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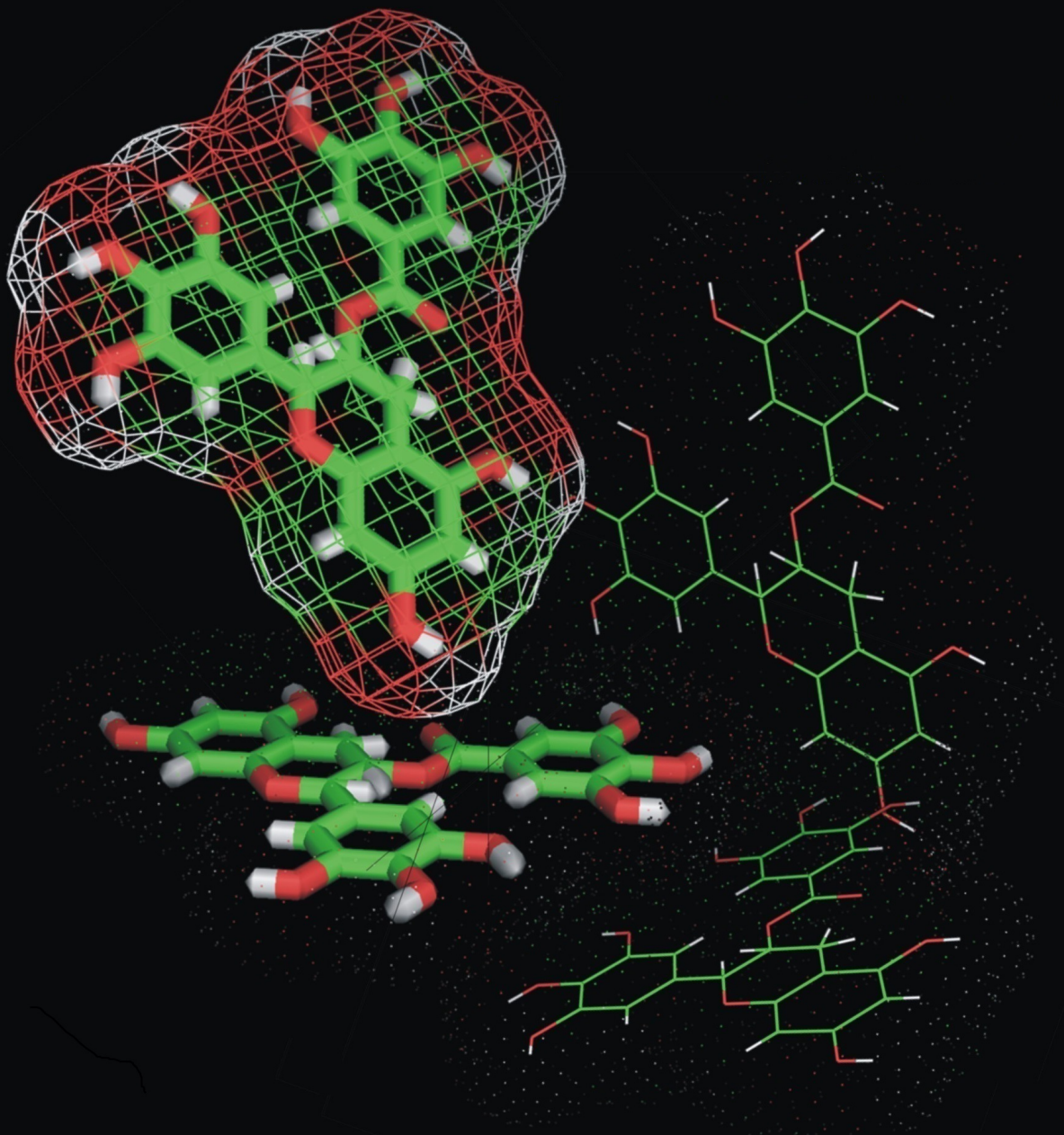

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Molecular Characterization of a Rigid Rod-Shaped Virus Isolated from Frangipani (*Plumeria* sp.) Showing Mosaic Symptom in Taiwan

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Abstract

Frangipani is an important succulent plant around the worlds and also in Taiwan, for example, *Plumeria rubra* is widely grown as a popular ornamental tree in parks and landscaped establishments in Taiwan. Recently, a new disease in frangipani with mosaic and distortion symptoms was found in Taiwan. No viruses caused frangipani disease has been reported in Taiwan and the references about frangipani disease are still limited and only *Frangipani mosaic virus* (FrMV) was found. In this study, the molecular properties of a virus isolated from symptomatic frangipani in south Taiwan, such as Pingtung, Kauhsiung and Tainan were investigated. The virus with rod-shaped particles of 300 nm long and 18 nm in diameter was examined inside diseased leaves by electron microscopy. The purified virus particles showed the typical UV spectrum of tobamoviruses with A_{260}/A_{280} value of 1.29 and maximum and minimum absorption at 260 nm and 249 nm, respectively. The molecular weight of 19.5 kDa as the size of coat protein of tobamoviruses was estimated by sodium dedocyl sulfate-polyacrylamide gel (SDS-PAGE). Furthermore, the degenerate primers for tobamoviruses were used to amplify 568 bp and 400 bp of the DNA fragments in RT-PCR and nested PCR, respectively. Based on these results, it was confirmed that the rigid rod-shaped virus isolated from mosaic symptom of frangipani leaves is an isolate of FrMV, belonging to the genus *Tobamovirus*. This is the first report thatFrMV infecting *Plumeria* sp. in Taiwan.

