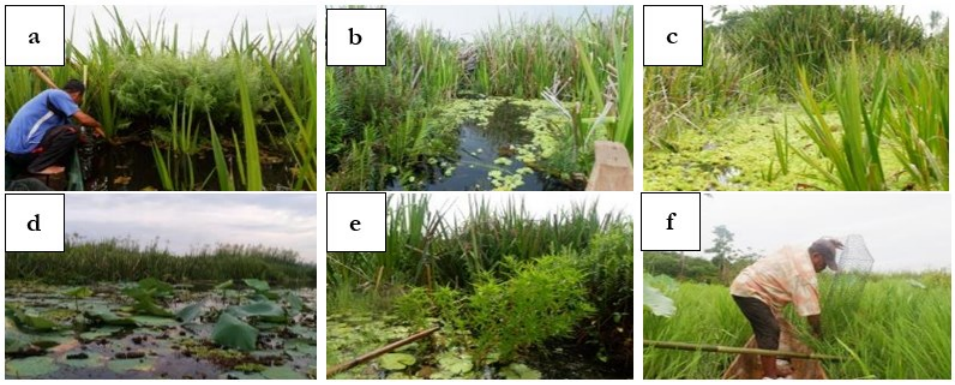


**Figure 3.** Habitat where *C. amboinensis* were mostly found in traps in Rawa Aopa (a and b), aquatic plants in trapping sites covered most of water level (c, d, e, and f).

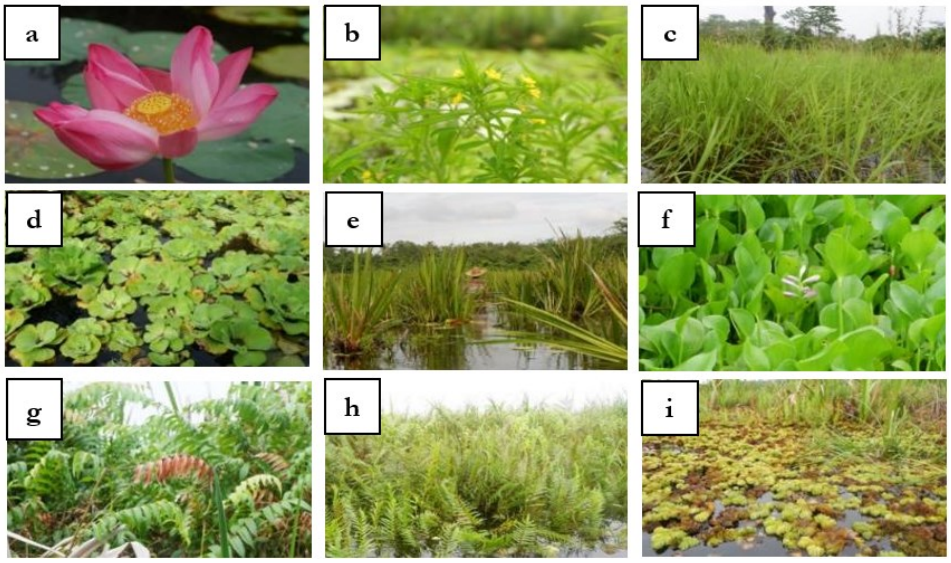


**Figure 4.** Several plants found in the habitat of *C. amboinensis* in Rawa Aopa: (a) lotus (*Nymphaea* sp.), (b) taboru-boru, (c) doida, (d) wolungatoleleo or water lettuce (*Pistia stratiotes*), (e) swamp pandanus (*Pandanus* sp.), (f) water hyacinth (*Eichhornia crassipes*), (g) tolueda (*Stenochlaena palustris*), (h) swamp fern, (i) kurangkasoro or watermoss (*Salvinia* sp.).

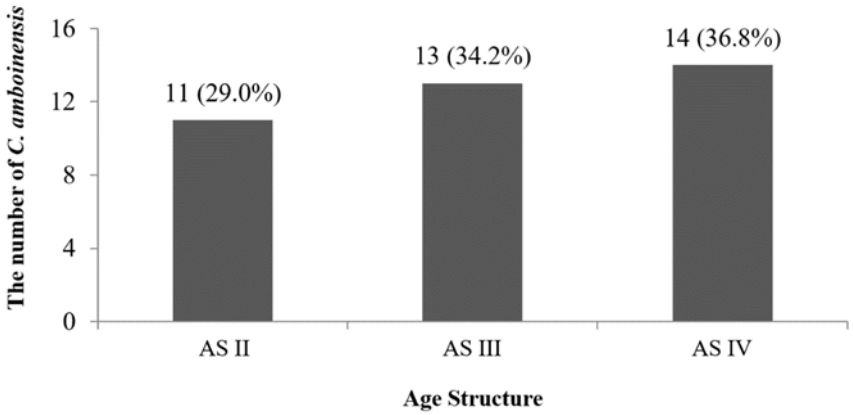


**Figure 5**. The age structure of *C. amboinensis* that were found from the bycatch in Rawa Aopa.

The size of *C. amboinensis* found from bycatches in Rawa Aopa varied highly, ranging from 7.4 cm to 18.5 cm (based on the SCL measurements; Figure 6). The largest size of female captured has SCL of 18.5 cm and for male with SCL 18.3 cm (Table 2).

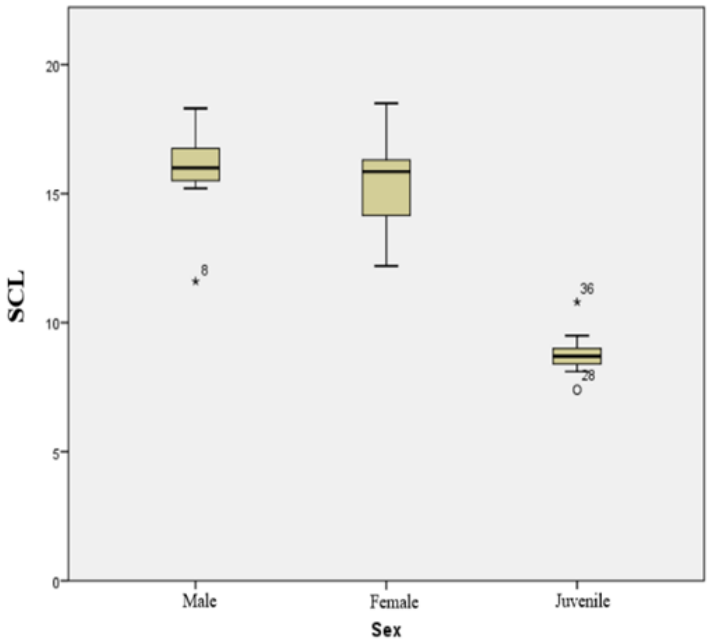


**Figure 6.** The boxplot of straight carapace length (SCL) from male (n = 11), female (n = 16), and juvenile (n = 11) of the *C. amboinensis* bycatch in Rawa Aopa.

The mean size (SCL) of the *C. amboinensis* in this study was smaller than the turtle found in Malaysia (SCL 21.5 cm) (Schoppe 2008), in temporary pools savanna in the national park from previous study (SCL 22 cm) (Widagti 2007), and in Myanmar (SCL 23 cm) (McCord & Philippen 1998). The difference might be caused by the equipment used in trapping the turtle. Fishing traps usually has a small opening gap (5-7 cm), thus bigger size turtles were not able to be trapped. Another study on four species of freshwater turtles (*Chrysemys picta, Graptemys geographica*, *Sternotherus odoratus*, dan *Chelydra serpentina*) in Canada also showed that the opening gap of the fishing gear was known to be affected on the size of the turtles caught (Cairns 2013). Turtles

**Table 2.** Morphological characteristics of adult *C. amboinensis* bycatch in Rawa Aopa.

| Measurement (cm) | Female (n=11) | | Male (n=16) | |
|---|---|---|---|---|
| | Average ± SD | Min-Max | Average ± SD | Min-Max |
| Straight carapace length (SCL) | 15.38 ± 1.81 | 12.2-18.5 | 15.95 ± 1.76 | 11.6-18.3 |
| Curved carapace length (CCL) | 17.63 ± 2.17 | 13.6-21.7 | 18.65 ± 2.76 | 11.2-22.0 |
| Straight carapace width (SCW) | 12.01 ± 1.71 | 10.1-17.7 | 11.59 ± 0.89 | 9.4-13.1 |
| Curved carapace width (CCW) | 17.10 ± 3.24 | 13.5-27.6 | 16.24 ± 1.59 | 12.4-17.8 |
| Straight plastron length (SPL) | 13.93 ± 1.69 | 11.4-17.3 | 13.45 ± 1.31 | 10.1-15.1 |
| Curved plastron length (CPL) | 15.06 ± 3.32 | 11.6-25.8 | 15.52 ± 4.55 | 10.5-24.6 |
| Straight plastron width (SPW) | 7.56 ± 0.80 | 6.3-9.0 | 7.16 ± 0.56 | 5.7-7.9 |
| Curved plastron width (CPW) | 7.66 ± 0.79 | 6.3-9.0 | 7.35 ± 0.62 | 5.8-8.1 |
| Height# | 6.53 ± 0.83 | 5.2-7.8 | 5.96 ± 0.59 | 5.1-7.2 |
| Tail length** | 4.05 ± 0.61 | 3.0-5.0 | 5.08 ± 1.05 | 3.3-6.9 |
| Weight (in g) | 556.19 ± 199.79 | 269-996 | 548.82 ± 127.59 | 248-785 |

**highly significant different at α=0.01, #slightly different at α=0.1

which had entered the trap need to have shorter carapace height than opening gap of the trap.

Except for the tail length (i.e., males had longer tail), other morphological characteristics showed no significant difference between males and females. Females slightly had higher body, although the differences was not quite apparent. The limited number of samples on large-sized *C. amboinensis* might hamper a firm conclusion on the body characteristics between males and females.

Fishing gear used by fishermen in Rawa Aopa was selective to the size of the *C. amboinensis* bycatch. There were three types of the fishing gear used by fishermen in Rawa Aopa: fishing line, seine, and *bubu* trap (Figure 7). *Bubu* traps of different size and shape were the most common fishing gear used by the fishermen. Almost all the turtles were trapped in the *bubu* (juveniles to adults), although no hatchling was found. The lack of hatchling might be caused by smaller carapace height of the hatchling compared to the opening gap of the *bubu* (5-7 cm), which enabled trapped hatchling to get out easily.

## Fishermen characteristics and activities

Most of the people in Pewutaa Village are fishermen. This might be influenced by the location of the village which is surrounded by Rawa Aopa swamp, thus harvesting fish was the main source of income of the people in the village. According to the fishermen, the use of *bubu* trap as the main fishing gear was due to the fact that most water level was covered by the aquatic plants. Time for checking *bubu* trap is relatively longer than other type of fishing gear, which is more effective and efficient because it does not need to be checked every day. In addition, fishes caught by *bubu* trap is still alive


**Figure 7.** Fishing gear used by fishermen of Rawa Aopa (a) fishing hook, (b) fishing line, (c) seine, (d) *bubu* trap size 20 × 130 cm, (e) *bubu* trap size 40 × 40 × 50 cm, (f) *bubu* trap size 40 × 40 × 100 cm, (g) *bubu* trap size 20 × 100 cm.

compared to other gears i.e. fishing rods or seine, where the fish caught will be mostly dead and rotting when harvested. This is considered to reduce the quality and the quantity of the catch.

Fish commodity from Rawa Aopa consisted of tilapia fish (*Oreochromis mossambicus*), climbing perch (*Anabas testudineus*), kissing gourami (*Helostoma temminckii*), snakeskin gourami (*Trichogaster pectoralis*), snakehead murrel (*Channa striata*), Java barb (*Barbonimus gonionotus*), and catfish (*Clarias batrachus*). Bycatch is the accidently capture of all other aquatic animals outside the main target catch including all other aquatic animals by fishing gear (Novrizal et al. 2018). Bycatch consisted of snails, crayfish (Cherax sp.), birds, snakes, water monitor (*Varanus salvator*), juvenile crocodile (*Crocodylus porosus*), armored catfish (*Glyptoperichthys gibbiceps*), and amboina box turtle (*C. amboinensis*). Some of the bycatch was consumed (i.e., armored catfish, crayfish, and snail), used as fishing bait (snails), or released by the fishermen (birds, snakes, water monitors, crocodile, and amboina box turtles). *C. amboinensis* are the most common bycatch compared to other species. The number of turtles found in one trap ranged from 1 to 10 individuals with varying sizes. The use of *bubu* traps might be one of the reasons for bycatch of *C. amboinensis* and there is a possibility that turtles were "attracted" by the content of the *bubu* trap. All respondents remarked that when they found the turtle they will immediately threw the turtle away and considered them as pests. This is because often fish catch decreased when turtle is found in the fishing gear (especially *bubu*). Although most fishermen said that they released all turtles alive, no fishermen stated that he specifically killed turtles found inside the *bubu* trap.

## Conservation implication of bycatch

Discard catch is part of bycatch that can be destructive, because the organism being discarded is almost never reported, resulting in distortion of the data used in stock assessments (Ardill et al. 2011). Reports on bycatch has focused more on marine fisheries i.e. marine turtles (Aucoin & Leon 2007, Moore et al. 2010; Lucchetti et al. 2018) marine snakes (Fry et al 2001), and marine mammals (Read et al. 2006; Moore et al. 2010; Mackay & Knuckey 2013). Meanwhile, reports of bycatch in freshwater fisheries are very few (Larocque 2011). Freshwater turtle bycatch reports are also very few and so far only been reported in Canada (McCord & Philippen 1998; Larocque 2011; Nguyen et al. 2013). Bycatch occurs as a result of overlaps in the spatial distribution between target (fish) and non-target (turtle) species, putting these turtles at risk of being caught accidentally (Larocque 2011). This research reveals the existence of freshwater turtle bycatch in Indonesia, even in Asia for the first time.

The impact of the bycatch is the mortality in non-target species. The mortality of non-target species bycatch can cause demographic shifts that lead to population decline and community change (Midwood et al. 2015). Bycatch is even major problem affecting turtle populations worldwide (Moore et al 2010).

This study revealed that bycatch of the *C. amboinensis* affected almost all class age, except hathcling. The population of *C. amboinensis* in Rawa Aopa might be threatened if the fishing gear used by fishermen is not selective (i.e., having a small opening) and if fishermen killed the turtle when the turtle trapped in their fishing gear. Therefore, the attitude of the fishermen toward the conservation of the *C. amboinensis* is crusial in maintaining the age structure and population in general.

On a large scale, the decreased population of this species can disrupt the balance of the ecosystem. *Cuora amboinensis* plays a central role in the ecosystem's food chain either as a predator of various invertebrates or as distributor of plants' seed (Karraker et al. 2020). The significant ecological impact of the loss of this species includes changes in energy flow, nutrient cycling, and food web structure (Schoppe 2008).

Most of the fishermen in Rawa Aopa have indirectly protected the existence of *C. amboinensis*. The fishing gear used was quite selective and most fishermen released the bycatch of *C. amboinensis* alive. This attitude needs to be maintained to protect the population of this species in Rawa Aopa. Ashanika people in Central Peru can serve as a good example on the conservation of turtle by using their traditional knowledge (Ferronato & Cruzado 2013).

## CONCLUSION

This research showed that the bycatch of *C. amboinensis* in Rawa Aopa occurred at all age class for both sexes. As fishing has been one of the major economic activities for the local people, the balance between the utilization of natural resources (i.e., fishing) and conservation (i.e., maintaining *C. amboinensis*) need to be achieved and maintained. Positive attitude of the fishermen to release the turtles unharmed is definitely needed to maintain the population of the *C. amboinensis* in Rawa Aopa.

## AUTHORS CONTRIBUTION

H.N. collected and analysed the data and wrote the manuscript. M.D.K. designed the research and supervised all the process including translating report into English. A.M. supervised all the process and assisted in manuscript revision.

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## CONFLICT OF INTEREST

No conflict of interest regarding the research or the research funding.

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Research Article

# Analysis of Soil Bacterial Diversity from Tropical Rainforest and Oil Palm Plantation In Jambi, Indonesia by 16S rRNA-DGGE Profiles

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## ABSTRACT

Oil palm plantations are the most invasive land use changes in Southeast Asia. It must have affected unique natural biodiversity. This study aimed to investigate the diversity of soil bacteria based on 16S rRNA gene profiles from tropical forest and oil palm plantation in Jambi Province, Indonesia. Soil sample was taken from tropical forest and oil palm plantation from Jambi province, Indonesia. The forest site is in Bukit Duabelas National Park, and the nearby oil palm plantation is in Sarolangun District, Jambi Province, Indonesia. The diversity of bacterial communities from topsoil was studied using Denaturing gradient gel electrophoresis (DGGE) of 16S rRNA gene and common biodiversity indices. PCR amplification of 16S rRNA gene was successfully conducted primers-using 33F/ 518R primers. Phylogenetic approach was used for revealing the community shift of bacterial phyla and genera in both areas. Phylogenetic analysis showed there were 4 phyla of bacteria i.e., *Firmicutes*, *Alphaproteobacteria*, *Gammaproteobacteria*, and *Actinobacteria*, respectively. *Actinobacteria* was the most dominant group in both areas. The composition of soil bacterial community in the oil palm plantation, based on total number of bands 16S rRNA generated from DGGE was richer than that in the Bukit Dua Belas National Park. It was probably caused by plantation year circle more than 10 years and routine activities during the plantation management, such as applications of agricultural lime, herbicide and fertilizer.



## INTRODUCTION

Tropical forests are unique ecosystems with a diverse range of endemic flora, animals, and microbes. The most abundant and diverse category of microorganisms in soil are prokaryotes (bacteria). These microorganisms are important for the majority of nutrient conversions in soil and are major

biogeochemical cycle drivers. On the top of the soil, the bacteria are vital in decomposition of the organic matters in soils. Due to the interdependence of the vegetation with the soil substrate, which relies on the maintenance of adequate water, canopy cover, organic litter inputs, and land use change, lowland forests are highly sensitive to the impacts of land use change (transformation) to agro-forest industry (such as oil palm plantation) (Ko et al. 2005; Yule 2010).

Indonesian forest had been damaged 3.5 million hectares per year since 1999 until 2006, 2 million hectares per year between 2006-2010, and 300,000 hectares per year between 2010-2012 (Wijayanti et al. 2014). Timber extraction is a major factor in the transition from primer to secondary forest. Migrants transform a portion of the forest into a temporary cropland. In Jambi, such land has the potential to evolve into permanent tree-based agricultural systems (agro-forests) (Palm et al. 2005). There were 420 thousand ha of production forest in Jambi heavily damaged by the high rate of conversion to industrial timber plantation, palm oil plantations, coal mines and the opening of other minerals, such as iron and gold. The extensive damage approximately 30% of the approximately 1.4 million ha remaining forest areas. The loss of environmental services provided by trees is a non-linear trend in Jambi Province, Sumatra, Indonesia. The oil palm plantation industry might be threatened a gradual simplification of complex agro-ecosystems in agro-forests with rising profitability (Mudiyarso et al. 2002).

Land-use change has more severe effects on the soil microbial community structure than agro forest age, and a change in microbial biomass is not necessarily accompanied by a change in microbial community diversity and activity. Several factors may be involved. The soil microbial community may have been impacted by land-use conversion that involved significant soil disruption, such as cultivation, compaction, and fertilizer application. Plant species have a significant selection influence on microbial communities in soil, including bacterial diversity, according to several studies such as by Xue et al. (2008).