Keywords: Frangipani plant, FrMV, mosaic disease, *Tobamovirus*.

INTRODUCTION

Frangipani (*Plumeria* sp.) is a small group of plant species native to tropical countries [1]. Frangipani is an important succulent plant around the worlds and some species are valuable sources as medicines, insecticides, fibers, and rubber [2]. Frangipani plants are also important in Taiwan.

For the example is *P. rubra* which widely grown as a popular ornamental tree in parks and landscaped establishments in Taiwan. It bears beautiful, big flowers of various colours and sizes that predominate especially during the summer [3].

Unfortunately, there are factors which inhibit frangipani growth, i.e. pest and pathogen attacked. One of pathogens which could attack frangipani plant is virus. Preliminary surveys in the fields of Pingtung, Kauhsiung and Tainan County showed that some frangipani plants are indicating attacked by viruses based on foliar symptoms about 30% severity (unpublished data, 2012). This condition encourages the research about molecular characterization (electron microscopy, virus purification, and RT-PCR,) of unknown reported mosaic disease of *Plumeria* sp. in Taiwan. The objective of this study was to identify the virus which attacked *Plumeria* sp.

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MATERIALS AND METHODS

Virus Source and Isolation

Frangipani plants showing symptom caused virus including mosaic and leaf distortion were collected in Pingtung County, Taiwan by random sampling method from 100 plants as sampel. Symptoms were recorded (photographed) at Plant Protection Laboratory, Department of Plant Medicine, National Pingtung University of Science and Technology (NPUST) Taiwan. Then, virus was isolated by mechanical inoculation from the infected frangipani leaf to indicator plant, *Chenopodium quinoa* Willd. and then local lesions of *C. quinoa* were collected and maintained on *C.quinoa* leaves by three passages [4]. Plants' leaves were dusted with carborundum (400 mesh) and 0.1 M phosphate buffer (PB) containing virus-stabilizing additivies of 0.1 % 2-mercaptoethanol, pH 7.0 was used [5]. The virus was subsequently multiplied on *C. quinoa* Plants because it's highly susceptible and easy to growth and maintained in the laboratory at room temperature with supplemented flourescene light providing a photoperiod at

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12L:12D. The virus isolate was temporary designated as Frangipani-Taiwan 1 (Fr-T1).

Electron Microscopy (Negative Staining)

Virus particles from *C. quinoa* and frangipani leaf extracts were one drop floated onto electron microscopy Formvar-fronted, carbon coated, 200 mesh copper grids and incubated for five minutes. Then, the grids were washed with 10 drops of distilled water, negatively stained with 6 drops of 2% aqueous uranyl acetate (pH 5.0) and incubated for five minutes. Then, all the fluid was removed by touching the edge of the grid carefully with a filter paper [6]. Then air dried before positioning the grid in a suitable container (grid storage box) and examination with Hitachi 7500 electron microscope. Particle size was determined by measuring the average of length and diameter of 100 virions.

The virus isolate Fr-T1 was partially purified according to the method by Dijkstra and de Jager [7] with slight modification as follows: infected *C. quinoa* leaves with local lesion symptoms were kept at deep freezer (-80°C) homogenized with one volume (v/w) 0.5 M phosphate buffer, pH 8.5, containing 0.01 M Na-EDTA. Then strained through 2 layer cheesecloth. After filtering through cheesecloth, the extract was clarified by adding 12% chloroform (4°C) and stirred for 2 minutes. Then continued by centrifuged at 8000 rpm for 20 minutes (R14A rotor, Hitachi CR21G, Japan). Collect the supernatant and layered on the top with 5 ml 20% sucrose in the bottom. Then centrifuged at 28.000 rpm for 2.5 hour (P70T rotor, Hitachi CP90WX, Japan).

The pellet was resuspended in borate buffer pH 8.2 containing 0.01 M Na-EDTA. Then centrifuge at 3,000 rpm for 10 minutes (R20A2, Hitachi CR21G, Japan). Collect the supernatant and centrifuged in swing-bucket rotor at 38,000 rpm for 23 hours (RPS40T-1180 rotor, Hitachi CP90WX) in 30% Cs_2SO_4 by density gradient centrifugation (Swing-bucket rotor). Dialysed the virus band with phosphate buffer 0.01 M, pH 7.0 and stirred for 16 hours at 4°C. Then, the purified virus was test by measuring wavelength absorbance at range 220-320 nm (Hitachi U-2001 spectrophotometer). Virus concentration (c in $mg.mL^{-1}$) were calculated by formula [7]:

$$c = A_{260}/E \times \text{dilution factor}$$

Determination of the Molecular Weight of Viral Coat Protein by SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Healthy *C. quinoa* leaf, infected *C. quinoa* leaf, healthy frangipani leaf, field mosaic frangipani leaf, and purified virus isolate Fr-T1 were homogenized with 1 : 4 (w/v) 0.5 M phosphate buffer, pH 7.0. Then centrifuged the samples on centrifuge 10,000 rpm for 10 min and collected the supernatant. The extracts were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with 12% running gel and 5% stacking gel. Mix the samples with dye on comparison 4:1 in microtube. Then heat the microtube on dry bath incubator (Violet Bioscience, Inc.) at 100°C for 3 min. Load Protech (Prestainde Protein Marker) 5µl as marker and 10 µl of each samples into the wells in the stacking gel. Start the electrophoresis with 70 voltage (V) during 30 minutes and continue with 120 V during 70 min. After finish running, the gel were stained with Coomassie Blue and shake gently on Orbital Shaker PSU-10i (Grant-Bio) with 70 rpm for 1 h. Replace the Comassie Blue with destaining buffer (100 ml methanol, glacial acetic 70 ml, add ddH_2O to 1,000 ml) and shake gently on Orbital Shaker PSU-10i (Grant-Bio) with 70 rpm for 24 h. Protein bands are stained and the molecular weight of the viral coat protein was determined by comparing migration of the viral protein in the gel with that of marker protein and run in parallel lanes [7].

Amplification and Analysis of Nucleic Acid Sequences

Extraction of Total RNA

Purified virus of virus isolate Fr-T1, healthy *Nicotina benthamiana* and *C. quinoa* leaf, *Tobacco mosaic virus* (TMV) infected tomato and *N. tabacum* samples and *Odontoglossum ringspot virus* (ORSV) were prepared. Each sample take 5 µl and use RNA extraction kit (Direct-zol™ RNA miniprep) to extract total RNA from the each sample according to the manufacturer's instructions (The Epigenetics Company).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

One-tube of RT-PCR reactions (25 µl) were performed. A final concentration of 1 µM for each tobamoviruses degenerate primers TobRT up1 (5'-GARTAYSCIGCIYTICARAC-3') and TobRT do2 (5' BGCYTCRAARTTCCA-3'), was used. The cycling profile was as follows : first step at 43°C for 60 min, second step at 50°C for 2 min, third

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step at 94°C for 4 min; five cycles segmented into step (a) 30 s at 95°C, step (b) 30 s at 43°C, step (c) 15 s at 72°C; 35 cycles segmented into step (a) 30 s at 95°C, step (b) 30 s at 46°C, step (c) 15 s at 72°C, followed by a final extension step at 72°C for 2 min [8]. Amplification was carried out in Px2 Thermal cycler (Thermo Electron Corporation).

Nested Polymerase Chain Reaction (PCR)

Nested PCR reactions (20 µl) were performed using 1µl of the first RT-PCR product mixed with PCR master kit (GeneMark, Taiwan) and 1 µM of each degenerate primer TobN up3 (5'–GGCGYTGCARACIATHGTITAYCA-3'), TobN do4 (5' GTRTTICCIATRAAIGTIGTIACRTC-3') and TobN do4G (5' GCCGATRAAGGTGGTGACRTC-3'). The cycling profile consisted of a denaturasing step at 95°C for 3 min, two cycles segmented into step (a) 20 s at 95°C, step (b) 15 s at 51°C, step (c) 5 s at 72°C; 26 cycles segmented into step (a) 20 s at 95°C, step (b) 15 s at 61°C, step (c) 5 s at 72°C, followed by a final extension step at 72°C for 2 min [8]. Amplification were carried out in Px2 Thermal cycler (Thermo Electron Corporation).

Electrophoresis Analysis

Electrophoresis analysis was used to ascertaining DNA product was amplified by RT-PCR and nested PCR. Get 1 µl loading dye with 5 µl PCR product uniformly mixing. Load 2 µl marker and each sample inside the horizontal electrophoresis analysis (1.5% agarose + 0.5 µl Save view DNA stain). Running on electrophoresis machine (Major Science®) with 120 V electricity for 20 min. Then, visualized under UV light and estimate the amplified product.

RESULT AND DISCUSSION

Virus Isolation

Frangipani plants exhibiting virus-like disease collected in Pingtung County (South Taiwan) which showed mosaic and leaf distortion on young leaf of frangipani plants (Fig. 1). Frangipani plants exhibiting virus-like disease was indicated on several city (Pingtung, Kauhsiung and Tainan). The symptom is similar according to previous study [9] noted that the leaves of frangipani infected by virus was showed chlorotic ringspots or mosaic and were often distorted. In addition, the virus induced chlorotic ringspot and mosaic, and often distortion on leaves of frangipani [10]. Mechanical inoculation to indicator plants (*Chenopodium quinoa*) and isolation by three time single lesion showing local lesion on leaves (Fig. 2).

Figure 1. Mosaic symptom of frangipani leaves on the field.

Mechanical inoculation to indicator plant (*C. quinoa*) and maintenance by three time single lesion showing local lesion (Fig. 2). The local lesion were produced on the inoculated leaves of *C. Quinoa* [9]. The virus induces chlolotic ringspots or mosaic, and oftendistortion on leaves of frangipani, and severe mosaic symptoms occur on leaves of virus-infected *Nicotiana benthamiana* [10].

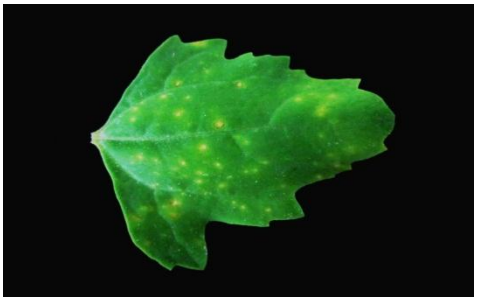

Figure 2. The local lesions caused by viru isolate Fr-T1 on *Chenopodium quinoa* leaf

Electron Microscopy

Rigid, rod-shaped particles with average length of 250-300 nm x 18 nm were seen in negatively stained preparations from infected frangipani leaves, infected C. quinoa leaves after three times single lesion and purified virus isolate Fr-T1 (Fig. 3). Electron microscopy test were continued for counting the average size (length and diameter) of 100 virions.