Bacterial diversity can be measured using two approaches, cultivable and non-cultivable. Only a small proportion of soil microbial diversity is cultivated, and the majority of soil microorganisms are unable to be cultured. Molecular biology and protein engineering technologies, such as metagenomic analysis, are being used to solve this problem (Glogauer et al. 2011). Metagenomic research of whole microbial communities inhabiting a certain niche is a culture-independent genomic analysis (Armougom & Raoult 2009). Recently, using denaturing gradient gelelectrophoresis (DGGE) of PCR amplified DNA fragments to examine the structural diversity of microbial communities has become a new strategy to approach the challenges of cloning and sequencing DNA fragments. DGGE was created for the purpose of detecting point mutations in medical research (Fischer & Lerman 1983), Muyzer et al. (1993) on the other hand, was the one who introduced it into the microbial ecology.

Denaturing gradient gel electrophoresis (DGGE) provides for the qualitative and semiquantitative profiling of microbial populations by determining the richness and evenness of dominant microbial species using 16S rRNA gene amplicons. The number of 16SrRNA gene sequence similarity groupings can be used to estimate diversity (*i.e.* the number of DNA bands on the DGGE gel and the intensity of DGGE bands). For the sake of simplicity, each band is considered to represent a species, which is a functional taxonomic unit. As a result, the current study used the DGGE rRNA 16S gene profile to analyze and compare the diversity of soil bacteria in tropical rainforests before and after conversion to oil palm plantations in Jambi Province.

## MATERIALS AND METHODS

### Soil Sampling and Laboratory Analysis

This study was carried out in the National Park of Bukit Dua Belas (NPBD) and the Humusindo Oil Palm Plantation in Jambi Province, Indonesia (Figure 1). Sampling sites of NPBD (latitude 01°56' 576" to 01-°56'502"S; longitude 102°34'879" to 102°34'836"E, altitude 87m to 116m asl) and oil palm plantation (latitude 01°56'491" to 01-°56'958"S; longitude 103°15'140" to 103°15'122"E, altitude 42m to 64m asl) were determined based on Purposive Random Sampling. The soil comprised relatively fertile, clay loam Acrisol soil in Bukit Duabelas and less fertile, loam Acrisol soil in Humusindo Oil Palm Plantation. Soil samples were collected from each two sampling sites in triplicate plots of 25 m². A total of 12 soil core samples were collected from each triplicate plots of the SW01 and SW02 (oil palm plantation aged 10-20 years) sites, TB03 and TB04 (tropical forest), and mixed to obtain a composite sample for each site. The soil samples were transported to the laboratory at room temperature and then stored at -20 °C until analysis. The

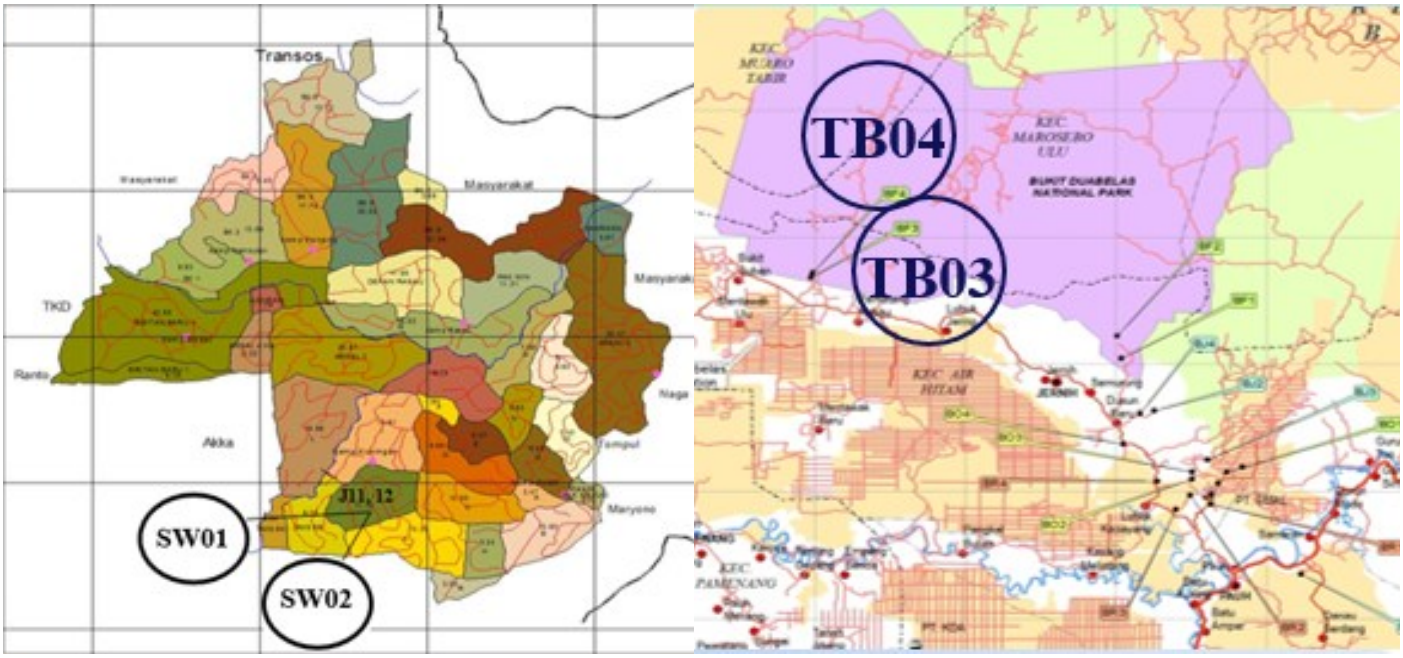


**Figure 1.** Map of Sampling location. Oil palm plantation of Humusindo (left) and National Park of Bukit Dua Belas (Right).

physico-chemical characteristics of soil were assessed using either field fresh soil or soil samples that had been air dried (Okalebo et al. 1993).

## DNA Extraction and Quantification from Soil Samples

Extraction of community soil DNA was conducted using the Fast DNA® SPIN Kit for Soil (BIO 101 according to manufacturer's instruction). Approx. 0.5 g of fresh soil (stored at -20°C) was taken in a 2 mL E-tube containing lysing mixture. 978 µL SPB (sodium phosphate buffer) and 122 µL MT buffer were added in the tube and homogenized at maximum speed for 1 min. The suspension was centrifuged for 1 min at 14000 x g and the supernatant was transferred into a clear 2 ml tube and 250µL PPS (protein precipitation solution) was added followed by mixing the tube for 2 min. Then the tube was centrifuged again for 5 min at 14000 x g and the supernatant was transferred into a 15 mL tube. 1 mL of binding matrix suspension was added and the tube was turned upside down for at least 5 times to allow binding of DNA to the matrix. About 500 µL of the supernatant at the surface layer of the tube was discarded and resuspended the remaining supernatant in the binding matrix. The slurry was transferred into a Spin Tube in two aliquots of 500 µL each and centrifuged to discard the waste liquids. Finally, the DNA in the Spin filter was washed with 500 µL of SEWS-M (salt/ethanol wash solution, DNase-free) at 14000 x g for 1 min and the filtrate was discarded. The Spin was removed from the tube and air dried for 5 min at room temperature. The Spin was replaced into a fresh catch tube and 75-100 µL of DNA eluting solution (DES, Dnase /TE Buffer) was added while gently stirring the filter membrane with the pipette tip. Then, the tube was centrifuged at 14000 x g for 1 min to elute the DNA into the catch tube. The DNA content of the extract was checked at 1% agarose gel and then quantified using Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, USA) (Laurent et al. 2001).

## PCR Amplification using 16S rRNA

T1 Thermocycler (BIOMETRA-Analytic Jena, Germany) was used to do PCR amplification of the 16S rRNA gene. P338F- GC (5' GCCCGC CGCGCGC GGCGGG CGG GGCGGGGGCACGGGGGGACTCC-TACGGGAGGCAGCAG-3') and P518R (5'-ATTA CCGCG GCTGCTGG -3') primers were used to amplify the 16S rRNA gene (Overeas et al. 1997) with PCR was set up for initial denaturation at 94°C for 5 minutes, then 35 cycles of 92°C denaturation for 30 seconds, 58°C annealing for 30 seconds, 72°C extension for 25 seconds, and 72°C extension for 3 minutes (Masrukhin et al. 2017). Electrophoresis in agarose gel (1% w/v) determined the PCR product, which was visualized in G:BOX gel documentation (Syngene, Frederick USA).

## DGGE analysis of 16S rRNA gene

DGGE was performed by using *D Code Universal Mutation Detection System*

(Bio-Rad, Hercules, CA, US). As much as 25 µL sample (20 µL DNA + 55 µL *loading dye*) was loaded into 8% (w/v) polyacrilamide gel with 30–70% gradient denaturant. For 100% denaturant was 7M urea and 40% formamide. The electrophoresis process was performed at 150 V, 60 ºC for 5.5 h in 1 × TAE buffer (Tris- acetate- EDTA). The gel was stained by 0.1% (w/v) Ethidium Bromide (EtBr) for 15 min. The gel image documentation was performed using G:BOX *Gel Documentation* (Syngene, Frederick, USA). CLIQS 1D software (Total Lab) was used to estimate the DNA band volume and total band in the gel using DGGE bands. On the basis of the CLIQS 1D analysis results, clustering analysis was performed. Each DGGE band on the gel was excised and stored in a microtube with 100 liters of nuclease-free water. Overnight, the microtubes were stored in the refrigerator. For the re-PCR process, two microliters of the excised bands were used as the template. The re-PCR conditions were identical to the previous PCR (Chen et al. 2011).

## Phylogenetic and Cluster Analysis

The PCR products were sequenced in a company laboratory providing sequencing services. For the assembling and trimming process, the sequences of the 16S rDNA gene were analyzed using ChromasPro (Technelysium, AU). BLASTN (Basic Local Alignment Sequence Tools for Nucleotide) was used to compare the sequences to the GeneBank database (blast.ncbi.nlm.nih.gov). The phylogenetic analysis was carried out using MEGA 6.0 software, and the phylogenetic tree was constructed using the neighbor-joining method according to the Bayesian information criterion (BIC) score. MEGA 6.0 software was used to perform a hierarchical cluster analysis based on UPGMA correlation (Tamura et al. 2013).

## Analysis of Shannon-Wienner Diversity Index (H') and Evenness Index (E')

Using the following equations, the Shannon-Wienner diversity index (H') and Pielou's equitability index (E') can be simply derived to represent hypothetical changes in the dominance among DGGE OTUs:

Bacterial diversity in the samples was estimated using Shannon-Wienner index of bacterial diversity (H'). The Shannon-Wienner diversity index was calculated as

$$H' = -\sum_{i=1}^{s} p_i Ln\, p_i$$

Based on Eichner's formula for relative band intensities (Eichner et al. 1999). Pi was defined as ni/N, where Ni is the area of a peak in intensity and N the sum of all peak areas in the lane profiles.

$$E_1 = \frac{H'}{H_{max}} = \frac{H'}{\ln(S)}$$

## RESULTS AND DISCUSSION

### Environmental Variables

Using the DGGE method to analyze the sequencing of the 16S rRNA gene, the composition of soil bacterial communities observed in two separate soil systems of tropical forest (TB) and oil palm plantations (SW) were tested and compared. The sequence of 16S rRNA gene was influenced by the chemical composition of two land types, such as total carbon (TC), C/N ratio, soil pH, total nitrogen (TN) and availability phosphate (AP) (Table 1). Organic matter content in the palm plantation tends to be lower, although not significant, compared to that in the Bukit Dua Belas National Park. This is most likely influenced by anthropogenic factors such as fertilizer use and land processing management.

Conversion of forestland into plantation can affect the soil chemical properties. This process changes the pH condition, carbon content, and modifies ratio of C/N and availability of phosphate in the soil. As a result of the conversion of forestland to plantation, one of the most important elements affecting the bacterial community is soil pH (Tripathi et al. 2012). Research conducted by Wan et al. (2014) showed that bacterial biomass increased with increasing pH and decreasing soil C/N ratio. pH is a major determinant of bacterial community diversity and composition, suggesting acidification has the potential to have a significant impact on bacterial-driven soil ecological processes (Wu et al. 2017). Soil pH in two oil palm plantation regions was higher than in NPBD, although the differences were not significant. This may be due to the soil acidic alleviation process during the land clearing for plantation. The tendency of ultisol forestland that is acidic, and poor of nutrients can be treated with ground limestone such as dolomite which contains Mg and Ca which are materials for soil acidic alleviation and have good effect toward soil properties such as increasing soil nutrients N and P, reducing the levels of Al and Mn, increasing soil pH and reducing the concentration of phytotoxins in highly acidic soil (Cristancho et al. 2014). Ca concentrations exhibited significant effects on active Rainforest Conversion Affects Active Bacteria communities and showed a positive correlation with increasing land use intensity from rainforest to oil palm plantation. This is most likely connected to liming practices and therefore to fertilizer application to counteract soil acidification (Tripathi et al. 2012). Previously observed changes of soil parameters after rainforest transformation to oil palm and rubber plantations indicated that the availability of N and other nutrients rely on continuous fertilization and liming (Allen et al. 2015). Thus, it is likely that the observed active bacterial community structure is highly dependent on ongoing treatment such as fertilizer application and liming.

According to Allen et al. (2015) that reduced carbon-to-nitrogen (C/N) ratio, which is known to be a significant additional driver of active bacterial communities, usually indicates a shift towards a more bacterial-dominated system. Ratio of C/N between oil palm plantation and tropical forests are not significantly different. Ratio of C/N ratio is an indicator that shows pro-

**Table 1**. Soil Variable Analysis.

| Sample | pH ($H_2O$) | pH (KCl) | Total Carbon (%) | Total Nitrogen (%) | Phosphate Availability (mg/100mg) | Rasio of C/N |
|---|---|---|---|---|---|---|
| NPBD (TB01, TB02) | 4.0± 0.70$^{b}$ | 3.7±1.20$^{b}$ | 1.33±0.23$^{a}$ | 0.11±0.48$^{a}$ | 18±0.70$^{e}$ | 12.1±0.77$^{c}$ |
| Oil palm plantation (SW01, SW02) | 4.6±0.42$^{b}$ | 4.0±0.70$^{b}$ | 0.98±0.33$^{a}$ | 0.09±0.01$^{a}$ | 15±0.70$^{d}$ | 10. ±0.98$^{c}$ |

Note: different letter notations indicate that the treatment given is significantly different, while the same letter notation indicates that the results obtained are not significantly different.

cess of mineralization and immobilization of N from organic material by microbial decomposers. The low C/N ratio indicates the soil containing more nitrogen, while the high C/N ratio indicates the low nitrogen content. It will make the competition between soil microbes and plants to obtain available nitrogen. The soil of palm plantations contains lower contents of nitrogen and carbon than that of NPBD, so its C/N ratio is also low. The soil with high C/N ratio needs the addition of nutrients to prevent it from nutrient deficiency, especially for nitrogen.

Generally, the change of forest land-use into plantations leads to decreasing level of organic C (Table 1). According to Allen et al. (2015) that tropical forest conversion causes biodiversity loss and promotes climate change, while also affecting the short- and long-term nutritional status of the converted land use systems. This condition was caused by some occurs much of the carbon been swept by erosion, runoff, lost in the form of gas, and also because of the absence of organic fertilization to supply of carbon in the soil. Organic carbon levels in soil cultivated with plantation crops range from low to moderate. Forestland has a higher carbon stock than plantation land, either in the body of vegetation, in the soil, or in the form of litter. It means that forestland is much more effective in absorbing and storing carbon than plantation (Monde 2009). The assessment criteria of physical and chemical properties of soil is based on Tropical Soil Quality Index (TSQI) (Arifin et al. 2012). Total carbon (0.98%) and total nitrogen (0.09 %) in oil palm plantation were classified as lowlevel category, and available phosphate (15 mg/100g) was classified as moderate level category, while total carbon (1.33%), nitrogen content (0.11%), were classified as moderate level category, and available phosphate (18 mg/100g) in forest land National Park of Bukit Dua Belas (NPBD) were classified as moderate level category. Phosphorus (P) is an essential element for all living organisms that makes up cell membranes, nucleic acids, proteins, and as the energy source such as GTP, ATP, and NADPH. This compound is also biological and environmental limiting factor and limiting nutrient for plants (Azziz et al. 2012). After nitrogen, phosphorus is the second most important critical element required by living organisms. Despite their abundance in nature, its availability is limited in the form insoluble phosphate. In nature, some bacteria species have the ability to dissolve phosphate. The abundance of phosphate in oil palm plantations decreases the amount of nutrients available to soil bacteria. The diversity and abundance of soil bacteria in oil palm plantations differs from that in the for-

est NPBD because of the availability of various nutrients. The low microbial biomass in oil palm plantations suggests that soil fertility and N availability may only temporarily decrease with the fertilization process and may not be as sustainable as in the original reference land use (Allen et al. 2015).

## Soil DNA extraction and amplification of 4 Locations

The purity ratio of DNA obtained from four locations is low, around 1.35 to 1.50 (Table 2). The A260/280 ratio of good-purity DNA should be between 1.8 and 2.0, and the A260/230 ratio should be above 2.0. Total DNA with low purity, pure DNA and RNA with an A260/280 ratio of 1.8-2.0 and an A260/230 ratio higher than 2.0 were obtained using the FastDNA SPIN KIT for soil (MP Biomedicals, USA) (Yeates et al. 1998; Sambrook & Russell 2001). A280 is the wavelength for protein, A260 is for DNA and A230 is for phenol, and also humic acid. Humic acid contamination in the soil can interfere in the DNA quantification process because humic acid absorbed well A230 and A260 nm (Yeates et al. 1998). In addition the A260/A30 ratio is very low at 0.07- 0.28, this value indicates the presence of humic acid or humic acid-like molecules detected in the A230 wavelength absorption. Using nested PCR and primers p338F and p518R, the amplification of the 16S rRNA gene of bacteria in soil from tropical forests and oil palm plantations was analyzed. This technique used two-time PCR, the first and second phase PCR produced 187 bp products.

**Table 2.** Quantity and Quality of DNA Extraction from 4 Soil Samples.

| No. | Sample | Nucleid acid Concentration (ng/µl) | 260/280 | 260/230 |
|---|---|---|---|---|
| 1. | SW01 | 85.0 | 1.37 | 0.14 |
| 2. | SW02 | 38.0 | 1.50 | 0.07 |
| 3. | TB03 | 236.3 | 1.35 | 0.28 |
| 4. | TB04 | 144.8 | 1.40 | 0.20 |

## The diversity of bacterial 16S rRNA Based on Method Non-Culture DGGE

This study used a non-culture approach, which not only can obtain bacteria that has been known for dominant and easily cultured, but also uncultured bacteria by genes of 16S rRNA obtained directly from environmental samples. The uncultured bacteria can be used to determine the breadth of bacterial distribution in an environment, as well as providing metabolic and biochemical information that has not before been discovered (Harris et al. 2004).

Based on the results of the DGGE, namely DNA band profile, diversity index of quantification of the BNA band intensity, dendogram analysis, OTU identity of DNA, and phylogenetic relationships among OTU(s). The results of 16S rRNA gene PCR product separation showed that the pattern of the bacterial communities varied in each sample and result of DGGE interpretation (Figure 2A). Band distribution pattern on polyacrylamide gel showed that the bacterial community in the soil samples of oil palm planta-

tion (16-24 bands) is more diverse than soil samples of NPBD (6-8 bands). Based on the results obtained, each band represents 1 species of bacteria which means that the diversity of bacteria in the oil palm plantation soil sample is more diverse than in the NPBD.

The results of cluster analysis, which compare similarities in all samples based on DGGE band location (Figure 2B), divided the samples into two groups. The soil bacterial community in the soil samples of palm plantations SW01 and SW02 have ± 99% similarity, according to an analysis of similarity patterns with binary data. Meanwhile, a community sample of soil patterns in tropical forests TB03 and TB04 also have similarities of ± 99% but it has its clusters differ with soil samples in oil palm plantations. This indicated the presence of different patterns of community with soil samples in oil palm plantations.