Rigid, rod-shaped particles with average length of 300 nm x 18 nm were seen in negatively stained preparations has constant results from infected frangipani leaves and purified virus isolate (Fig. 3). Particle morphology which has rod-shaped, usually straight with the size about 300 nm long and 18 nm in diameter are characteristic similar to FrMV [7,11,12].

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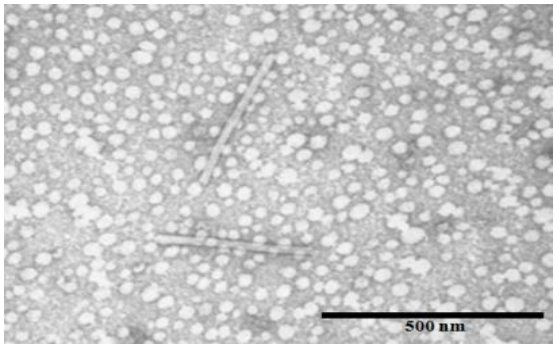

Figure 3. Electron micrograph of negatively stained (2% uranyl acetate) virus particles from crude sap of frangipani leaves showing mosaic symptom (Bar=500nm).

The virus were checked the absorbance value by spectrophotometer and showed the curve spectrum rises rather slowly as the wavelength decreases from 300 to 250 nm, and then rapidly as the wavelength decreases below 250 nm. The purified virus showed the typical UV spectrum of nucleoprotein with $A_{260}/_{280}$ value is 1.29 and maximum and minimum absorption at 260 nm and 249 nm, respectively (Fig. 4). There is also evidence that at least at wavelength longer than 250 nm, the virus protein and RNA is partially protected from damage by UV [13].

The molecular weight of coat protein of the virus was estimated 19.5 kDa by electrophoresis in sodium dedocyl sulfate polyacrylamide gel (SDS-PAGE) (Fig. 5). Lane 1 and 3 are healthy leaf samples (*C. quinoa* and field mosaic frangipani leaf) that did not show band on 19.5 kDa, but infected leaf samples (lane 2 and 4) were showing band with identical molecular weight compare to purified virus isolate Fr-T1 (19.5 kDa). This data is slighty different from known molecular weight of tobamoviruses genus, such as the coat protein of TMV has molecular weight of 17–18 kDa [14,15]. The slight difference of coat protein molecular weight between virus isolate Fr-T1 and other tobamoviruses may be as a result when draw horizontal line for comparing marker and samples bands on gel.

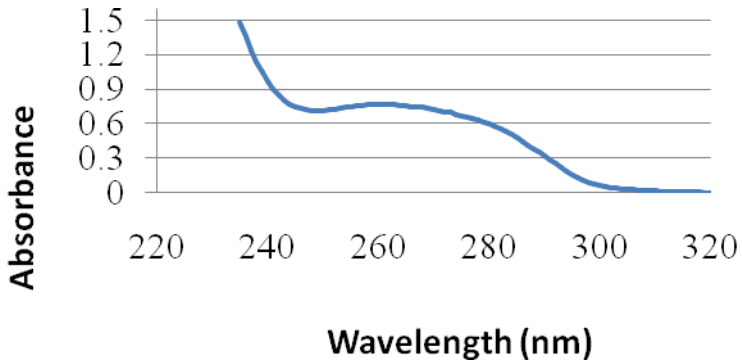

Figure 4. The ultraviolet absorption spectrum of purified preparation of virus isolate Fr-T1.

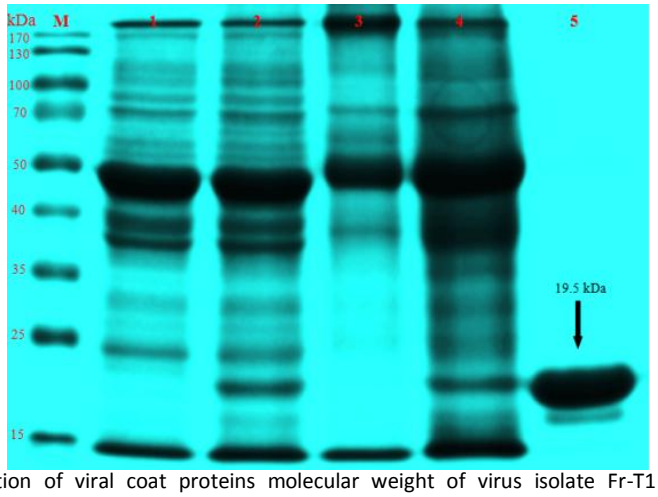

Figure 5. The determination of viral coat proteins molecular weight of virus isolate Fr-T1 by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). (M : Marker, 1 : Healthy *Chenopodium quinoa* leaf, 2 : Infected *Chenopodium quinoa* leaf, 3 : Healthy frangipani leaf, 4 : Field mosaic frangipani leaf, 5 : purified virus isolate Fr-T1).