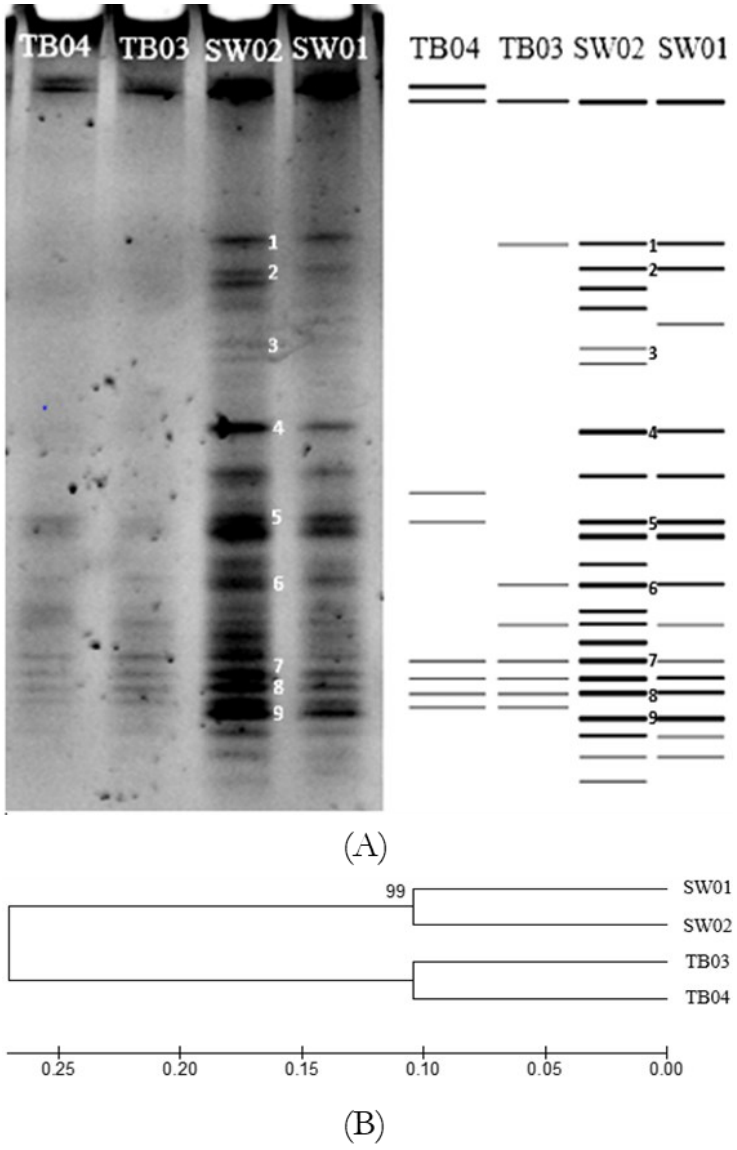


**Figure 2.** (A) DGGE profile of 16S rRNA from soil samples (left). DGGE illustration by CLIQS 1D software (right). Nine bands were excised for further analysis. (B) Clustering analysis of soil bacterial diversity, based on 16S rRNA gene showed different cluster between oil palm plantation of Humusindo (SW) and National Park of Bukit Dua Belas (TB) area.

## Phylogenetic Analysis and BLAST

From four soil samples, DGGE analysis of the whole bacterial community identified 9 different bands. Purified DNA of DGGE bands were amplified with the same primer without GC clamps with a size of ± 180 bp product (Figure 3). Sequence alignment were conducted using Mega 6 software. The alligned sequence is a 16S rRNA sequence with a base length of about 173bp using 338f and 518r primers which amplified the third region of 16S rRNA genes (Overeas et al. 1997). The nine bands that have been successfully sequenced are then compared among the OTU sequences to see the same sequence sequence and the different base pairs sequences of the nine DGGE OTU sequences (Figure 4). The results obtained are that the nine OTU sequences have sustainable areas in some sequences marked with black areas. The gray area shows sequences between OTUs that have the same region (semi conserved region) whereas the white area is a variable region showing the difference of the basic sequences of each OTU from DGGE.

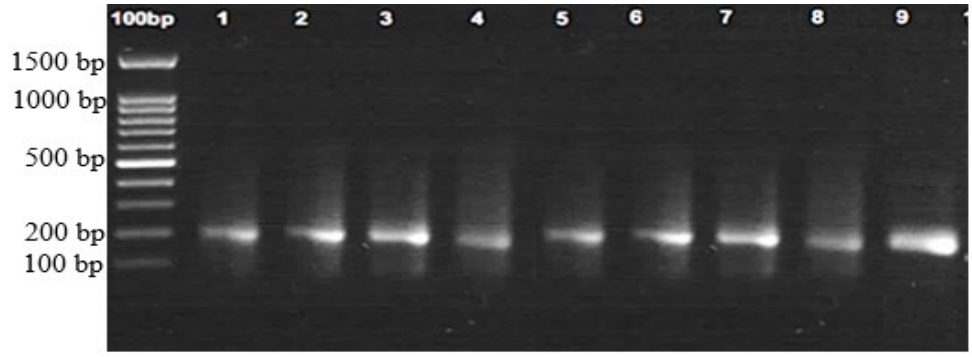


**Figure 3.** Amplification of 16S rRNA gene soil bacteria from four sample locations with product size ±180 bp using primer p338F (Non GC Clamps) and p518R. Lane 100 bp as a marker, lane 1 to line 9 : clone band of DGGE.

Based on sequence analysis and database in Genebank, the obtained results showed 6 bands of DGGE were identified as uncultured bacterium. However, there were 3 DGGE band identified as *Enterococcus canis* (band 1) with the similarity percentage of 94%, *Pseudomonas aeruginosa* (band 2) by 100% and the percentage of similarity *Weisselia confusa* (band 3) was about 93% (Table 3). Generally, the percentage of sequence similarity of DGGE results with Genebank database were about 89-100%.

Neighbor joining tree with bootstrap 1000x was used to construct the phylogenetic tree. The sequence analysis of 16S rDNA DGGE bands showed 3 different phylla of bacteria which were found in both of location i.e Actinobacteria, Proteobacteria (Bethaproteobacteria, and Gammaproteobacteria), Firmicutes (Figure 5). Betaproteobacteria**,** Gammaproteobacteria, and Firmicutes are 3 subphylum appearing on DGGE results from soil samples of oil palm plantations. While Actinobacteria are a group that always appear in both samples and the most dominating in both soil samples both TNBD forest and oil palm plantations.

DGGE results showed that group of species that have been identified

**Table 3.** Blast N result of DGGE bands base on 16S rRNA gene.

| No. | Closely relative description | Quary Cover | E-Value | Identity | Accesion number | Family |
|---|---|---|---|---|---|---|
| Clone band 1 | *Enterococcus canis* strain NBRC 100695 | 99% | 3e-62 | 94% | NR_113931.1 | Bacili/Firmicutes |
| Clone band 2 | *Pseudomonas aeruginosa* P-hrb07 | 99% | 2e-98 | 100% | GU214819.1 | Gamma-Proteobacteria |
| Clone band 3 | *Weisselia confusa* | 99% | 4e-76 | 93% | AM117158.1 | Bacili/Firmicutes |
| Clone band 4 | Uncultured Bacteria QM-73-158 | 92% | 6e-84 | 100% | Ku928795.1 | Beta-proteobacteria |
| Clone band 5 | Uncultured bacterium clone A15C08 | 95% | 6e-38 | 91% | JQ379115.2 | Actinobacteria |
| Clone band 6 | Uncultured bacterium clone VHA39_ | 99% | 2e-69 | 95% | KX753094.1 | Actinobateria |
| Clone band 7 | Uncultured bacteria clone BButL 8P5CO2 | 98% | 2e-83 | 99% | LK024724.2 | Actinobacteria |
| Clone band 8 | Uncultured bacteria clone MA-537 | 99% | 2e-83 | 99% | KM 207493.1 | Actinobacteria |
| Clone band 9 | Uncultured bacteria clone Out 26067 | 54% | 3e-16 | 89% | KX878538.1 | Actinobacteria |

were almost belong to the uncultured species in abundance. In oil palm plantations, the Actinobacteria group was the most dominant, with a higher relative abundance than forest soils. According to Lee-Cruz et al. (2013), concluded the group Actinobacteria was more abundant in palm oil than on forest land. Actinobacteria Anthropogenic activities have impacted the existence of oil palm plantations through the fertilization process. Actinobacterial shift communities on oil palm plantations, as well as ectomycorrhizal fungal communities, have been characterized as a result of anthropogenic intervention and forest conversion (Kerfahi et al. 2014). The increasing the number of Actinobateria especially actinomycetales group on deforested land explain why the increased availability of nutrients in supporting the growth of the Actinobacteria as copiotropic bacteria (Wagner & Horn 2006).

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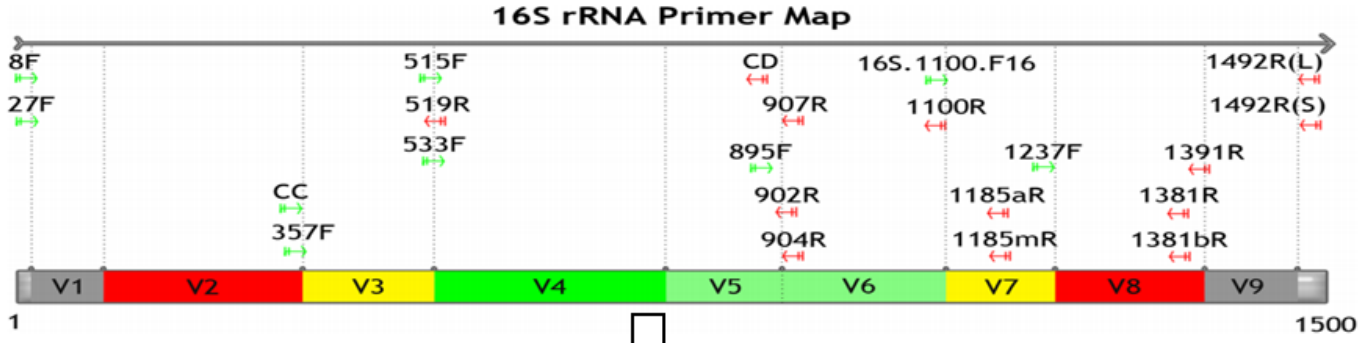
Species were detected in DGGE results that belong to a group Firmicutes / bacilli were *Enterococcus canis* and *Weisselia confuse*. Most of the species that belong to Firmicutes phylum have a high and good resistance varied to dryness and extreme environmental factors. Because these microorganisms

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Band_1  CAATGG-ACG AAAGCCTGAT GGAGCACCGC CGCGTGAGTG ATGAAGGTTT [ 50]
Band_2  CAATGG-GCG AAAGCCTGAT CCAGCCATGC CGCGTGTGTG AAGAAGGTCT [ 50]
Band_3  TTCTGGTAAG ATACCGTCAA ACAGTCAACA GTTACTCTCA CTGTCGTTCT [ 50]
Band_4  CAATGG-GGG AAACCCTGAC GCAGCAACGC CGCGTGGAGG ATGAAGTCCC [ 50]
Band_5  CAATGG-GCG AAAGCCTGAC GCAGCGACGC CGCGTGGGGG ATGAAGGCCT [ 50]
Band_6  ---------- TGAGAATGAC GCAGCTACGC TGCGTGG-TA ATGAAGGCCT [ 50]
Band_7  CAATGG-GGG AAACCCTGAC GCAGCAACGC CGCGTGGAGG ATGAAGTCCC [ 50]
Band_8  ---------- --AGCCTGAC GCAGCGACGC CGCGTGG-GG ATGAAGGCCT [ 50]
Band_9  CAATGG-GCG AAAGCCTGAC GCAGCGACGC CGCGTGGGGG ATGAAGGCCT [ 50]

Band_1  TC--GGATCG TAAAACTCTT GTTGTTAGAGA -AGAACGACA GTGAGAGTAA [100]
Band_2  TC--GGATTG TAAAGCACTT TTAAGTTGGGA -GGAAGGGCA GTAAGTTAAT [100]
Band_3  TCTCTAACAA CAGAGTTTTT ACGAGCCG--- -AAGACCTTC ATCACTCACG [100]
Band_4  TT--GGGATG TAAACTCCTT TTCGATC---- -GGGACGATA ATGAC----- [100]
Band_5  TC--GGGTTG TAAACCCCTT TTCAGCAGGGA CGAAGCGAAA GTGAC----- [100]
Band_6  TC--GGATTG TAAACCTCTT TTCGGCA---- -GGGACGAAG ATGAG----- [100]
Band_7  CTTAGGGGTG TAAACTCCTT TTCGATCC--- -GGGACGATT ATGACC---- [100]
Band_8  TC--GGGTTG TAAACCCCTT TTCGGCA---- -GGGACGAAG CTGAA----- [100]
Band_9  TC--GGGTTG TAAACCCCTT TTCAGCAGGGA CGAAGCGAAA GTGAC----- [100]

Band_1  CTGTTCACTG TTTGACGGT ATCTAACCAGA AAGCCACGGC -TAA---ATA [150]
Band_2  ACCTTGCTGT TTTGACGTT ACCAACAGAAT AAGCACCGGC -TAA---CTT [150]
Band_3  CGGCGTTGCT CCATCAGGC TTTCGCCCATT GGATGGAAGA TTCC---CTA [150]
Band_4  ---------- ------GGT ACCGGAAGAAG AAGCACCGGC -TAA---CTC [150]
Band_5  ---------- ------GGT ACCTGCAGAAG AAGCGCCGGC -TAA---CTA [150]
Band_6  ---------- ------GTG ACCGTTCCTGG AAACGAAGGA -TAA---CTT [150]
Band_7  ---------- ------GGT ACCGGATGAAG AAGCACCGGC GTAAACCCCC [150]
Band_8  ---------- ------GGG ACCGTACCTGC AAACGACGGG -CCA---GTT [150]
Band_9  ---------- ------GGT ACCTGCAGAAG AAGCGCCGGC -TAA---CTA [150]

Band_1  CGTGCCAGCA GCCGCGGTAATAA [173]
Band_2  CGTGCCAGCA GCCGCGGTAATAA [173]
Band_3  C-TGCTGCCT CCCGTAGGAGTAG [173]
Band_4  TGTGCCAGCA GCCGCGGTAATAT [173]
Band_5  CGTGCCAGCA GCCGCGATAATAA [173]
Band_6  CACTACAACC CACGCGCCAATAC [173]
Band_7  TGTGCCAGCA GCCGCGGTA---- [173]
Band_8  AACTACGTGC CCCCAGCCGCGAA [173]
Band_9  CGTGCCAGCA GCCGCGATAATA- [173]
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**Figure 4.** Sequence Alignment of 9 16S rRNA gene sequences in the third region (V3) of soil bacteria obtained from DGGE analysis.

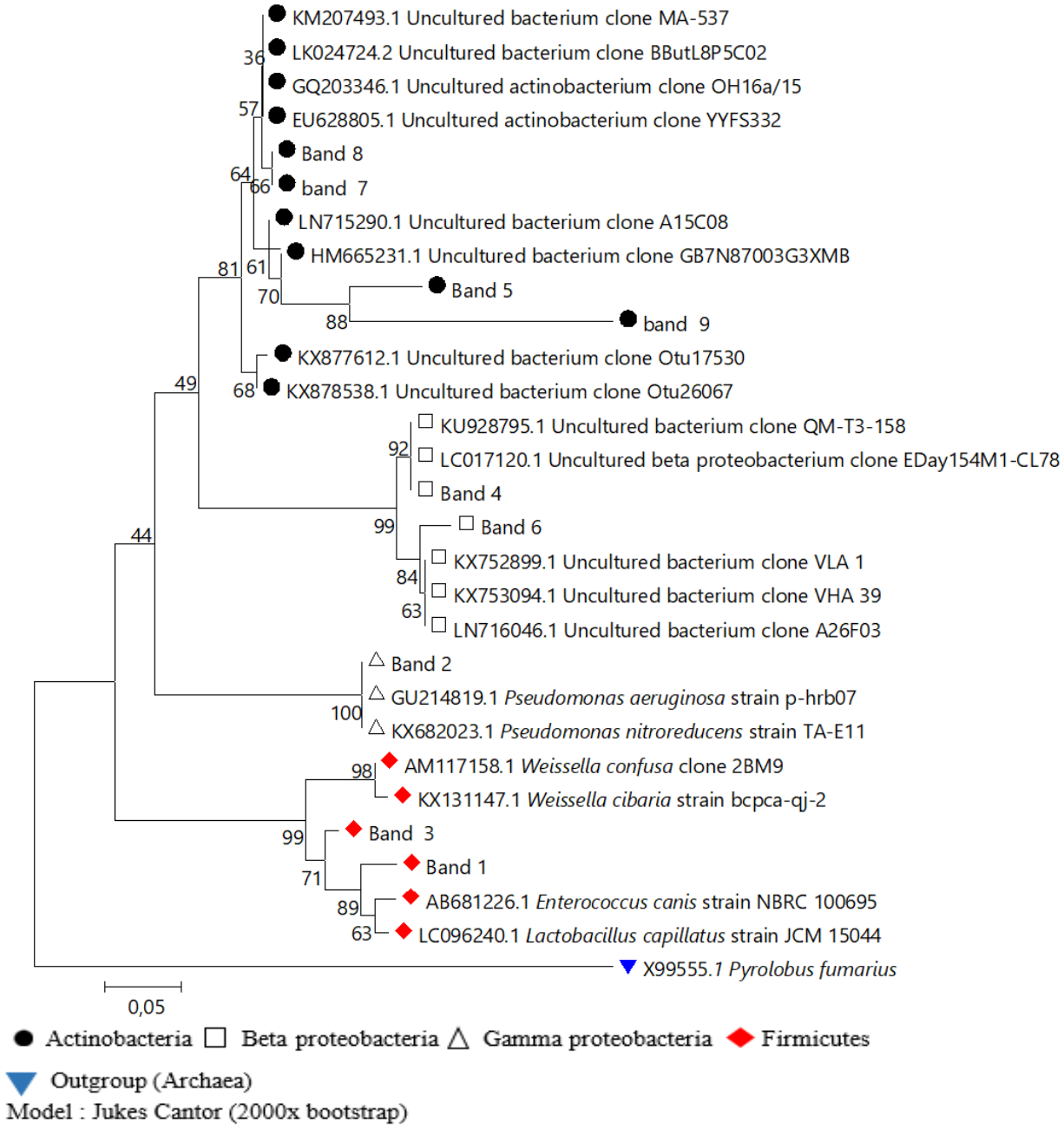


**Figure 5.** Phylogenetic tree of 9 DGGE bands of 16S rRNA. The tree was constructed using neighbour joining method through 2000x bootstrap.

are known to thrive in carbon-rich environments and land surface temperatures vary throughout the day, a significant rise in the relative abundance of Firmicutes sequences observed following land conversion is detected.

Species detected in DGGE results were classified in Gammaproteobacteria ie *Pseudomonas aeruginosa*, from the group Betaproteobacteria included into uncultured bacteria. This study found that there is no species belong to the group Alfaproteobacteria, Deltaproteobacteria and Epsilonproteobacteria. These results are consistent with the study by Janssen et al. (2002) which stated that the bacterial phylum Proteobacteria was the highest abundance. Alphaproteobacteria was the most abundant class followed by Gammaproteobacteria, Betaproteobacteria, Deltaproteobacteria, and Epsilonproteobabteria. The higher comparison of proteobateria in oil palm plantations compared to primary forest land because of the large influx of fertilization and

organic matter which showed the soil fertility and could decrease of competition of the microbs. The increase of bioavailable nitrogen through fertilization in intensively managed soils provides other bacterial taxa with improved growth conditions. This observation is confirmed by studies on N availability in the investigated land use systems, which decreased along the described land use gradient (Allen et al. 2015). The C to N ratio, a predictor for nitrogen availability, was lower in managed soils due to fertilizer usage (Schneider et al. 2015). Betaproteobacteria were copiotrophic, and their relative abundances were highest in soils with high C availability, either as a nutrient or as an essential ingredient of the soil. The relative abundances of Betaproteobacteria and Bacteroidetes increased while the relative abundances of Acidobacteria decreased (Fierer et al. 2007). Total carbon in oil SW sites was found to be lower than in TB sites in this study.

## Shannon-Wienner (H') Diversity and Equitability (e') Index

Shannon-Wienner (H') diversity index was used to estimate the diversity of microbes in each sampling site. The results showed that the bacterial soil community diversity index (H') based on 16S rRNA gene at four locations ranged at 1.90 – 2.93. Meanwhile the equitability index (E') at four locations ranged at 0.914 – 0.939 (Figure 6).


**Figure 6.** Shannon-Wienner Diversity Index and Equitability Index of Bacterial community based on 16S rRNA gene.

The composition of soil bacterial community revealed in the DGGE profile showed SW01 and SW02 have relatively high abundance of bacteria compared with TB03 and TB04. This result was also confirmed by the diversity index of Shannon-Wienner. The differences of the bacterial diversity among the samples can be influenced by some factors both abiotic and biotic (Hallman et al. 1997). The soil results of cultivation produce a diversity of microbial higher than the forest soil, this can be happen due to the activity of cultivation that do like tilling the soil and run off from fertilizers produced will decrease competition nutrients that will produce more diverse of microbes (Lee-Cruz et al. 2013).

## CONCLUSION

Conversion of forestland into oil palm plantation increased constantly over the last decades, which led to massive deforestation, especially on Jambi Province. It also can affect the chemical properties of the soil so it changes pH conditions may affect the soil bacterial diversity in two locations of oil palm plantations and tropical forests. Based on the number of bands 16S rRNA obtained through DGGE, the soil bacterial population in oil palm plantation seems to be more diverse than that in Bukit Dua Belas National Park. The identification of 16S rRNA and phylogenetic analysis of 16S rRNA found out four different phyla in the oil palm plantation, i.e. *Proteobacteria* (*Betaproteobacteria* and *Gammaproteobacteria*), *Firmicutes*, and *Actinobacteria*. Diversity analysis showed that Shannon-Wienner diversity index (H') of soil bacterial community in four locations ranged between 1.90 and 2.93, while the equitability index (E') ranged between 0.914 and 0.934. We stated there is an increase of soil bacteria diversity from rainforest to oil palm plantations. Some specific bacterial and archaeal taxa are present in rainforest soils and absent in other land use systems. Thus, although there is an increase in diversity in managed systems, but the conversion of rainforests to managed systems leads to a loss of bacterial biodiversity, which is coupled with a loss of traits. The long-term effect of this condition is not known and has to be determined in long-term studies, e.g., analysis of the recovery potential of soil bacterial communities after reforestation or in the absence of management treatments like fertilization.

## AUTHORS CONTRIBUTION

All authors have reviewed the final version of the manuscript and approved it for publication. R.H.W., I.R. and N.R.M. designed the research, N.R.M., I.R. and M.T.S. supervised all the process, S. and M. collected and analyzed the molecular data and R.H.W. wrote the manuscript.

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## CONFLICT OF INTEREST

The authors state no conflict of interest from this manuscript.

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Research Article

# The Effect of Liquid Organic Fertilizer "Bio Ferti" Application on the Growth Rate of *Spirulina platensis* by Using Haldane Model

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## ABSTRACT

This experimental research was performed to observe the influence of an agricultural liquid organic fertilizer called Bio Ferti on the growth and biomass of *Spirulina platensis*, aiming at replacing inorganic fertilizer with the liquid organic one. The cultivation of the microalgae was conducted over seven days at Nogotirto Algae Park. The liquid organic fertilizer, namely Bio Ferti, was obtained from the Faculty of Biology, Universitas Gadjah Mada, and prepared to have doses of 2, 4, 6, 8, and 10 mL. For comparison, an inorganic fertilizer with the same doses was also prepared. The variables to be observed were cell density, dry cell weight, and growth kinetics. The culture medium conditions observed were temperature, pH, and salinity (the optimum salinity was 20 ppt). The growth kinetic analysis was performed mathematically using numerical simulations using the Contois and the Haldane models. This research's results showed that Bio Ferti affected the growth rate of *Spirulina platensis*. With a dose of 2 mL, it became the optimum medium which produced the highest density and dry weight of $1.78 \times 10^6$ cells/mL and 160 mg/mL, respectively. Meanwhile, the inorganic fertilizer with a dose of 10 mL produced the highest density and dry weight of $2{,}13 \times 10^5$ and 80 mg/mL, respectively. The temperature ranged from 28 to 31°C, while the pH ranged from 8.01 to 9.02 for each medium. The suitable model to describe the growth kinetics of *Spirulina platensis* was the Haldane model.



## NOMENCLATURE

| | | |
|---|---|---|
| $\mu_m$ | [day$^{-1}$] | maximum specific growth rate |
| $K_s$ | [mg/L] | half saturation constant |
| $Y_{x/s}$ | [mg cell] | yield of cell formation |
| $k_d$ | [day$^{-1}$] | death rate constant |
| $K_I$ | [mg/L] | inhibition constant |

## INTRODUCTION

Microalgae are biomass potential to develop. As fast-growing unicellular or simple multicellular microorganisms, they provide several advantages, including higher photosynthetic efficiency, higher growth rates, and higher biomass production, compared to other energy crops (Li et al. 2008; Gouveia et al. 2009). *Spirulina platensis,* known as the Blue-Green alga, is multicellular, filamentous, and unbranched blue-green microalgae, which is naturally grown in alkaline waters in warm regions (Bandara & Arunakumara 2020). It can colonize in a wide range of environments that are not suitable for many other living organisms (Madkour et al. 2012). Although possessing a simple prokaryotic cell structure, it has no plant cell wall but the photosynthetic ability and glycogen-containing cellular membrane, making it like the bacterial, plant, and animal kingdoms, respectively (Usharani et al. 2012).

*Spirulina platensis* can produce valuable metabolites, such as proteins, fatty acids, and pigments for feed additives and nutritional purposes (Guerin et al. 2003; Hu. 2004). Among filamentous Cyanobacteria, *S. platensis* is preferred for biomass production mainly due to its relatively high cell growth rate and easy biomass recovery (Pioreck et al. 1984). In addition, its ability to grow in highly alkaline media reduces the risk of being contaminated by other microorganisms (Walach et al. 1987).

*S. platensis* has high adaptability, which makes it easier to be cultivated in various nutrient conditions. Generally, Zarrouk and Walne media are used to cultivate it on a large scale. However, both media are relatively complex, expensive, and limited, bringing the production cost to rise. For this reason, some researchers have begun to search for other media to minimize the production cost and increase revenue.

There are very few studies on the use of agricultural fertilizers for cultivating macro and microalgae (Ilknur 2011). In recent years, consumers preferred to use organic media such as Zarrouk, Walne, and many more, as generally preferred by *S. platensis* cultivators. Some investigators reported the replacement of several elements contained in the Walne and Zarrouk media like organic matters (Andrade &Costa 2009) and wastewater (Mezzomo et al. 2010) to be used as sources of nutrients for *S. platensis*. In this research, the liquid organic fertilizer used is Bio Ferti (Figure 1). Bio Ferti is a liquid organic fertilizer made from fermented vegetable and fruit waste with help of larva HI (*Hermetia illucens)*. It contains some minerals functioning as enzymes such as phosphates, esterase (C4), esterase lipase, lipase, leucine arylamidase, valine arylamidase, and cysteine arylamidase (Kim et al. 2011). Bio Ferti also contains macronutrients such as N, P. K, Ca, Mg, and S around 103.771 mg/L (all value) and micronutrients such as Fe, Mn, Zn, and Cu around 0.2-0.62 mg/L (BPTP2007).

This study aimed to replace inorganic fertilizer with liquid organic fertilizer and to determine the influence of fertilizer on the growth and dry weight (biomass) of *S. platensis.*


**Figure 1.** Liquid organic fertilizer Bio Ferti (source: Faculty of Biology UGM).

## MATERIALS AND METHODS

### Microorganism and Media

The material needed in this research was the microalga *Arthrospira ('Spirulina') platensis*. The culture of *Spirulina platensis* was taken from Nogotirto Algae Park UGM which had previously been cultivated as a research culture stock. The liquid organic fertilizer used was Bio Ferti to be compared with the inorganic fertilizer consisting of urea, NPK, $Na_2SO_4$, and soda ash. For urea was taken as much as 0.05 g, NPK 0.03 g, 0.015 g, and soda ash 0.075 g (Source: Nogotirto Algae Park). The inorganic fertilizer needed was then weighed and dissolved in 1 L of water. The doses of each Bio Ferti and the inorganic fertilizer were 2, 4, 6, 8, and 10 mL or in percent volume/volume was 0.4, 0.8, 1.2, 1.6 and 2 % v/v. The other materials needed were bottles, lamps, pumps, straw, and cotton.

### Cultivation

The cultures were grown in 500 mL bottle volume. For the work volume, 350 mL of water and 100 mL cells of *Spirulina platensis* were inoculated with three replicates. The bottles were equipped with supporting tools such as straw and cotton. The bottles were continuously aerated using pumps and illuminated by lamps. The cultivation was applied for seven days with 24 hours of light.

### Water Quality Parameters

The parameters observed in the culture media were temperature, pH, and salinity (the optimum salinity was 20 ppt). Measurement of temperature and pH were carried out every day to determine the quality of *Spirulina platensis* cultivation media.

### Analytical and Statistical Procedures

Cell density measurement was done by sampling 0.1 mL of *Spirulina platensis* using a dropping pipette and dropping it on a hemocytometer put under a light microscope. The number of cells located on the four corners and at the

center was recorded and then the total number of the cells was calculated using Eq.1 (Kawaroe et al. 2015). The dry weight of cells was measured by taking 10 mL of each of the samples every day during cultivation to be filtered using filter papers. Afterward, the filter paper is heated in the oven to evaporate the water at temperature of 60 °C for approximately 1 hour and then weighed to find out the sample weight. The dry cell weight means the difference between the weight of microalga-containing filter paper and that of the empty one.

$$A = Nx\frac{25}{5}x\ 10 \quad (1)$$

Where:
A: Cell density (cell/mL)
N: Observed number of cell (cell)

The kinetic study was done by synthesizing the differential equations for batch conditions. Two models, namely the Contois (Eq. 2) and the Haldane (Eq. 3) models were proposed to study the growth kinetics. The differential equations were solved numerically in pairs, and the corresponding kinetic constants were determined by minimizing the sum of squares of errors (SSE) between the calculated and experimental data of the organic matter concentrations as dry weight of cell (X) and substrate (s). The differential equation pairs would be each of Eq. 2 and Eq. 3 paired with Eq. 4.

$$\mu = \mu_{max}\frac{s}{KsX+s} \quad (2)$$

$$\mu = \mu_{max}\frac{s}{s+K_s+\frac{s^2}{K_1}} \quad (3)$$

$$\frac{ds}{dt} = -\frac{1}{Y_{x/s}}\frac{dX}{dt} \quad (4)$$

Where:

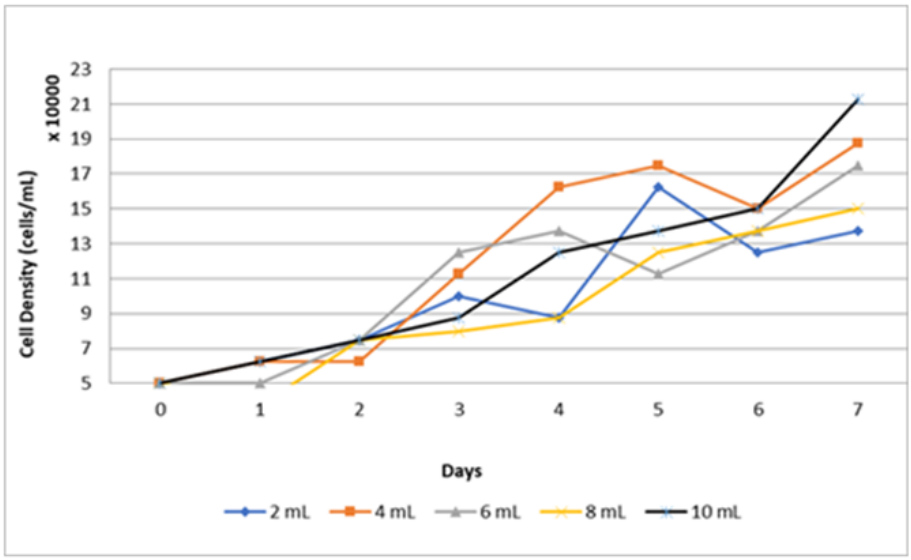
| | |
|---|---|
| $\mu$ | : specific growth rate of microalgae (hours$^{-1}$ or days$^{-1}$) |
| $\mu_{max}$ | : maximum growth rate of microalgae (hours$^{-1}$ or days$^{-1}$) |
| Ks | : half saturation coefficient (mg/L) |
| s | : concentration of nutrients in the medium at time (mg/L) |
| ds/dt | : substrate degradation rate with time |
| $Y_{x/s}$ | : yield of dry cell formation to substrate decrease |
| dX/dt | : dry cell formation rate with time. |

## RESULTS AND DISCUSSION

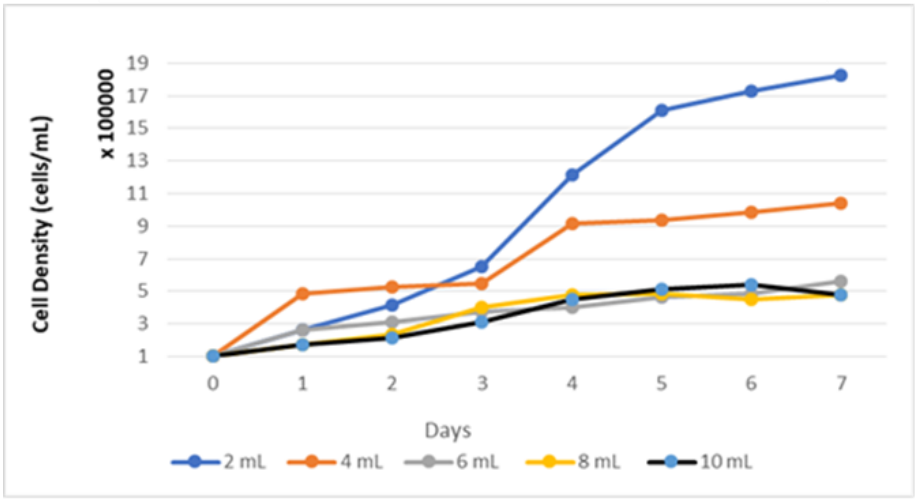
### *Spirulina platensis* Density

Microalgae growth is influenced by a variety of culture parameters, such as light intensity, pH, salinity, nutrients availability, temperature, CO2, and dissolved oxygen concentration (Chowdury et al. 2020). Microalgae growth consists of four phases, and those are the lag phase, exponential phase, stationary phase, and death phase. Nutrients are also needed by phytoplankton for growth. The density calculation of the *Spirulina platensis* was conducted to de-

termine the growth of *S. platensis*. This density calculation was conducted using a microscope and a hemocytometer as a tool to calculate the total density of *Spirulina platensis* cells. Observation was done for seven days for each concentration of fertilizer. From the daily results of the observed cell abundance, the peak time of *S. platensis* density can be discovered. Based on observation, due to differences in concentration in the used medium, the density of *S. platensis* cultured in each used medium was different. The results of the *S. platensis* cell density after using Bio Ferti liquid organic fertilizer and inorganic fertilizers are presented in Figures 2 (a and b).




a




b

**Figure 2**. Differences in cell density growth using (a) inorganic fertilizers and (b) Bio Ferti. The value is obtained by calculating using the cell density formula. This research was conducted using different nutrient concentrations, with qualitatively different results in each concentration.

The differences in the *S. platensis* density between using Bio Ferti and the inorganic fertilizer in this study are presented in Figures 2 (a and b). On the day after the doses were given, the density increased in the doses of 2, 4, and 6 mL. In Bio Ferti with the doses of 8 and 10 mL, it was stable or in oth-

er words it hasn't grown yet. Meanwhile, in the inorganic fertilizer, it decreased at the dose of 8 mL and was stable at the dose of 10 mL. On the second day after the doses were given, *S. platensis* in each treatment showed the ability to adapt to the culture media environment, except in the treatment using inorganic fertilizer, in which the growth was still slow. In the doses of 8 and 10 mL, the cells grew slowly because, at the time, it was at the adjustment phase to the culture media (Zahidah et al. 2012).

The growth of *S. platensis* during the cultivation is strongly influenced by the availability of nutrients, cell age, and light intensity in growing media (Sanchez-Saveedra & Voltolina 2005). Then, it was suspected that the factors inhibiting the growth of *S. platensis* in the adaptation phase in the dose of 8 mL using inorganic fertilizer were the equality of the inoculum used when stocking and the condition of culture media.

Based on Figures 2 (a and b), Bio Ferti with the dose of 2 mL produced the densest population of *S. platensis* on the 7$^{th}$ day with a density of 1,775,000 cells/mL, while the doses 4 and 6 mL reached it on the same day with densities of 987,500 and 512,500 cells/mL. The dose of 8 mL produced the densest population on the 5$^{th}$ day with a density of 437,500 cells/mL, and the dose of 10 mL reached it on the 6$^{th}$ day with a density of 487,500 cells/mL (Figure 2b).

Of the inorganic fertilizer, the doses of 2 mL produced the densest population on the 5$^{th}$ day with a density of 162,500 cells/mL, while the doses of 4, 6, 8, and 10 mL produced the densest population on the 7$^{th}$ day with densities of 187,500, 175,000, 150,000, and 2,122,500 cells/mL, respectively (Figure 2a).

The experiments showed the highest density of 1,775,000 cells/mL was in the application of Bio Ferti with the dose of 2 mL. In the inorganic fertilizer, the highest density, namely 212,500 cells/mL, was shown by the dose of 10 mL (Figure 3).

The experiments showed the highest density of 1,775,000 cells/mL was in the application of Bio Ferti with the dose of 2 mL. In the inorganic fertilizer, the highest density, namely 212,500 cells/mL, was shown by the dose of 10 mL (Figure 3).

The lowest density in the application of Bio Ferti was shown by the dose of 10 mL, namely 425,000 cells/mL, while that in the application of inorganic fertilizer as indicated by the dose of 2 mL, namely 137,500 cells/mL (Figure 3). The high and low growth rates in the density of *S. platensis* cells were due to differences in nutrients in each treatment and the absorption of excess nutrients used for cell movement and growth processes.

The differences in cell density in each treatment show that *S. platensis* can use the nutrients contained in Bio Ferti and the inorganic fertilizer for its growth. One of the factors that influence the growth of *S. platensis* is the nutrients available, including macronutrients and micronutrients, which also are the factors that influence the biochemical composition of algae (Utomo et al. 2005). The differences in cell density are caused by the effect of differ-

ent doses of the organic fertilizer given. The quality of the nutritional contents of *S. platensis* is related to the composition of nutrients in the culture media and water quality parameters (Widianingsih et al. 2008).