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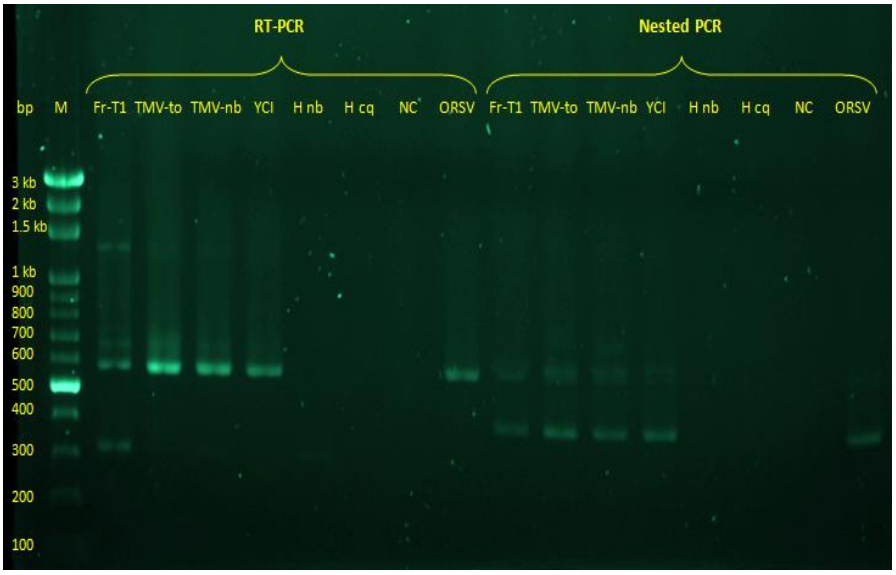

Figure 6. Agarose gel electrophoresis analysis of RT-PCR and Nested PCR products obtained from different tobamovirus isolates. (M : Marker, Fr-T1 : *Frangipani virus*isolateTaiwan 1, TMV-to : *Tobacco mosaic virus* infected tomato, TMV-nb : *Tobacco mosaic virus* infected *Nicotiana tabacum*, YCI : Yocai virus isolate from company, H nb : Healthy *Nicotiana tabacum,* H cq : Healthy*Chenopodium quinoa*, NC : Negative control, ORSV : *Odontoglossum ringspot virus*).

Agarose gel electrophoresis analysis of RT-PCR (Fig. 6 left) and Nested PCR (Fig. 6 right) products obtained from different tobamovirus isolates were conducted for detection and partial characterization of unknown species. The virus isolate Fr-T1 could be amplified by RT-PCR using degenerate primers TobRT up1 and TobRT do2 and resulted 568 bp. This length are similar comparing to other tobamoviruses isolates (TMV-to,TMV-nb and ORSV) [8].

Nested PCR assays using Fr-TI isolate from RT-PCR product yielded about 400 bp and this amplification products is similar as expected for all tobamovirus isolates tested (TMV-to,TMV-nb and ORSV), but not shown on control (Healthy *Nicotina benthamiana* and *C. quinoa*) (Fig. 7 right). Degenerate primers for detection of tobamoviruses by RT-PCR can be more efficient for the amplification of most members of a gene family, also allowing for the detection of new and unidentified virusspecies, followed by a nested PCR amplification that increased specificity and sensitivity of detection [8].

CONCLUSION

Based on these results, it was confirmed that the rigid rod-shaped virus isolated from mosaic symptom of frangipani leaves has length of 300 nm x 18 nm and could be identified as FrMV, belongs to *Tobamovirus*upon from its molecular (SDS-PAGE, RT-PCR, Nested PCR and nucleotide sequence comparison) characterizations. This is the first report that FrMV infecting frangipani (*Plumeria* sp.) in Taiwan.

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Extract of *Caesalpinia sappan* L. as Antibacterial Feed Additive on Intestinal Microflora of Laying Quail

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Abstract

Caesalpinia sappan L. is a phytobiotic plant that serves as an antibacterial. Active compound such as flavonoids in the *C. sappan* L. extract acts as an antibacterial. The protein food sources in Indonesia including poultry such as quail. Several studies demonstrated that quail are very susceptible to bacterial infections including *Escherichia coli* and *Salmonella*. Thus *C. sappan* has been used as food additive for quail in laying eggs. This research objective was to study the effects of *C. sappan* L. extract against intestinal miclofora of quail in laying eggs. This study used 168 quails with 4 treatments and 5 replications, each treatment used 7 quails. This study used 4 Wood Extract *C. sappan* L. treatment with different concentrations including 0%, 0.2%, 0.4%, 0.6%. The results showed that administration of Extract Wood *C. sappan* L. was not significantly different (P> 0.05) to the total number of bacteria *E. coli* with the average P_0 (6.0903 Log CFU), P_1 (6.0903 log CFU), P_2 (6.0887 Log CFU), and P_3 (6.0868 Log CFU). Meanwhile it was significantly different (P <0.05) to the total number of *Salmonella* bacteria in the gut laying with the average number of bacteria P_0 (5.4059 Log CFU), P_1 (5.4048 Log CFU), P_2 (5.4045 Log CFU), and P_3 (5.4039 Log CFU). It is concluded that flavonoids compounds in *C. sappan L.* extract could decrease the number of *Salmonella*, but not *E. coli*, in the intestine of quail.

Keywords: *Caesalpinia sappan* L., *Escherichia coli*, Flavonoid, *Salmonella*.

INTRODUCTION

Caesalpinia sappan L. is a medicinal plant that has been used as a healthy drink product in Indonesia. The boiled wood shavings of *Caesalpinia* is usually used and mixed with other ingredients/herbs serves as a hot drink. In the previous experiment, *C. sappan* L. qualitatively contained flavonoid [1]. Other active compounds included tannins, polyphenols, kardeno-lin, anthraquinone, sappan chalcone, caesalpin, resin, resonin, brazillin, d-alpha phallandren, osaemenan, and essential oil [2]. The active compounds of *C. sappan* L. have anti-inflammatory properties, antiproliteratif, anti-coagulants, anti-virus, anti-oxidants [3], immunostimulant, anticonvulsants [4] and anti-microbial properties [5]. Thus, the flavonoid of *C. sappan* L. could be used as a feed additive to modify intestinal microflora of quail at laying periods.

Quail is one of the productive poultry species which could produce 250-300 eggs per year [6], a nutritious protein source for human. Bad environmental condition and biosecurity cause quail is easily infected with pathogenic bacteria. The synthetic antibiotics are usually used to solve the problem. However, the prolong use of antibiotics may have a negative effect for the quail. The previous study reported that the long term use of antibiotics causes of bacterial resistant [7]. One alternative is to replace the use of synthetic antibiotic by using wood extract of *C. sappan* L. Therefore, the current research was intended to determine flavonoid content and effect of *C. sappan* L. on intestinal micro-flora of laying Japanese quail.

MATERIALS AND METHODS

Preparation of *Caesalpinia sappan L.* Extract

One kg powder of *Caesalpinia sappan L.* produced by UPT of Materia Medica, Batu Malang was diluted with 5 L of 90% ethanol. It was then macerated for 4 x 24 hours. Furthermore, the filtrate was separated, and extracted by using soxhlet destilation apparatus for 18 hours. Unlike what has been done previously [5], this current destilation was done once and the result obtained was 20 ml wood extract of *C. sappan* L.

Procedure to Determine Flavonoid Compounds in the Wood Extract of *Caesalpinia sappan L.*

Wood extract of *C. sappan* L.as much as 5 ml was heated for 5 minutes, then was added with a few drops of concentrated HCl and a little Magnesium powder, stirred well by hand. A

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positive test result was indicated by the appearance of a dark red or pink.

Experiment on Intestinal Microflora of Quail

This materials of study consisted of 168 laying quails of 10 weeks old, wood extract of *C. sappan* L. In addition, some feed ingredients also used namely yellow corn, soybean meal, fish meal (CP 50%), MBM, $CaCO_3$, Methionine, L-Lysine, Coconut Cake meal, Pollard, Premix, and Di Calcium Phospate. The result of proximate analysis of feed used in the study was described in Table 1.

Table 1. Feed Chemical Analysis Test Results

Chemical contents	Contents
Crude Protein	21.90 %
Gross Energy	3692 Kcal/Kg
Fiber	3.18 %
Fat	6.51 %
Calsium	2.55 %
Phospor	0.70 %

Source: Division Livestock Nutrition Laboratory of Blitar (2017).

The feed was given twice a day, morning and evening, with the respective proportion of 40% to 60%. The feed was offered 26 $g.bird^{-1}day^{-1}$, while water was given *ad libitum*. The used method was experiment, arranged in Completely Randomized Design (CRD) with 4 treatments and 5 replications. Each treatment consisted of 7 quails. The treatments were quail as follows:

P_0 = Basal feed (Control)
P_1 = Basal feed + 0.2% extract of *C. sappan* L.
P_2 = Basal feed + 0.4 % extract of *C. sappan* L.
P_3 = Basal feed + 0.6% extract of *C. sappan* L.

Variables observed in this study were the total number of *Escherichia coli* and *Salmonella* taken from ileum of quail at 16 weeks of age. The numbers of bacterial colonies were counted by using Gariga method [8].

Data Analisys

Data were statistically analyzed by ANOVA test of Completely Randomized Design and if significantly different effect appears then followed by Duncan's Multiple Range Test.

RESULT AND DISCUSSION

Qualitative Determination of Flavonoid

Qualitative analysis of flavonoid compounds showed that wood extract of *C. sappan* L. positively contained reasonable concentration of flavonoid indicated by appearance of dark red color. The previous study also reported similar result [1], and also reported that strong antioxidant activity of extracts of *C. sappan* L. is positively correlated to the concentration of total phenol and flavonoid. It is showed in Figure 1.

Figure 1. A dark brown colour showed when detected flavonoid was qualitatively analysis

Effect of Treatment on Microbial Population

Effect of different levels of wood extract of *C. sappan* L. on microbial population in the quail intestinal tract was described in Table 2.

Table 2. Effect of Treatment on *Escherichia coli* and *Salmonella* Population

Treatment	Total Colonies of Bacteria (Log CFU)	
	Escherichia coli	*Salmonella*
P_0	6.090 ± 0.003	$5.406 \pm 0{,}001^a$
P_1	6.090 ± 0.003	$5.405 \pm 0{,}001^b$
P_2	6.089 ± 0.002	$5.404 \pm 0{,}001^c$
P_3	6.087 ± 0.003	$5.404 \pm 0{,}001^c$

Description: different Superscrib in the same column indicated significant different (P<0.05)

Feeding different levels of *C. sappan* L. wood extract as feed additive did not significantly change (P>0.05) total colonies of *E. coli*, but significantly decreased (P<0.05) the number of *Salmonellla* in ileal digesta of quail.