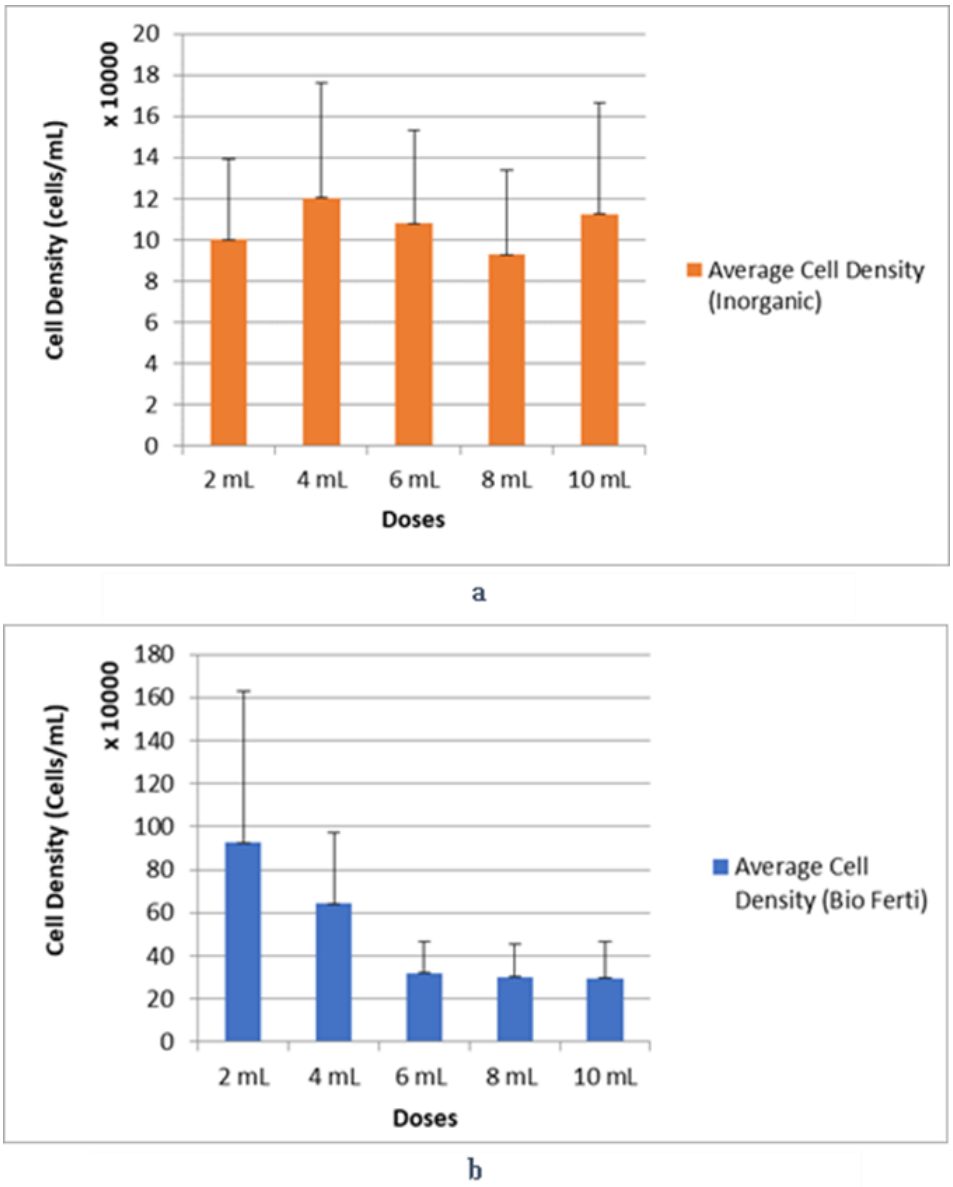


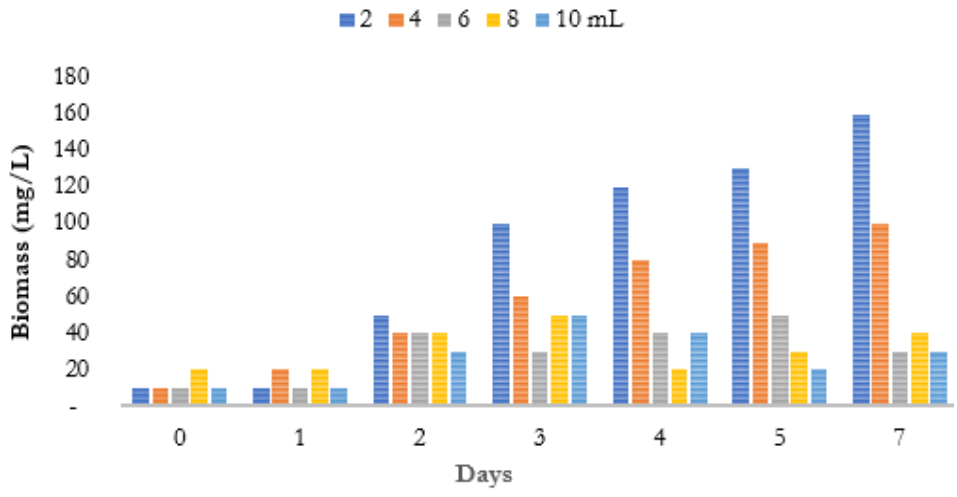

**Figure 3.** Average cell density of *Spirulina platensis* using (a) Inorganic fertilizer and (b) Bio Ferti. The value is obtained by calculating using the cell density formula. This research was conducted using different nutrient concentrations, with qualitatively different results in each concentration.

## Dry Weight (Biomass) of *Spirulina Platensis*

Dry cells are obtained from the yield of the growth of *Spirulina platensis* cells by assimilating the nutrient content contained in the medium into the cells. Based on the research, the biomass (dry cell) yield of *S. platensis* using Bio Ferti liquid organic fertilizer and inorganic nutrients are presented in Figures 4 (a and b).

The daily response of *Spirulina platensis* biomass to the inorganic fertilizer and Bio Ferti in this study is presented in Figures 4 (a and b). The increasing biomass in all media during the cultivation days showed different growth rates. There was no difference in Bio Ferti until the 1st day after the doses

were given. On the 2nd to the 7th days, the rate for the dose of2 mL was higher than that of the other doses. The highest biomass achieved by Bio Ferti was 160 mg/L, with a dose of 2 mL, while that by the inorganic fertilizer was 80 mg/L with a dose of 10 mL. The decreasing rate of *S. platensis* biomass in each media was thought to be due to the depletion of nutrient availability and the increasing cell mass.

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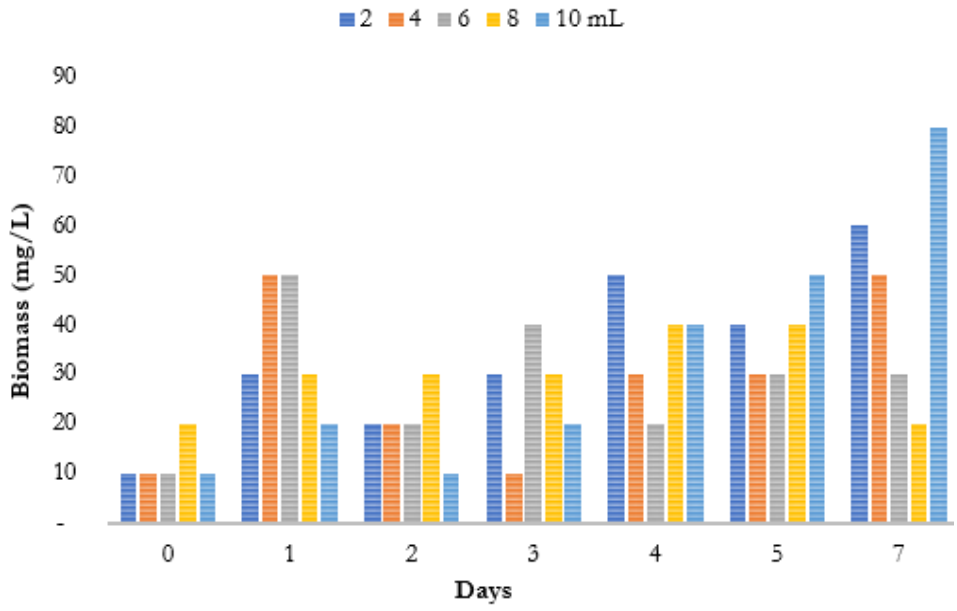



b

**Figures 4**. *Spirulina platensis* biomass in (a) Bio Ferti and (b) Inorganic Fertilizer. The dry cell weight value means the difference between the weight of microalga-containing filter paper and that of the empty one. This research was conducted using different nutrient concentrations, with qualitatively different results in each concentration.

The results of the analysis of some minerals contained in Bio Ferti showed that the lower the dose given, the more adequate the nutrients needed for *S. platensis* to grow. The higher the dose of Bio Ferti given; the less biomass of *S. platensis* produced. Meanwhile, for inorganic fertilizer, the higher the dose given, the higher the biomass produced. As mentioned above,

Bio Ferti contains N, P. K, Ca, Mg, and S around 103,771 mg/L. Several investigators reported that the growth and productivity of *S. platensis* were determined by phosphorus concentration in the cultivation media (Xin et al. 2010; Dammak et al. 2017). Meanwhile, nitrogen plays a role in the formation of macromolecules and cell biomass (Perez-Garcia et al. 2011). With a concentration of 2.5 g/L, it is optimum and recommended for cultivating *S. platensis* (Celekli & Yavuzatmaca 2009).

## Growth Kinetics

Based on the obtained result of the research, a dose of 2 ml is the optimum medium. Kinetic study was conducted at a dose of 2 ml to identify the growth kinetics of *Spirulina platensis* using two different mathematical models. The used models are the Contois model (Equation 2) and the Haldane model (Equation 3). This mathematical approach is used to predict the growth rate of microalgae and design microalgae mass culture. The numerical calculations were carried out using MATLAB.

The Contois and Haldane equations are corrected by considering another factor, and that is the death phase. The death phase factor is assumed to be one of the causes of the decrease in cell mass in microalgae culture.

$$decay\ rate = -K_d.X \tag{5}$$

In a continuous system, the growth rate of microalgae can be determined by calculating the specific growth rate and concentration of microalgae.

$$\frac{dx}{dt} = \mu.X \tag{6}$$

Thus, the Contois and Haldane equations in equations 2 and 3 become,

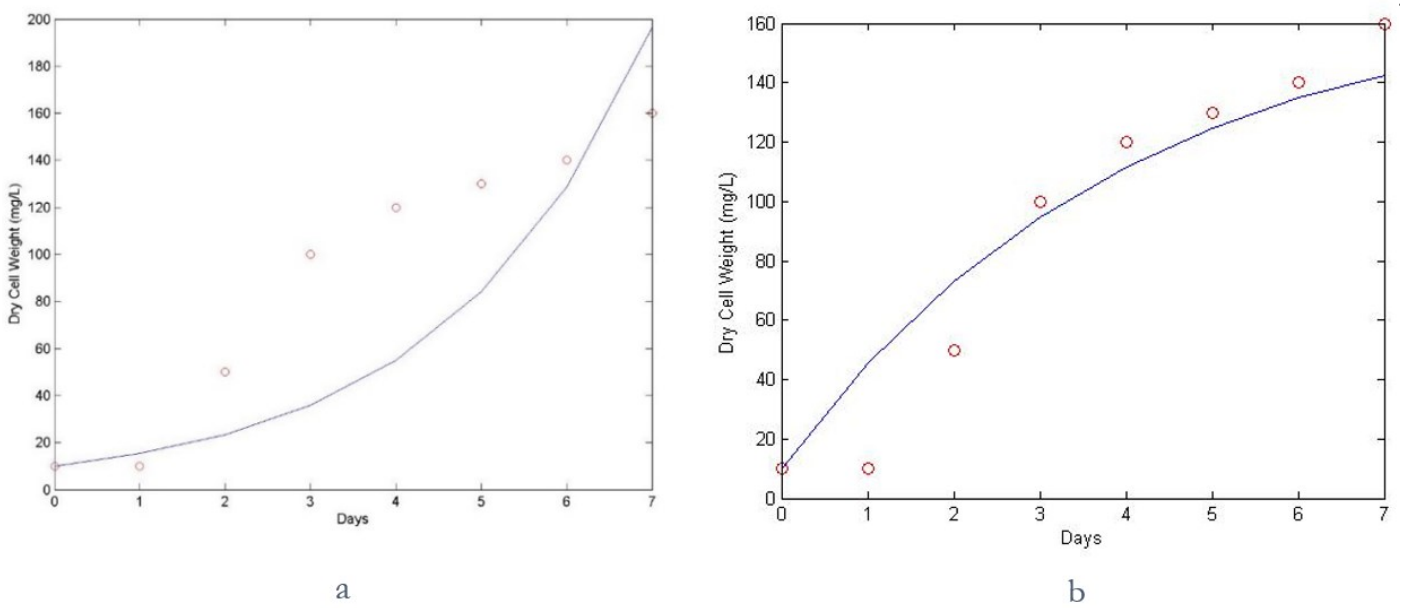
$$\frac{dx}{dt} = \mu_{max}\frac{s}{K_sX+s}X - k_d.X \tag{7}$$

$$\frac{dx}{dt} = \mu_{max}\frac{s}{s+K_s+\frac{s^2}{K_1}}X - k_d.X \tag{8}$$

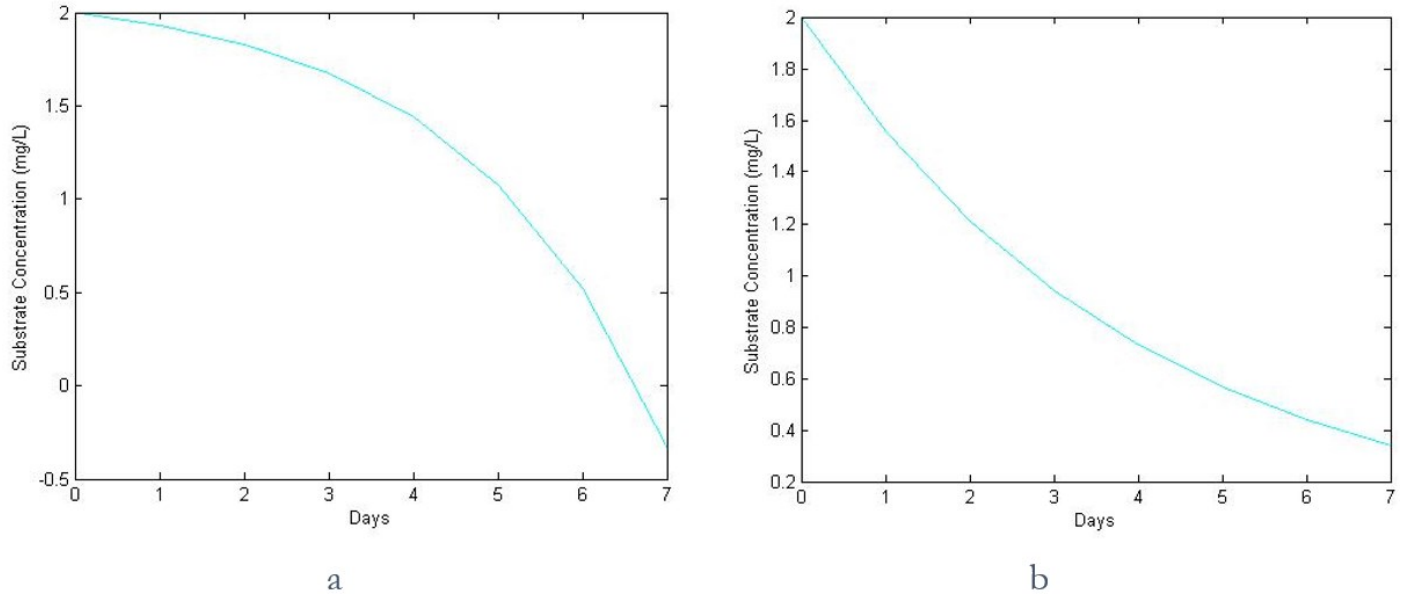
The results of the numerical simulation using the Contois and Haldane equations are presented in the graph of the relationship between experimental data and simulation data against time, a graph of the decrease in substrate during the cultivation process and the kinetics parameters of the microalgae *S. platensis* growth rate.

The result of the kinetic simulation using the Contois and Haldane models are presented in Figures 5 (a and b). The graph describes the relationship between dry cell weight and time period that shows a comparison of the estimated biomass to the experimental data. Based on the figure, there is a deviation that occurs in the dry cell weight curve. The significant deviation between the experimental and simulation data occurred on the second to the fifth day of each model.

Figures 6 (a and b) are curves of simulation results that show the relationship between substrate concentration and time period. Based on those




**Figures 5**. Comparison between experimental data and concentration estimates of dry cell weight using (a) the Contois and (b) the Haldane model. Blue line represents X simulation; Red line represents X experiment.




**Figures 6**. Comparison between substrate concentration and time period using (a) the Contois and (b) the Haldane models. Figure 6(a) and (b) are curves of simulation results that show the relationship between substrate concentration and time period.

two figures, there was a decrease in substrate concentration at a dose of 2 ml during the cultivation process. This occurred because the substrate was consumed by microalgae to reproduce and produce biomass and other components.

Quantitative analysis was conducted by looking at the parameter calculated from the mathematical modeling and kinetic simulation of the growth rate of *S. platensis* microalgae as presented in Table 1. The parameter values obtained from the simulation are maximum specific growth rate ($\mu_{max}$, day$^{-1}$), half-saturation constant (Ks, mg/L), inhibition constant ($K_1$, mg/L), and death coefficient (Kd day$^{-1}$).

From Table 1, the result of numerical calculations using MATLAB, the constant values generated in each model show different results. The value of the specific growth rate (μ) indicates the growth rate of *S. platensis* cells per unit of time. The kinetics of the growth rate was at its peak point in the exponential phase, the growth rate reached a maximum (μmax) and the maximum density was reached. The μmax value obtained with the Contois model is 69.9558 $day^{-1}$, while in the Haldane model of 187.2500 $day^{-1}$, the low specific growth rate value describes the slow growth of microalgae.

**Table 1**. Kinetic constants of the two proposed models generated from MATLAB simulation.

| Parameter | Model | |
|---|---|---|
| | Contois | Haldane |
| $\mu_{max}$ | 69.9558 | 187.2500 |
| $K_s$ | 127.852 | 92.719 |
| $K_d$ | 0.1169 | 0.01006 |
| $K_1$ | - | 343.2452 |
| SSE | 126,530,000 | 22,695,000 |

The Ks value obtained in the Contois model is 127.852 mg/L, while in the Haldane model the value is smaller, which is 92.719 mg/L. Half-saturation constant (Ks) describes the affinity of microorganisms for the substrate (Kim et al. 2003). The low Ks value using the Haldane model shows the result that microalgae have high efficiency or are faster in using substrates at low concentration. This can be seen in Figures 6 (a and b) where the substrate subsidence curve depicted by the Haldane Model is more positive than that of the Contois Model.

In determining the appropriate model to describe the growth kinetics of *S. platensis* microalgae culture, it was done by looking at the results of the lowest Sum of Squared Error (SSE) value. Sum of Squared Error is the total difference from experimental data to simulation data. The smaller SSE value indicates that the experimental data obtained are different from the data of the MATLAB simulation.

From Table 1, the SSE value generated using the Contois model is 126,530,000 while the SSE value obtained by the Haldane model is 22,695,000 which is smaller than the Contois model. Therefore, in this research, the appropriate model to study the growth kinetics of *S. platensis* at a dose of 2 ml is the Haldane model.

## Water Quality Parameters

Factors that influence the growth of *Spirulina platensis* cells during cultivation are water quality. Water quality parameters that were cultured as supporting factors for this research were temperature and degree of acidity (pH). Measurement of water quality parameters is carried out using digital tools. Each device has a sensor which will be immersed in the culture and then read the temperature and pH values on each screen.

## Temperature

Temperature is one of the factors that affect the growth of *S. platensis* cells. Measurement was made daily during cultivation. Temperature measurement ranged from 26-32 °C for each media. Changes in temperature during the research period were still within the tolerance limit, which still allows *S. platensis* to grow. The results of temperature measurements using Bio Ferti liquid organic fertilizer and inorganic fertilizer are presented in Table 2 and 3.

**Table 2.** Temperature data of *Spirulina platensis* using Bio Ferti liquid organic fertilizer.

| Bio Ferti (mL) | Days | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| 2 | 26.9 | 29.2 | 28.5 | 30.1 | 29.4 | 28.9 | 29.8 | 28.7 |
| 4 | 28.2 | 28.5 | 28.3 | 30.5 | 30.2 | 29.6 | 29.2 | 29.2 |
| 6 | 27.9 | 30.5 | 27.6 | 29.8 | 28.9 | 29.3 | 30.2 | 28.4 |
| 8 | 28.9 | 30.1 | 29.2 | 28.8 | 29.4 | 30.1 | 28.8 | 29.3 |
| 10 | 28.5 | 29.7 | 28.9 | 29.2 | 30.9 | 29.8 | 30.4 | 28.9 |

**Table 3.** Temperature data of *Spirulina platensis* using inorganic fertilizer.

| Inorganic fertilizer (mL) | Days | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| 2 | 30.2 | 28.5 | 30.5 | 31.4 | 30.2 | 29.8 | 29.3 | 30.3 |
| 4 | 31.2 | 30.5 | 30 | 30.4 | 31.9 | 30.6 | 29.7 | 29.2 |
| 6 | 31.5 | 29.5 | 31 | 32.4 | 31.2 | 31.8 | 29.8 | 30.5 |
| 8 | 30.9 | 30.2 | 31.5 | 31.8 | 30.9 | 31.4 | 29.2 | 30.6 |
| 10 | 31.8 | 30.4 | 31.3 | 31.9 | 31.6 | 30.8 | 29.3 | 30.3 |

Temperature directly affects the efficiency of photosynthesis and is a determining factor in the growth of *S. platensis*. In this research, it can be seen in Tables 2 and 3 that the temperature changes in each treatment during the cultivation period seemed to fluctuate. The temperature for the use of Bio Ferti liquid organic fertilizer ranged from 26-30˚C. The temperature in the culture medium using Bio Ferti liquid organic fertilizer increased and decreased in each treatment with the highest temperature 30.5˚C and the lowest temperature of 26.8˚C, while for the use of inorganic fertilizer the temperature value ranged from 28-31˚C, with the highest temperature concentration of 31.5˚C and the lowest of 28.5˚C. Normally in laboratory condition, temperature change is influenced by room temperature and light intensity.

The optimum temperature range for the growth of *S. platensis* is 20-30˚C (Hariyati 2008). The temperature changes in this research were still within the optimum temperature range for the growth of *S. platensis*. According to Odum (1973), although the temperature doesn't vary in water as much as in air, temperature is one of the limiting factors for aquatic organisms, condition below or above the optimum temperature will result in inhibition of organism growth and development. In fact, at extreme temperatures, organisms

will die (Wahyudi 1999). Thus, keeping the temperature of the culture medium in optimum condition is necessary for the optimum growth of *Spirulina platensis*.

### PH Value

The acidity degree (pH) is one of the important factors aside from temperature. pH measurement was conducted every day for seven days of the cultivation. The results of the pH measurements are presented in Tables 4 and 5.

pH is the solubility of minerals in the culture medium and directly affects the metabolism of microorganisms (Borowitzka1988). According to (Prihantini et al. 2005), drastic changes in pH can affect the work of enzymes and can inhibit the photosynthesis process and the growth of microalgae. Based on the table, from the pH measurement in each medium the pH values ranging from 8-9 are obtained. The pH value is still within the tolerance limit for *S. platensis* growth media because one of the characteristics of this alga is that it can live in slightly alkaline water condition. Cifferi (1983) stated that optimum pH for the growth of *S. platensis* ranged from 7-11. Blue-green algae grow well at neutral pH and tolerate more alkaline than acidic conditions because algae can utilize carbon dioxide efficiently although it is available at a very low concentration (Hariyati 2008).

**Table 4.** pH data of *Spirulina platensis* using Bio Ferti liquid organic fertilizer.

| Bio Ferti (mL) | Days | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| 2 | 8.01 | 8.30 | 8.26 | 8.27 | 8.27 | 8.26 | 8.50 | 8.22 |
| 4 | 8.08 | 8.26 | 8.24 | 8.27 | 8.29 | 8.31 | 8.30 | 8.17 |
| 6 | 8.11 | 8.30 | 8.25 | 8.28 | 8.28 | 8.26 | 8.40 | 8.18 |
| 8 | 8.11 | 8.33 | 8.26 | 8.29 | 8.26 | 8.28 | 8.40 | 8.18 |
| 10 | 8.15 | 8.31 | 8.26 | 8.28 | 8.27 | 8.30 | 8.40 | 8.2 |

**Table 5.** pH data of *Spirulina platensis* using inorganic fertilizer.

| Inorganic fertilizer (mL) | Days | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| 2 | 8.5 | 8.67 | 8.53 | 8.85 | 8.89 | 8.83 | 8.08 | 8.42 |
| 4 | 8.53 | 8.7 | 8.91 | 8.78 | 8.65 | 8.67 | 8.05 | 8.46 |
| 6 | 8.47 | 8.71 | 8.51 | 8.58 | 8.51 | 8.71 | 8.13 | 8.38 |
| 8 | 8.56 | 8.7 | 8.6 | 8.67 | 8.85 | 8.52 | 8.11 | 8.36 |
| 10 | 8.49 | 8.85 | 9.02 | 8.75 | 8.61 | 8.48 | 8.06 | 8.18 |

## CONCLUSION

The conclusion of this research showed that liquid organic fertilizer Bio Ferti can be used as an alternative to commercial fertilizers and inorganic fertilizers because the resulting production is higher in cell density and dry weight. The 2 ml dose is the optimum dose with the highest growth rate and highest cell density. The highest cell density was on day 7which was 1.78 x $10^6$. The appropriate growth kinetics model to study the growth kinetics of *Spirulina platensis* at the optimum dose of 2 ml is the Haldane model. It yielded a maxi-

mum specific growth rate of 187.2500 day$^{-1}$, a half-saturation constant of 92.719 and SSE value of 22,695,000. Therefore, in this research, the appropriate model to study the growth kinetics of *S. platensis* at a dose of 2 ml is the Haldane model.

## AUTHORS CONTRIBUTION

MIMG conducted the research and the data analysis and wrote the manuscript, EAS, MDK, and LTS supervised the research and manuscripts writing, while UJS and AB supervised and designed the research.

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## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publishing of this article. The authors certify that the manuscript is original work and is not under review at any other publication.

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Review Article

# Pharmacological Maneuver of Mangrove Endophytic Fungi in the South China Sea – A review

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## ABSTRACT
Conventional products have a role in addressing the thriving universal demands for biologically active substances. Since the South China Sea is a prodigious province of geostrategic and mercantile importance, it meets the basic needs of people who dwell there. The South China Sea is dominant in mangrove biodiversity which, represents 11.4% of the world's 15.5 million hectares of mangrove forest. Mangroves are harbored by multifaceted fungal communities that represent the second colossal ecological breed of marine fungi. The symbiotic association between the plants and fungi stimulates the bioactive components such as alkaloid, depsipeptides, cyclic peptides, quinone, terpenes, lactones, terpenoid, flavonoid, phenolic acid, steroids. These components have multifaceted pharmacological activities likely, anti-inflammatory, antidiabetic, anticancer, antioxidant, and antimicrobial. This review article attempts to present a piece of insightful information currently being explored on the biologically active components generated by mangrove endophytic fungi of the South China Sea.



## INTRODUCTION
People experience multifaceted maladies that devastate their health. Several synthetic and natural therapy are available to curb and treat these diseases. Utmost people globally entrusted natural medicine than synthetic due to its minimal side effects and cost-effectiveness. Hitherto, plants and microbes are appraised to be a root source of natural medicine. Ethnopharmacological proficiency provides the unrivaled groundwork for the future endeavor of medicinal expedients in traditionally used plants. With the aid of advanced technologies, researchers can identify, isolate and extract an enormous number of new components from natural resources. From 1981 to date, merely 50% of anti-microbial drugs are in the practice of the pharmacy market. Roughly 75% of it is from natural or derivates of natural products (Salini 2015). However, increased demand for commercially successful natural products causes an overwhelm of plant material to produce an adequate quantity of drugs, which has engendered threat concerns like species extinction, biodiversity loss, and environmental depletion. These jeopardized situations kindle the researcher to scrutinize and isolate Taxol (cancer drug) producing endo-

phytic fungi *Taxomyces andreanae* which gave an alternative route to acquire an inexpensive and readily affordable product *via* fermentation of microorganisms (Bibi et al. 2020). Based on the existing literature survey signify that plant endophytes can inherit the secondary metabolites of the host. Enormous metabolites extracted from the endophytic fungi of mangrove species emerged mainly from the South China Sea (SCS). These components show various remarkable biological activities, namely anticancer, antimicrobial, anti -inflammatory, antidiabetic, and antioxidant, which have biotechnology exploitation in the field of pharmacy. In this survey article, we endeavor to explore the insightful benefits of the biologically active components generated by mangrove endophytic fungi of the SCS.

## MANGROVES - SCS REGION

The SCS is one of the vast marginal seas of the Western Pacific Ocean, bordering a region of approximately 3,500,000 km² (1,400,000 sq mi) (Anon 2008). It is a province of prodigious mercantile and geostrategic that meets the basic need of two hundred and seventy million people dwelling on the border nations of SCS. Mangroves, seagrass meadows, and coral reefs are the most dominant coastal ecosystems of the SCS. South China Sea is considered one of two global hotspots of mangrove biodiversity. The total area of mangrove species on the SCS coast of all countries (Brunei, Cambodia, China, Indonesia, Malaysia, Philippines, Singapore, Taiwan, Thailand, and Vietnam) are consolidated as 1.77 million hectares, representing 11.4% of the world's mangrove forest (FAO 2007; Vo et al. 2013). Mangrove, known as halophytes, grows in the coastal saline or brackish waters in rocky or muddy soils. The presence of pneumatophores (*Bruguiera gymnorhiza* (L.) Lam.) and rhizophores system (*Rhizophora mucronata* Lam.), provides structural support for the plants to adapt to the harsh coastal condition (Tomlinson 1994). Mangrove produces outstanding natural resources on their own due to the inhabitants of the transition zone between land and sea.

Mangrove forests are rich in different types of flora and fauna. The prime genera are *Lumnitzera* and *Laguncularia* (Combretaceae), *Nypa* (Palmae), *Avicennia* (Avicenniaceae), *Kandelia* and *Rhizophora* (Rhizophoraceae), and *Sonneratia, Bruguiera, Ceriops* (Sonneratiaceae) (Tomlinson 1994). Due to frequent flooding of the tidal wave, mangrove resilience with environmental cues and also loaded with the surplus of nutrients to serve a myriad of heterogenetic habitats including microorganisms such as epiphytes and endophytes (Sridhar 2019; Thatoi et al. 2020). Until now, plant species of mangrove have greatly been conventionally used for therapeutical purposes as a treatment against a broad range of ailments namely snake bites, hypertension, toothache, oral infection, diabetes, hematuria, hepatitis, diarrhea, constipation, fever, dysentery, rheumatism, dyspepsia, among others. Carotenoids, catechins, procyanidins, gibberellins, gallic acid, isorhamnetin, isocoumarin, among others, are the several phytoconstituents segregated from mangrove plants that were explored for diverse biological activities (Salini 2015).

## MANGROVE ENDOPHYTIC FUNGI

The microscopic community of microorganism is hidden and reside within the plant known as endophytes. Endophytes (actinomycetes, bacteria, fungi) spend their life cycle entirely or partially in the tissues of the plants that cause no menace to the plant. Plants harbor a varied cluster of endophytes ubiquitously distributed in roughly 300,000 plant species (Sridhar 2019). The endophyte symbiotically associated with the plants are knowns as symbionts. Moreover, the endophytic colonies are beneficial to our surroundings. For instance, endophytes aid the plants to flourish by generating growth hormones, nutrient cycling, biodegrading. It also takes part in phytoremediation that reduces detritus load in our landscape (Das et al. 2018).

Mangroves are harbored by versatile fungal communities, which account for the second colossal ecological breed of marine fungi. Mangrove endophytic fungi are robustly resistant to oceanic environments, temperature for example, *Alternaria*, *Aspergillus*, *Cladosporium, Clolletotrichum*, *Fusarium, Paecilamyces, Penicillium*, *Pestalotiopsis*, *Phomopsis*, *Phyllosticta*, and *Trichoderma* (Bibi et al. 2020). Repeated flooding and drastic climatic fluctuation make the mangrove plant resilient both physiologically and morphologically. This acclimatization engenders the plant to generate a broad spectrum of biologically active secondary metabolites. Endophytes were grown in such an ecosystem with a unique nature that produces remarkable secondary metabolites such as flavonoids, quinones, alkaloids, terpenoids, tetralones, benzopyranones, xanthones among others. The plant-endophyte association generates bioactive components with promising pharmacological effects such as antioxidant, anticancer, and antimicrobial properties (Das et al. 2018).

## BIOACTIVE METABOLITES OF MANGROVE ENDOPHYTES

Unrivaled research of plants and microbes generates a trove of novel natural products that act as happening and growing demand to curb diverse aliments. Multifarious biologically active metabolites have been extracted and identified from fungi habitats around countries bordering the South China Sea. Thatoi et al. (2020) stated that enzymes extracted from the mangrove endophytes have an ecological role in the decomposition and a bioactive role towards the medical environment. Secondary metabolites obtained from these endophytes have an efficient role in mitigating various ailments which have bioactive potential in both the medical and pharmaceutical fields. Camptothecin, Irinotecan, Podophyllotoxin, Taxol, Vinblastine, Vincristine are the commercially available metabolites among them (Bibi et al. 2020).

With the evolution of the latest modern technologies, such as chromatographic and spectroscopic techniques, CD spectra analysis, in-vitro bioassay methods, and nuclear magnetic resonance (NMR), researchers can skillfully perform mangrove fungal isolation, cultivation, and extraction of captivating beneficial bioactive metabolites (Perera et al. 2019). Endophytes produce versatile secondary metabolites such as isocoumarins, xanthones, lactones, and ergosterl with antimicrobial properties that preclude the plants

from pathogens (Sridhar 2019). Several comprehensive types of research have been carried out in the last decades, related to the characterization and isolation of metabolites from the mangrove endophytic fungi. Liu et al. (2016) recognized polyketides from *Penicillium* sp. ZJ-SY2 endophytic fungus of mangrove which shows an immunosuppressive activity. Li et al. (2019) extracted polyketide alkaloid derivatives namely phomopsols A and B from the *Phomopsis* sp. xy21 endophytic fungus of mangrove. Liu et al. (2018) obtained polyketide (1) from the endophyte A*scomycota* sp. SK2YWS-L conducts an anti-inflammatory activity. One new natural amide alkaloid and two new benzophenones are identified by Zheng et al. (2019) from *Penicillium citrinum* mangrove fungus that has an antibacterial property against *Staphylococcus aureus* and shows strong cytotoxic action against A549 human cell lines. Chen et al. (2019) obtained ascomylactams A-C metabolites from the endophyte *Didymella* sp. CYSK-4 of mangrove that performs average cytotoxicity towards HCT116, MDA-MB-435, PC-3, NCI-H460, MDA-MB-231, and SNB19. From these discussions, we can drive that those endophytic fungi extracted from the mangrove species are the prime origin of bioactive metabolites and requires further studies for other feasibilities.

## BIOLOGICAL ACTIVITIES OF MANGROVE ENDOPHYTES

An endophytes-plants symbiotic relationship activates the inheriting properties of the bioactive compounds of the host. They are the warehouse of novel bioactive secondary metabolites that possess various biological applications in the pharmaceutical field, likely anticancer, anti-inflammatory antidiabetic, and antioxidant. Table 1 represent the metabolites generated from the mangrove endophytic fungi of the SCS and their bioactive significance. Figure 1 depict the pharmacological benefits of secondary metabolites of mangrove endophytes.