The result data showed that extract of *C. sappan* L. could decrease total colony of *E. coli* and *Salmonella*. The total decrease of bacterial colony is caused by flavonoid compound in *C. sappan* L. [3]. Flavonoids undermine the permeability of bacterial cell membranes that result in the membrane sitplasma into lysis [9].

Effect of Graded Levels of *Caesalpinia sappan L*. Wood Extract on Population of *Eshericia coli*

The result presented in Table 2 showed that the respective average values of TPC of *E. coli* from the highest to the lowest, i.e. P_0, P_1, P_2 and P_3. The highest number of colonies of *E. coli* was for P_0 (6.090 ± 0.003 Log CFU) and the lowest was for P_3 (6.087 ± 0.003 Log CFU). Statistical analysis showed that wood extract of *C. sappan* L. did not

significantly change (P>0.05) the population of *E. coli*.

Theoretically, the antibacterial compound of flavonoid in the wood extract of *C. sappan* L. could combat the pathogenic bacteria such as *E. coli.* But the research data in Table 2 showed no reduction in *E. coli* population in the small intestine of quail. One of the reasons was the cell wall of *E. coli* is thicker than *Salmonella*, leading to difficulty in disrupting the wall because flavonoids could not penetrate the cell wall of *E. coli* [10]. Lipid content of the cell wall of *E. coli* (11-24%) was thicker than than of *Salmonella* (1-4%) may contribute to the difficulty of flavonoids to penetrate the cell wall. So, it might need higher concentration of flavonoid to kill *E. coli* [10]. This is also supported bu another study that formation of inhibition zone against *E. coli* because of giving the same concentration of *C. sappan* L. wood extract solution was smaller than that of *Salmonella* (9.0 ± 0.7 mm vs 20 ± 1.3 mm) [11].

Effect of Graded Levels of *Caesalpinia sappan L*. Wood Extract on population of *Salmonella*

Table 2 showed that the use of wood extract of *C. sappan L.* as quail feed additive significantly increased (P <0.05) the number of colonies of *Salmonella* in the intestine. The averages of colonies of *Salmonella* were consecutively P_0 (5.406 ± 0.001^{a}); P_1 (5.405 ± 0.001^{b}); P_2 (5.405 ± 0.001^{b}); and P_3 (5.404 ± 0.001^{C}). The results showed that the highest level of wood extract of *C. sappan* L. gave the lowest number of colonies of *Salmonella* in the intestine of quail. This indicated that at the highest concentration of the extract which also means highest level of flavonoid, could effectively disrupt the cell wall of *Salmonella* leading to decrease its population in the intestine of laying quail. Changes in organic components and transport nutrients metabolism by the bacteria occurred due to disruption of the hydroxyl groups of flavonoid compounds. Flavonoid compounds also inhibit topoisomerase II (DNA gyrase) enzyme, an important enzyme in the process of bacterial DNA replication and transcription [12].

The decline in the number of colonies of *Salmonella* bacteria in the intestine caused by the content of quail flavonoids, phenols, and brazilin on wood extract *C. sappan* L. flavonoids decrease the number of colonies of bacteria by destroying the bacterial cell wall, microsomes and lysosomes as a result of the interaction of flavonoids with bacterial DNA. Flavonoids inhibit the growth of bacterial cells by disrupting the bacterial cell nutrient transport processes that lead to decreased metabolism so that the proliferation and growth of bacteria becomes inhibited [13]. Flavonoid activity can damage the cell wall of bacteria is also due to be lipophilic, which work to form complex bonds with extracellular proteins [14].

CONCLUSION

It is concluded that wood extract of *Caesalpinia sappan* L. is qualitatively contains flavonoid and showed antibacterial effect due to ability of reducing colonies of *Salmonella* in the laying quail intestine. This may attribute to the flavonoid may act to disrupt cell wall of *Salmonella*.

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The Effect of Electroporation Method towards the Motility and Viability of Java Barb Fish (*Puntius javanicus*) Sperm

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Abstract

Electric shock treatment of Java Barb Fish (*Puntius javanicus*) sperm using electroporation method on sperm as transfer gen (Sperm Mediated Gen Transfer) has not been implemented in Indonesia. This study was conducted to know the effect of electric shock using gene pulser at different voltage level toward motility and viability of Java barb fish sperm. This research was conducted at Fish Breeding Labroratory, Faculty of Marine and Fisheries and *LSIH* of Brawijaya University-Malang in May 2017. The trial design used Complete Randomized Design with 3 different treatments and 1 control which each treatment repeated 3 times. The treatments used the Gene pulser with 3 different voltages: A (20 V), B (30 V), C (40 V). The result showed that the electric shock treatment with different voltages level affected motility and viability of Java barb fish sperm. Based on the data analysis used polynomial orthogonal, a linear-form of the relationship among the treatments in the form of equation was found (*Puntius javanicus*) with R^2=0.9815 and equation y = -40.5x + 130.83 with R^2= 0.8626. Based on the result of this research, electroporation for Java barb fish' sperm as gene transfer media should be done with voltage 20 V, pulse number 4 times and pulse length 1 ms.

Keywords: electroporation, motility, *Puntius javanicus,* sperm, viability.

INTRODUCTION

Sperm is spermatozoa fluid found in the seminal fluid produced from the testicle hydration or a part of fish' reproductory organ [1]. The amount of sperm produced by male fish has various volume and quality which are influenced by the age, size and the ejaculation frequency [2].