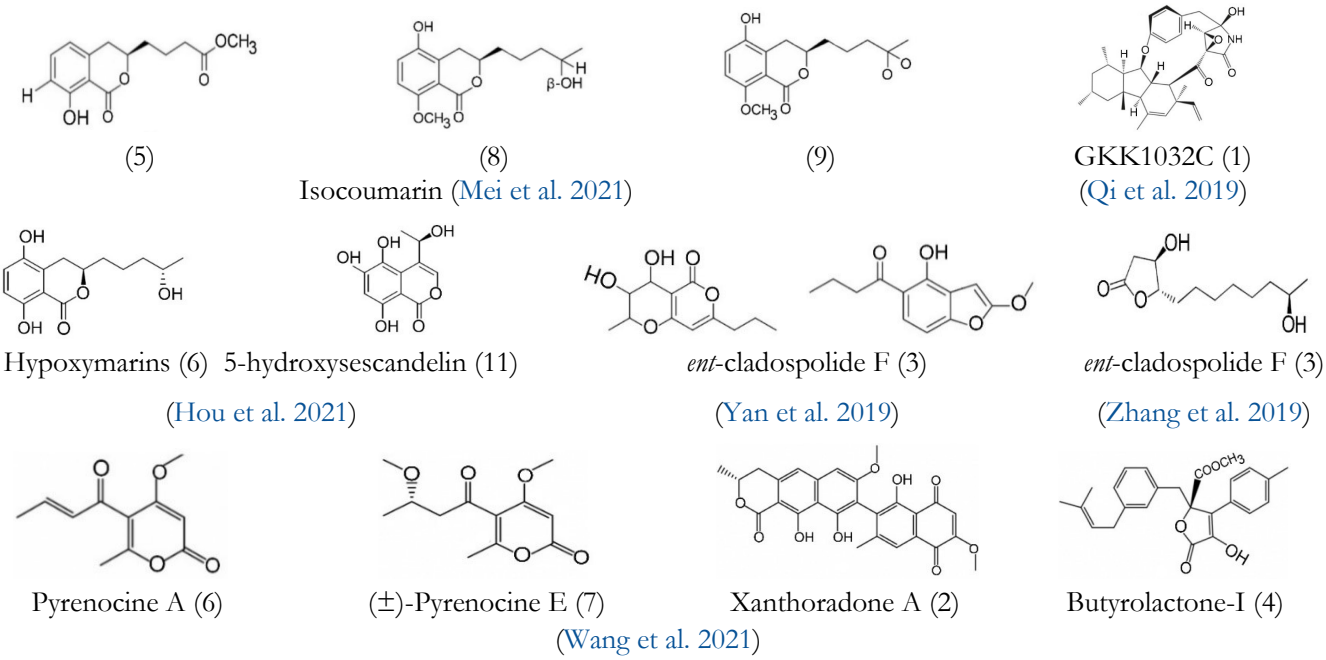


**Figure 1.** Bioactive metabolites isolated from mangrove endophytic fungi.

**Table 1.** Bioactive metabolites and their pharmacological applications omangrove endophytic fungi.

| Endophytic fungi | Mangrove plant | Bioactive metabolites and Activities | References |
|---|---|---|---|
| | | Cytotoxic effects | |
| *Aspergillus* sp. HN15-5D | *Acanthus ilicifolius* | Aspergisocoumrins A (1) ($IC_{50}$ = 5.08 ± 0.88 μM) and aspergisocoumrins B (2) ($IC_{50}$ = 4.98 ± 0.74 μM) showed cytotoxicity in oppose to cancer cell line of MDA-MB-435. | Wu et al. 2019 |
| *Aspergillus sydowii* #2B | *Aricennia marina* | Butyrolactone I (4) ($IC_{50}$ = 1.92 ± 0.82 μM), pyrenocine S (5) ($IC_{50}$ = 20.06 ± 2.01 μM), pyrenocine A (6) ($IC_{50}$ = 7.92 ± 0.86 μM) and pyrenocine E (7) ($IC_{50}$ = 10.13 ± 0.88 μM), (þ)-3,30 ,7,70 ,8,80 -hexahydroxy-5,50 -dimethyl-bianthra-quinone (3) ($IC_{50}$ = 33.36 ± 1.42 μM), xanthoradone A (2) ($IC_{50}$ = 4.19 ± 1.02 μM), showed potent cytotoxicity's towards prostate cancer VCaP cell line. | Wang et al. 2021 |
| *Penicillium citrinum* HL-5126 | *Bruguiera sexangula* var. *rhynchopetala* | Penibenzophenones B (2) ($IC_{50}$ = 15.7 μg/mL) has cytotoxicity that resists human cell line A549. | Zheng et al. 2019 |
| *Penicillium* sp. J-54 | *Ceriops tagal* | Penicieudesmol B (2) ($IC_{50}$ = 90.1 μM) exhibits minimal cytotoxicity inhibition of the human K-562 cell line. | Qiu et al. 2018 |
| *Cladosporium* sp. HNWSW-1 | *Ceriops tagal* | Cladosporitins B (2) exhibit cytotoxicity resist towards cell lines BEL-7042 ($IC_{50}$ = 29.4 ± 0.35 μM), K562 ($IC_{50}$ = 25.6 ± 0.47 μM) and SGC-7901 ($IC_{50}$ = 41.7 ± 0.71 μM), whereas talaroconvolutin A (4) against cancer cell lines Hela ($IC_{50}$ = 14.9 ± 0.21 μM) and BEL-7042 ($IC_{50}$ = 26.7 ± 1.1 μM). | Wang et al. 2018 |
| *Cytospora* sp. | *Ceriops tagal* | Integracin A (4) ($IC_{50}$ = 5.98 ± 0.12 μM) and Integracin B (5) ($IC_{50}$ = 9.97 ± 0.06 μM) has a cytotoxicity effect that opposes HepG2 cancer cell line. | Wei et al. 2020 |
| *Trichoderma* sp. 307 | *Clerodendrum inerme* | Botryorhodine H (1) has a potent cytotoxic that oppose cell lines of rat pituitary adenoma GH3 ($IC_{50}$ = 3.64 μM) and rat prolactinoma MMQ ($IC_{50}$ = 3.09 μM). | Zhang et al. 2018 |
| *Cladosporium* sp. OUCMDZ-302 | *Excoecaria agallocha* | New polyketide compound (6) ($IC_{50}$ = 10.0 μM) has cytotoxicity that hinders the H1975 cell line. | Wang et al. 2018 |
| *Didymella* sp. CYSK-4 | *Pluchea indica* | Ascomylactams A (1) and C (3) have average cytotoxicity towards the human cancer cell lines such as HCT116, MDA-MB-435, PC-3, MDA-MB-231, SNB19, and NCI-H460 ($IC_{50}$ range from 4.2–7.8 μM). | Chen et al. 2019 |
| *Aspergillus fumigatus* HQD24 | *Rhizophora mucronata* | 1,11-dideacetyl-pyripyropene A (2) cytotoxic activities. | Zou et al. 2021 |
| *Phoma* sp. SYSU-SK-7 | *Kandelia candel* | Colletotric B (2) and analog (1) ($IC_{50}$ values range between 16.82 - 37.73 μM) also showed cytotoxicity against human cancer cell lines A549 and MAD -MB -435. | Chen et al. 2019 |
| *Streptomyces* sp. | Rhizosphere of *Kandelia candel* and *Excoecaria agallocha* | Non-ribosomal peptide synthetase, type-I polyketide synthase, and type-II polyketide synthase are the enzymes that show cytotoxic activities against CNE-2 and Hela. | Gong et al. 2018 |
| *Pseudopithomyces* sp. 1512101 | *Sonneratia caseolaris* | Phospholipase $A_2$ ($PLA_2$) Anti-cancer activity against A549, SH-SY5Y, and HeLa cells. | Wei et al. 2021 |
| | | Antimicrobial effects | |
| *Phomopsis* sp. HNY29-2B. | *Acanthus ilicifolius* | α-pyrone derivative, compounds (2) (MIC = 25 μM) and (3) (MIC = 50μM) reveal the least antibacterial activities against *Pseudomonas aeruginosa*, *Staphylococcus aureus,* and *Bacillus subtilis*. | Cai et al. 2017 |

**Table 1.** Contd.

| | | | |
|---|---|---|---|
| *Talaromyces stipitatus* SK-4 | *Acanthus ilicifolius* | Talaromyones B (2) (MIC = 12.5 µg/mL) shows a potent antibacterial activity against *Bacillus subtilis*. | Cai et al. 2017 |
| *Epicoccum nigrum* SCNU-F0002 | *Acanthus ilicifolius* L | New polyketides compounds (6 and 7) showed antibacterial effects against *Bacillus subtilis* (ATCC 6538), *Escherichia coli* (ATCC 8739), and *Staphylococcus aureus* (ATCC 6538), with MIC ranging between 25–50 µg/mL. | Yan et al. 2019 |
| *Phomopsis longicolla* HL-2232 | *Brguiera sexangula* var. *rhynchopetala* | New biphenyl derivative 5,5'-dimethoxybiphenyl-2,2'-diol (1) show a moderate inhibition on *Vibrio parahaemolyticus* (MIC = 10 µg/mL), altersolanol B (4) antibacterial activities against *Vibrio parahaemolyticus* (MIC = 2.5 µg/mL) and *Vibrio anguillarum* (MIC = 5 µg/mL). | Li et al. 2017 |
| *Daldinia eschscholtzii* HJ001 | *Brguiera sexangula* var. *rhynchopetala* | New cytochalasin (1) (MIC = 50 µg/mL) as [11] cytochalasa-5(6),13-diene-1,21-dione-7,18-dihydroxy-16,18-dimethyl10-phenyl-(7S*,13E,16S*,18R*) shows least antibacterial towards *Vibrio alginolyticus, Vibrio parahaemolyticus, Bacillus cereus Staphylococcus aureus,* and *Escherichia coli.* | Yang et al. 2018 |
| *Ascomycota* sp. CYSK-4 | *Pluchea indica* | Dichlorodiaportintone (1), desmethyldichlorodiaportin (5), and dichlorodiaportin (6) (MIC ranging between 25- 50 µg/mL) show an antibacterial property against *Klebsiella pneumoniae, Staphylococcus aureus, Bacillus subtili, Acinetobacter calcoaceticus, and Escherichia coli.* | Chen et al. 2018 |
| *Cladosporium cladosporioides* MA-299 | *Bruguiera gymnorrhiza* | Polyketides *ent*-cladospolide F (3) (MIC = 8.0 µg/mL) exhibit average inhibitory against human pathogenic bacteria *Staphylococcus aureus,* cladospolide G (4) (MIC = 1.0 µg/mL) strong inhibitory against plant pathogen *Fusarium oxysporum and Glomerella cingulat.* Compounds (7) - exhibited antimicrobial activity against plant pathogen *Glomerella cingulat* (MIC = 4.0 µg/mL) and aquatic bacterium *Edwardsiellaictarda* (MIC = 1.0 µg/mL). | Zhang et al. 2019 |
| *Penicillium citrinum* HL-5126 | *Bruguiera sexangula* var. *rhynchopetala* | Penibenzophenones A (1) (MIC = 20 µg/mL) - antibacterial against *Staphylococcus aureus.* | Zheng et al. 2019 |
| *Cladosporium* sp. JJM22 | *Ceriops tagal* | (3S)-3,8-dihydroxy-6,7-dimethyl-αtetralone (3) (MIC = 20 µM) - reveal a wide range of antibacterial against *Vibrio parahemolyticus, Escherichia coli, Staphylococcus aureus, Bacillus cereus,* and *Vibrio alginolyticu.* | Wu et al. 2019 |
| *Cladosporium* sp. JS1-2 | *Ceriops tagal* | Pentenoic acid derivative compounds (1) (MIC = 25.0 µg/ml), (2) (MIC = 12.5 µg/ml), (4) (MIC = 6.25 µg/ml), (6) (MIC = 1.25 µg/ml) and (7) (MIC = 6.25 µg/ml) showed average antibacterial activity oppose to *Staphylococcus aureus.* | Bai et al. 2019 |
| *Cytospora* sp. | *Ceriops tagal* | Cytospomarin (2) (MIC = 0.35 µM) feeble inhibition of *Escherichia coli* (GIM1.201). | Wei et al. 2020 |
| *Penicillium commune* QQF-3 | *Kandelia candel* | Peniisocoumarins G (7) ($IC_{50}$ = 20.7 µM) Mycobacterium tuberculosis protein tyrosine phosphatase B (MptpB). | Cai et al. 2018 |
| *Aspergillus* sp. ZJ-68 | *Kandelia candel* | Asperophiobolins H (8) ($IC_{50}$ = 19 µM) was found to have significant inhibition of Mycobacterium tuberculosis protein tyrosine phosphatase B (MptpB). | Cai et al. 2019 |
| *Aspergillus* sp. 085242 | Mangrove plant | Racemate (3) showed antibacterial activity against *Salmonella.* | Guo et al. 2020 |

**Table 1.** Contd.

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|---|---|---|---|
| Aspergillus sp. xy02 | *Xylocarpus moluccensis* | (7S,10S)-7,10-epoxysydonic acid (2), (7R,11S)-7,12-epoxysydonic acid (3), 7-deoxy-7,14-didehydro-12-hydroxysydonic acid (5), (E)-7-deoxy-7,8-didehydro-12-hydroxysydonic acid (7), and analogues (9-12) with $IC_{50}$ range between 31.5 - 41.9 µM that exhibit medium resist action towards *Staphylococcus aureus* ATCC 25923. | Wang et al. 2018 |
| *Talaromyces* sp. HZ-YX1 | *Kandelia obovata* | Talaramide A (1) ($IC_{50}$ = 55 µM) - showed active inhibitory of mycobacterial PknG. | Chen et al. 2017 |
| *Aspergillus* sp. ZJ-68 | *Kandelia candel* | 2-(hydroxymethyl)-3-propylphenol (8) and brassicadiol (11) indicate an opposing activity to strains *Escherichia coli, Bacillus subtilis* and *Staphylococcus aureus*, with MIC values ranging between (4.15 - 12.5 µg/mL). | Cai et al. 2019 |
| *Pestalotiopsis* sp. HHL101 | *Rhizophora stylosa* | Pestalotiopisorin B (1) exhibit average antibacterial inhibition of *Escherichia coli* (MIC =12.5 µg/ml) and *Pseudomonas aeruginosa* (MIC =50 µg/ml). | Xu et al. 2020 |
| *Streptomyces* sp. | Rhizosphere of *Kandelia candel* and *Excoecaria agallocha* | Nonribosomal peptide synthetase, type-I polyketide synthase, and type-II polyketide synthase reveal antibacterial against *Bacillus cereus* ATCC 14579 and *Escherichia coli* ATCC 13706, *Staphylococcus aureus* SA115 and *Enterococcus faecium* EF009 | Gong et al. 2018 |
| *Leptosphaerulina* sp. SKS032 | *Acanthus ilicifolius* | Leptospyranonaphthazarin A (1) (MIC = 25.0 µg/mL) and leptosnaphthoic acid A (2) (MIC = 50.0 µg/mL) and compound (6) (MIC = 50.0 µg/mL), exhibit minimal antibacterial activities resist to *Staphylococcus aureus*. | Cui et al. 2017 |
| *Phoma* sp. SYSU-SK-7. | *Kandelia candel* | Colletotric B (2) and analog compounds (1) (MIC range between 1.67–6.28 µg/ml) have an efficient antimicrobial inhibition of MRSA, *C. albicans* and *P. aeruginosa*. | Chen et al. 2019 |
| *Penicillium* sp. CPCC 400817 | *Ceriops tagal* | GKK1032C (1) (MIC = 1.6 µg/ml), manifest strong antibacterial activity against MRSA. | Qi et al. 2019 |
| *Cladosporium* sp. JJM22 | *Ceriops tagal* | (3S)-3,8-dihydroxy-6,7-dimethyl-αtetralone (3) (MIC = 20 µM) - indicate wide range of antibacterial action against MRSA. | Wu et al. 2019 |
| | | Anti-diabetic effects | |
| *Talaromyces stipitatus* SK-4 | *Acanthus ilicifolius* | Talaromyones B (2), purpactin A (4), and tenellic acids A (5) have an inhibition action towards α-glucosidase enzyme activity $IC_{50}$ range between 48.4 - 99.8 µM | Cai et al. 2017 |
| *Penicillium commune* QQF-3 | *Kandelia candel* | Peniisocoumarins C (3), G (7), I (9), and J (11) reveal average α-glucosidase inhibition with $IC_{50}$ range between 38.1 - 78.1 µM. | Cai et al. 2018 |
| *Lasiodiplodia* theobromae ZJ-HQ1 | *Acanthus ilicifolius* | Lasiodiplactone A (1) **(**$IC_{50}$ = 29.4 µM**)** shows a potent α-glucosidase enzyme inhibition. | Chen et al. 2017 |
| *Hypoxylon* sp. Hsl2-6 | *Bruguiera gymnorrhiza* | Diterpenoids and isocoumarin derivatives hypoxyterpoids (1) ($IC_{50}$ = 741.5 ± 2.83 µM) manifest a moderate α-glucosidase inhibitory activity. | Hou et al. 2021 |
| *Mycosphaerella* sp. SYSU-DZG01 | *Bruguiera gymnorrhiza* | Asperchalasine I (1) ($IC_{50}$ = 17.1 µM), epicoccolide B (8**)** **(**$IC_{50}$ = 26.7 µM**)** and asperchalasine A (9) ($IC_{50}$ = 15.7 µM) indicate potent α-glucosidase enzyme inhibition. | Qiu et al. 2019 |
| *Trichoderma* sp. 307 | *Clerodendrum inerme* | Botryorhodines H (1), C (2), and D (3) ($IC_{50}$ range between 8.1 - 11.2 µM) reveal α-glucosidase enzyme inhibition. | Zhang et al. 2018 |
| *Cladosporium* sp. JJM22 | *Ceriops tagal* | Cyclohexene derivatives cladoscyclitols B (2) ($IC_{50}$ = 2.95 µM) and 4- *O* -α-D-ribofuranose-2-pentyl-3-phemethylol (5) ($IC_{50}$ = 2.05 µM) signify α-glucosidase enzyme inhibition. | Zhang et al. 2021 |

**Table 1.** Contd.

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| *Penicillium* sp. YYSJ-3 | *Heritiera littoralis* | Isocoumarin derivatives compounds (3) ($IC_{50}$ = 100.6 µmol/L), (6) ($IC_{50}$ = 133.4 µmol/L) and (7) ($IC_{50}$ =130.9 µmol/L) indicate promising inhibitory activity against α-glucosidase. | Qiu et al. 2020 |
| *Aspergillus* sp. 085242 | Mangrove plant | Asperisocoumarin G (1) **(**$IC_{50}$ = 392.4 µmol/L**)** and pergillin (4) ($IC_{50}$ = 428.1 µmol/L**)** have a medium *a*-glucosidase enzyme inhibitory action. | Guo et al. 2020 |
| *Aspergillus flavus* QQSG -3 | *Kandelia obobata* | Compounds (3, 5, 10, and 11) ($IC_{50}$ values range between 1.5 - 4.5 µM) are phenolic bisabolane sesquiterpenoid possess strong α-glucosidase inhibitory effects. | Wu et al. 2018 |
| *Aspergillus* sp. ZJ-68 | *Kandelia candel* | Phenylpropanoid derivatives compound (6) ($IC_{50}$ = 12.4 µM) reveal potent inhibitory activity against α -glucosidase. | Cai et al. 2019 |
| *Talaromyces* sp. CY-3 | Semi-mangrove *Hibiscus tiliaceus* | Sambutoxin derivatives sambutoxin A (1), sambutoxin B (2), sambutoxin C (3), ilicicolin H (4), deoxyleporin B (5) with $IC_{50}$ =12.6 ± 0.9 - 57.3 ± 1.3 µM possess an α-glucosidase inhibitory action. | Yang et al. 2021 |
| *Cladosporium* sp. HNWSW-1 | *Ceriops tagal* | Talaroconvolutin A (4) ($IC_{50}$ = 78.2 ± 2.1 µM) and anthraquinone (5) ($IC_{50}$ = 49.3 ± 10.6 µM) have an enzyme inhibitory action towards α-glycosidase. | Wang et al. 2018 |
| *Penicillium* sp. MGP11 | *Xylocarpus granatum* | New isocoumarins namely penicimarin I (5) ($IC_{50}$ = 776.5 µM), penicimarin G (8) ($IC_{50}$ = 683.7 µM) and penicimarin H (9) ($IC_{50}$ = 868.7 µM) reveal an α -glucosidase enzyme inhibition. | Mei et al. 2021 |
| *Phoma* sp. SYSU-SK-7 | *Kandelia candel* | Phomosterols A (1) ($IC_{50}$ = 51.2 µM), B (2) ($IC_{50}$ = 46.8 µM) own a potent *a*-glucosidase enzyme inhibition. | Chen et al. 2020 |
| Phoma sp. SYSU-SK-7 | *Kandelia candel* | Colletotric B (2), colletotric C ( 4), 3 -hydroxy5 -methoxy-2,4,6 -trimethylbenzoic acid ( 3), and analog (1 and 5) ($IC_{50}$ values range between 36.2–90.6 µM) signify α-glucosidase enzyme inhibition. | Chen et al. 2019 |
| | | Anti-inflammatory effects | |
| *Aspergillus* sp. GXNU-MA1 | *Acanthus ilicifolius* | New sesquiterpene, gxsespene A (1), and compounds (2-5) **(**$IC_{50}$ range between 16.15 - 27.08 µM) possess an anti-inflammatory property by preventing NO secretion in LPS triggered RAW264.7 cells. | Zhou et al. 2020 |
| *Aspergillus* sp. GXNU-A9 | *Acanthus ilicifolius* | Guanxidone A (1) ($IC_{50}$ value of 8.22 µM) owns an anti-inflammatory property by reducing the NO generation in LPS influenced RAW 264.7 macrophage. | Hao et al. 2020 |
| *Aspergillus sydowii* #2B | *Aricennia marina* | Compounds 2-(12S-hydroxypropyl)-3-hydroxymethyl-6-hydroxy-7-methoxychromone (1) ($IC_{50}$ = 40.15 µM, (þ)-3,30 ,7,70 ,8,80 -hexahydroxy-5,50 -dimethyl-bianthra-quinone (3) ($IC_{50}$ = 28.69 µM), pyrenocine A (6) ($IC_{50}$ = 25.25 µM), and pyrenocine E (7) ($IC_{50}$ = 43.08 µM) signify minimal inhibitory effects on NO secretion in LPS influenced RAW 246.7 cells. | Wang et al. 2021 |
| *Aspergillus* sp. ZJ-68 | *Kandelia candel* | Asperophiobolins H (8) I (9) J (10) and analogs (14–17) (IC50 range between 9.6 - 25 µM) has an opposing action of NO generation in LPS triggered RAW 264.7 macrophage cells. | Cai et al. 2019 |
| *Ascomycota* sp. SK2YWS-L | *Kandelia candel* | Ascomindone D [(+)-1 ($IC_{50}$ = 17 µM) and (-)-1 ($IC_{50}$ = 17.1 µM) reveals a significant anti-inflammatory by hindering against the NO oozing in LPS promoted RAW 246.7 mouse. | Liu et al. 2018 |

**Table 1.** Contd.

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|---|---|---|---|
| *Phomopsis* sp. SYSU-QYP-23 | *Kandelia candel* | Compounds (3–9) namely, farinomalein H (3) indole alkaloid phomoamide (8), and analog compounds (4-7) and (9) ($IC_{50}$ values ranging between 4.5 - 25 µM) of five new maleimide derivatives reveal inhibitory action against NO generation in LPS stimulated RAW 264.7 cells. | Chen et al. 2020 |
| *Phomopsis* sp. SYSU-QYP-23 | *Kandelia candel* | Eight new sesquiterpene derivatives compounds (1 –7) ($IC_{50}$ values ranging between 8.6 to 14.5 µM) potent resistance to NO discharge in LPS influenced RAW 264.7 cells. | Chen et al. 2021 |
| *Talaromyces amestolkiae* YX1 | *Kandelia obovata* | Amestolkolide (B) ($IC_{50}$ = 1.6 ± 0.1 µM) exhibits sturdy anti-inflammatory activity by inhibiting NO production in LPS triggered in RAW264.7 cells. | Chen et al. 2018 |
| *Diaporthe* sp. QYM12 | *Kandelia candel* | Diaporpenoids A (1) ($IC_{50}$ = 21.5 µM) and diaporpyrones A (4) ($IC_{50}$ = 12.5 µM) showed effective anti-inflammatory activities by resisting the generation of NO in LPS- triggered RAW264.7 cells. | Chen et al. 2021 |
| *Penicillium citrinum* QJF-22 | *Kandelia candel* | Benzopyran derivatives 3R,4S)-3,4,8-Trihydroxy-3,4-dihydronaphthalen-1(2H)-one ($IC_{50}$ = 44.7 µM) signify feeble resistance effects on LPS-induced NO production in RAW264.7 cells. | Yang et al. 2020 |
| *Ascomycota* sp. CYSK-4 | *Pluchea indica* | Desmethyldichlorodiaportintone *(2) (*$IC_{50}$ = 15.8 µM*)* indicattes effective inhibiting of the generation of NO in LPS stimulated RAW 264.7 cells. | Chen et al. 2018 |
| *Phomopsis* sp. 33# | *Rhizophora stylosa* | Chromenopyridines derivatives (3) *(*$IC_{50}$ = 49.0 µM*)* and (4) *(*$IC_{50}$ = 51.0 µM*)* showed average inhibition of nitric oxide generation in LPS induced RAW 264.7 cells. | Chen et al. 2018 |
| *Aspergillus fumigatus* HQD24 | *Rhizophora mucronata* | 1,11-dideacetyl-pyripyropene A (2) immunosuppressive activity. | Zou et al. 2021 |
| *Phomopsis* sp. xy21 | *Xylocarpus granatum* | Phomopsol A (1) and polyketide-derived (3) neuroprotective effects. | Li et al. 2019 |
| *Phoma* sp. SYSU-SK-7 | *Kandelia candel* | Phomosterols A (1) ($IC_{50}$ = 13.5 µM), B (2) ($IC_{50}$ = 25.0 µM) and neocyclocitrinol B (5) ($IC_{50}$ = 12.5 µM) hinder NO production in LPS triggered RAW 264.7 cells. | Chen et al. 2020 |
| | | Anti-oxidant effects | |
| *Epicoccum nigrum* SCNU-F0002 | *Acanthus ilicifolius* L | Compounds (10) ($IC_{50}$ = 13.6 µg/mL), (11) ($IC_{50}$ =12.1 µg/mL), (12) ($IC_{50}$ =18.1 µg/mL) and (13) ($IC_{50}$ =11.7 µg/mL) showed potent antioxidant activity. | Yan et al. 2019 |
| *Epicoccum nigrum* MLY-3 | *Bruguiear gymnorrhiza* | Furobenzotropolones B (2), 3-hydroxyepicoccone B (3), and compounds (5 and 8) with $IC_{50}$ values ranging between 14.7 - 29.3 µM indicate promising DPPH antioxidant scavenging. Above compounds along with compound (7) exhibit efficient ABTS antioxidant scavenging. | Zou et al. 2021 |
| *Phomopsis* sp. 33# | *Rhizophora stylosa* | Chromenopyridines derivatives (4) *(*$IC_{50}$ = 34.0 µM*)* antioxidant cap*ability to scavenge DPPH radical.* | Chen et al. 2018 |
| Aspergillus sp. xy02 | *Xylocarpus moluccensis* | Phenolic bisabolane sesquiterpenoids analog compound (12) ($IC_{50}$ =72.1 µM) has a minimal antioxidative scavenge DPPH radical activity. | Wang et al. 2018 |
| *Hypoxylon* sp. Hsl2-6 | *Bruguiera gymnorrhiza* | Diterpenoids and isocoumarin derivatives hypoxymarins (6) ($IC_{50}$ = 15.36 ± 0.24 µM) and 5-hydroxysescandelin (11) ($IC_{50}$ = 3.69 ± 0.07 µM) own DPPH scavenging antioxidant activity. | Hou et al. 2021 |

**Table 1.** Contd.

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| *Mycosphaerella* sp. SYSU-DZG01 | *Bruguiera gymnorrhiza* | Compounds (1), 2-methoxycarbonyl-4,5,6-trihydroxy-3-methyl-benzaldehyde (4), 1,3-dihydro -5-methoxy-7-methylisobenzofuran (6) and epicoccolide B (8) ($EC_{50}$ values range between 16.3 - 85.8 µM) reveal antioxidant activity by scavenging DPPH· | Qiu et al. 2019 |
| *Penicillium* sp. MGP11 | *Xylocarpus granatum* | New isocoumarin *penicimarin G* (8) ($IC_{50}$ = 4.6 µM) has significant antioxidant activity. | Mei et al. 2021 |
| Phoma sp. SYSU-SK-7. | *Kandelia candel* | Compound (7) ($EC_{50}$ = 11.8 µM ) possesses inhibition of radical scavenging activity as opposed to DPPH. | Chen et al. 2019 |
| | | Anti-acetylcholinesterase | |
| *Cladosporium cladosporioides* MA-299 | *Bruguiera gymnorrhiza* | Polyketides ent-cladospolide F (3) ($IC_{50}$ = 40.26 µM) potent enzymatic inhibition of AChE. | Zhang et al. 2019 |
| *Aspergillus* sp. 16-5c | *Sterculia apetala* | Isoaustinol (3) ($IC_{50}$ = 2.50 µM), dehydroaustin (7) ($IC_{50}$ = 0.40 µM), and dehydroaustinol (8) ($IC_{50}$ = 3.00 µM) showed AChEIs | Long et al. 2017 |
| *Aspergillus terreus* H010 | *Kandelia obovata* | 1,2-dehydro-terredehydroaustin (1) ($IC_{50}$ = 42.3 ± 0.20 µM) inactivates AChE enzyme. | Liu et al. 2018 |

## Anticancer

Cancer or malignant is an abnormal growth of cells found either in organs or tissue of several types of ailments. It grows beyond the boundaries and spreads uncontrollably to other adjoining parts of the organs. It is the second supreme engender of mortality globally (approximately 9.6 million deaths in 2018). It ruined the lifestyle of humans physically, emotionally, and financially. Chemotherapy is a synthetic therapy applied to cure cancer that causes several side effects such as hair loss, fever, loss of appetite, diarrhea, fatigue, damage to lung tissue, heart problems, and kidney problems. Advance research in inventing anticancer drugs shows new and improved varieties of compounds derived from natural sources. Investigation of Taxol, a commercially anticancer drug that was procured from endophytic fungi *Taxomyces andreanae of Taxus brevifolia plant*, captivated the mycologists to explore the benefits of metabolites from diverse endophytic fungi (Bibi et al. 2020). The secondary metabolite components yielded by the endophytes have commercial importance towards humans as an anticancer drug. Mangrove plants place an active role in producing more novel phytochemical components, mainly due to the adaptable nature of the environment they survive. Mangrove plants are the storehouse of endophytes. The endophytes of mangrove plants have a symbiotic relationship that inherits the secondary metabolites from their host (Salini 2015). It produces metabolites for example, *Acanthus ilicifolius* mangrove store the endophyte *Diaporthe phaseolorum* SKS019 that produce metabolites deoxybostrycoidin (8) has cytotoxic activity that inhibits the cancer cell lines NCI-H460 ($IC_{50}$ = 5.32 µM) and MDA-MB-435 ($IC_{50}$ = 6.57 µM), and fusaristatin A (9) shows growth inhibition of cancer cell line MDA-MB-435 ($IC_{50}$ = 8.15 µM) (Cui et al. 2017). *Xylocarpus granatum* mangrove plant has *Trichoderma* sp. Xy24 secretes 9R, 10R-dihydroharzianone (1) exhibit cytotoxic action that resists cell lines of MCF-7 ($IC_{50}$ = 30.7 µmol/L) and HeLa. ($IC_{50}$ = 30.1 µmol/L) (Zhang et al. 2016). *Penicillium*

*chermesinum* endophytes of *Hertiera littoralis* produce polyketide derivative compound (2-chloro-3,4,7-trihydroxy-9-methoxy-1-methyl-6H-benzo[c] chromen-6-one (1)) possess cytotoxicity opposing the cancer cell lines MOLT-3 ($IC_{50}$ = 14.94 µM), HuCCA-1 ($IC_{50}$ = 56.39µM), A-549 ($IC_{50}$ = 115.71µM), and HepG2 ($IC_{50}$ =55.06 µM) (Darsih et al. 2017). *Lasiodiplodia* sp. 318 from *Excoecaria agallocha* which generates lasiodiplodins 2,4-dihydroxy -6-nonylbenzoate (4) shows the most potent cytotoxicity resist of GH3 ($IC_{50}$ =13.05 µM) and MMQ ($IC_{50}$ =5.29 µM) cell lines (Huang et al. 2017).

## Antimicrobial

Pathogenic microbes such as fungi, bacteria, and viruses can cause contiguous diseases that disseminate through different modes of transmission. It can be cured either by the administration of synthetic or herbal antimicrobial drugs. Synthetic drugs are well known for curing several communicable ailments but have high side effects on human health. Chronic uses of antibiotic drugs cause the influence of new multi-drug resistant microbes ongoing threats to the world. This threat urges an investigation of alternative sources of naturally available antimicrobial drugs. The discoveries of drugs from diverse natural sources (plants, animals, and non-pathogenic microbes) may have fewer side effects and more effective antimicrobial properties. Based on the proof of scientific research, mangrove endophytes render potential and notable robust sources of antimicrobial activities. Phytochemical compounds of mangrove endophytes can act as antimicrobial agents to cure communicable diseases (Kuzhalvaymani et al. 2020). *Penicillium citrinum* HL-5126 spotted from *Bruguiera sexangula var. rhynchopetala* produce benzopyran derivative compound (6) (MIC = 6.94 µg/mL) exhibit the most efficient inhibition of *Micrococcus tetragenus, Bacillus subtilis,* and *Bacillus cereus* (Zheng et al. 2016), *Penicillium* sp. GD6 spotted from *Bruguiera gymnorrhiza* has the metabolites 2-deoxy-sohirnone C (1) with MIC = 80 µg/ml that has an average resistance of Methicillin-resistant *Staphylococcus aureus (MRSA)* (Jiang et al. 2018). *Penicillium aculeatum* (No. 9EB) endophytes present in *Kandelia candel* has chromone metabolites derivative such as bacillisporin A (2) (MIC = 0.13 ± 0.02 µM), bacillisporin B (3) (MIC = 0.13 ± 0.02 µM) with significant antibacterial inhibition of *Bacillus subtilis*, whereas metabolite (1) known as (2′S*)-2-(2′-hydroxypropyl)-5-methyl-7, 8-dihydroxy-chromone, exhibit antibacterial activity oppose to *Salmonella* with a MIC = 2.00 ± 0.02 µM (Huang et al. 2017).

## Antidiabetic

Diabetes is a chronic hyperglycemia disease that results from a disorder in insulin action and insulin secretion. It has common symptoms such as increased thirst, increased urinary discharge, ketonemia, and ketonuria. Diabetes is one of the globally occurring diseases, predicted to affect approximately a 693 million adults by 2045. Microvascular and macrovascular are the complications are provoked by diabetes. It causes several defects in the kidneys, nerves, eye, and cardiovascular, which elevates the mortality rate and ruin the

quality of life of diabetic patients. Alpha-glucosidase inhibitors treat type2 diabetic patients. The inhibitors slow down carbohydrates' absorption in the small intestine and mitigate the postprandial level of insulin and blood glucose (Van De Laar et al. 2005). Mangrove endophytic produce trove of metabolites that have inhibited α-glucosidase enzyme role, for example, *Alternaria* sp. SK6YW3L derived from mangrove plant *Sonneratia caseolaris* has a compound namely altenusin derivatives compounds (2) ($IC_{50}$ = 78.2 μM), (3) ($IC_{50}$ = 78.1 μM), and (9) ($IC_{50}$ = 64.7 μM) exhibit medium α-glucosidase enzyme inhibition (Liu et al. 2016). *Sonneratia ovata* mangrove has an endophyte *Nectria sp. HN001* that contains a Nectriacids B (2) ($IC_{50}$ = 23.5 μM) and C (3) ($IC_{50}$ = 42.3 μM) indicate effective inhibitory activity toward α-glucosidase (Cui et al. 2016). *Penicillium aculeatum* (No. 9EB) identified from *Kandelia candel* produces compound (2) ($IC_{50}$ = 33.55 ± 0.63 μM) and (3) ($IC_{50}$ =95.81 ± 1.12 μM) possessed significant inhibitory activity against α-glucosidase (Huang et al. 2017).

## Anti-inflammatory

Inflammation is the immune response to toxic stimuli involved in the wound healing process. Three prime pathways NF-ϰB, MAPK, and JAK-STAT, stimulate the inflammatory signal process. Any variance caused in these pathways might provoke inflammation-related diseases, including cancers, rheumatoid arthritis, type 2 diabetes, cardiovascular diseases, and atherosclerosis. Anti-inflammatory drugs impede the pathophysiological process of inflammation, thus reducing tissue damage and protecting patients from injuries. Therefore, it is impressive to mention that the availability of natural sources (plants and endophytic fungi) and bioactive components generated from them act as anti-inflammatory drugs. *Botryosphaeria* sp. SCSIO KcF6 identified from *Kandelia candel* can secrete a phenyl derivative (3) ($IC_{50}$ = 1.12 μM), exhibited a specific cyclooxygenase-2 (COX-2) inhibitory activity (Ju et al. 2016). *Penicillium* sp. ZJ-SY2 endophytes from *Sonneratia apetala* produce metabolites peniphenone (1) and xanthones (3, 5, 7) ($IC_{50}$ range between 5.9 - 9.3 μg/mL) owns an immunosuppressive activity (Liu et al. 2016). *Lasiodiplodia theobromae* ZJ-HQ1 endophytes from *Acanthus ilicifolius* produce metabolites lasiodiplactone A (1) ($IC_{50}$ = 23.5 μM) signify anti-inflammatory property by precluding the nitric oxide (NO) generation in lipopolysaccharide (LPS) promoted RAW264.7 cells (Chen et al. 2017).

## Antioxidant

Any disproportion between the production of reactive oxygen species (ROS) and antioxidants induces oxidative stress, which damages the tissue. Free radicals are highly reactive and unstable molecules generated in the body either by the normal metabolic process of cells as a by-product of oxidation or by exposure to carcinogens in our environment (smoke, air pollution, some viruses, chemical exposure, ultraviolet radiation, and medical radiation). Cells get damaged by antioxidant deficiency that engenders more accumulation of

free radicals, leading to various kinds of ailments such as cancer, asthma, diabetes, atherosclerosis, and many others. An antioxidant is a compound available in diverse forms (natural or artificial) that inhibits or delays the accumulation of free radicals (Pizzino et al. 2017). Phenolic compounds present in the endophytes were the primary sponsor of the antioxidant properties. *Cladosporium sp. OUCMDZ-302* endophytes are spotted from *Excoecaria agallocha* has an antioxidant activity of secondary metabolite polyketide compounds (4) ($IC_{50}$ = 2.65 µM), (8) ($IC_{50}$ = 0.24 µM), (9) ($IC_{50}$ = 5.66 µM) and (10) ($IC_{50}$ = 6.67 µM) showed promising DPPH antioxidant scavenging (Wang et al. 2018). *Ascomycota* sp. SK2YWS-L obtained from *Kandelia candel* produces an antioxidant metabolite ascomindones A (1) ($IC_{50}$ = 18.1 µM) possesses efficient scavenging activities that inhibit DPPH (Tan et al. 2016).

### Anti-Acetylcholinesterase

Acetylcholinesterase (AChE) is the prime enzyme of the cholinergic nervous system. Predominant in the neuromuscular junctions and cholinergic synapses. AChE ceases neurotransmission at cholinergic synapses by rapid hydrolysis of acetylcholine to choline and acetate this enzyme activity is inhibited by Acetylcholinesterase inhibitors (AChEIs), which in turn augment the accumulation of acetylcholine in neuromuscular junctions, central nervous system, and autonomic ganglia, where acetylcholine receptors are surplus (Cheung et al. 2012). Hence, AChEIs treat dementia with Lewy body, Parkinson's, and Alzheimer's diseases. Plants are considered a most efficient and enormous source for AChEIs enzyme but, microbes' production of these enzymes indicates an eco-friendly, cost-effective, efficient, and alternative approach easily manipulated (Zhang et al. 2019). *Kandelia candel* is a mangrove plant that stores a plethora of endophytes one of them is *Penicillium* sp. SK5GW1L which has *a*-pyrone meroterpenoids compounds (3) ($IC_{50}$ = 3.03 µM), (4) ($IC_{50}$ = 0.23 µM) and (5) ($IC_{50}$ = 0.028 µM) shows sturdy inhibition of acetylcholinesterase (AchE) (Ding et al. 2016). Liu et al. (2018), reported that compounds 1,2-dehydro-terredehydroaustin (1) ($IC_{50}$ = 42.3 ± 0.20 nM) resist the AChE enzyme produced by *Aspergillus terreus* H010 endophytes of *Kandelia obovata* mangrove plants.

## CONCLUSION

This review accentuates that mangrove endophytic fungi of SCS has more capacity to generate an impressive range of metabolites. The plant-endophyte symbiotic concomitant activates the production of bioactive components likely alkaloid, depsipeptides, terpenes, lactones, allenolic, cyclic peptides, quinone, chinone or terpenoid, flavonoid, phenolic acid, steroid, with propitious biopharmaceutical potential including anticancer, antimicrobial, antioxidant, anti-inflammatory and antidiabetic. Out of 84 mangrove species recorded currently, only 27 of them have been pharmacologically corroborated in terms of endophytic fungi. Still, there is a meager of knowledge that insists more investigation is needed to explore the relationship and mechanism of

association between the endophytic fungi and host in order to procure enormous metabolites that have a significant role in medical field.

## AUTHORS CONTRIBUTION

All authors reviewed the literature and compilation of this manuscript. All authors have read and approved the final manuscript.

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## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this article.

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