Java Barb fish belongs to the commodity of freshwater fish as the member of the silver carp family. The spermatozoa of silver carp fish have length around ±24 µm. It has round head with the length of ±1.5-2 µm and width of ±1.5-1.8 µm [3].

The motility and viability of sperms are important parameters that determine the success of fertilization process. Sperm motility shows the ability of the spermatozoa in fertilizing the ovum. The higher the motility value, the higher the survival percentage (viability) of the spermatozoa [4].

Gene transfer technology in Java Barb Fish (*Puntius javanicus*) using sperm as a gene transfer media has not been implemented in Indonesia. Microinjection was commonly used as gene transfer method. One of the weekneses of this method is take a long time process. The eggs must be injected one by one and not practical to mass produce fish, further needs another alternative that is electroporation method. The advantages of electroporation methods can produce mass transgenic fish and electroporation methods can be combined with Sperm Mediated Gen Transfer (SMGT), where sperm was used as a transfer media. Further research is needed on the effect of electric shock treatment of fish sperm through electroporation method. Previous study added the advantages of the electroporation method that can insert most of the DNA copies into the recipient fish genome. Electroporation method can be combined with Sperm Mediated Gen Transfer (SMGT), where sperm as its transfer media [5].

Electroporation method is a gene transfer method using a set of short electric shocks to stimulate temporary pores to grow within the phospholipid bilaer. After being given electric shock, the pores of the cell membrane will be tightened again [6]. Factors that influence the transfection efficiency using the electroporation method are the voltage, duration, temperature, DNA adjustment, DNA concentration and ion composition within the transcription mediators [7]. Electric shock given to muscle tissue has two effects which are; changing the structure of

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muscle tissue permeability, besides electroporation also helps transferring the DNA throughout the membrane permeability [8]. The objective of this study was to see the effect of the electric shock given, using the gene pulser at different voltages toward the motility and the viability of Java Barb Fish' sperm.

MATERIALS AND METHODS

The experiment design used in this research was the complete random design. There were 3 treatments and 1 control in which each was repeated 3 times. The treatments employed in this study were:

Treatment A = electric shock at 20 V
Treatment B = electric shock at 30 V
Treatment C = electric shock at 40 V
Control = without any electric shock

Determination treatment with different voltages level (20 V, 30 V, 40 V) in this study based on the result of previous research on Japanese carp (*Cyprinus carpio var.Koi*) sperm using electroporation method with voltage level 30 V [9]. In each of the treatment, the electric shock was given for 1 ms repeated 4 times, except for the control [9].

Preparing the Sperm Striping

Preparation on the male fish with mature gonad was to produce the sperm. The male fish with mature gonads were then measured its length and weight. The sperm was taken using the stripping method which was later kept in the eppendorf. The sperm was then diluted by adding it up with physiologic fluid at the comparison 1:1. Then, the researchers prepared the electroporation tools [10].

Electroporation Process

Total 25 µl of sperm was put into the cuvette, before the cuvette was put into the shock pod. After that, the voltage of *square wave* was chosen and adjustment on the strength of the electricity, shock duration and the shock frequency were made before the *pulse* button was pushed to start the shock. This treatment was repeated 3 times for each of the voltage [9].

After being shocked with the electricity, the liquid sperm was added with Physiologic Natrium in the cuvette. After theat, the sperm was poured into the appendorf and each of the 25 µl of sperm was observed to see its motility and its viability [9].

Research Parameters

Motility of the Spermatozoa

Spermatozoa motility is a beneficial parameter that is used to predict the life time of the spermatozoa. Alive sperms are the ones that show fast movement, slow movement or show any movement on their heads or tails. Meanwhile, dead sperms are those that do not show any movement either in the head or in the tail [11].

The percentage of motile sperm is measured using a microscope which is completed with video recorder. Motile sperm is counted when the sperm show progressive movements. Sperm that shows no movement or sperm that goes around only in certain point are considered dead ones. The scoring (Table 1) was made based on the method proposed by McMaster [12]. To assure the validity of the data and to assure the objectiveness, the score was only given after observing the video with some repetitions [12].

Table 1. Criteria of Sperm Motility Categorization

Criteria	Score
Very poor (only 0-20% Progressive motile)	1
Poor (only 20-40% Progressive motile)	2
Good (only 40-60% Progressive motile)	3
Very good (60-80% Progressive motile)	4
Excellent (80-1000% Progressive motile)	5

Source: McMaster [12]

Viability of the Sperm

The viability of the sperm was observed using a technique that employed the eosin hue and negrosin. A small drop of sperm and a drop of eosin negrosin fluid were put in an object glass to be mixed until it looked homogeneous. After that, in a petri dish, the sperm was heated in order to drain it which process lasted for 15 seconds. The sperm was then observed using a microscope at 400x magnification [13].

$$\text{Sperm viability} = (\sum \text{live sperm} \times 100\%)/200$$

Data Analysis

The data obtained in this study were statistically analyzed using the homogeneity test (ANOVA) in accordance to the complete random design used in this study. Based on the variance of the data, it is found that there was an obvious significant difference up to highly significant difference after the treatment. Thus, in order to compare the values of each treatment, a Least Significant Difference and regression test were administered.

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RESULT AND DISCUSSION

Spermatozoa Motility

The result of the motility test administered to the spermatozoa of the Java Barb fish in each of the treatment after the electroporation and after the repetition is presented in Table 2. Based on the data of the average motility value, it is shown that the highest motility percentage was found in the control group (without the electroporation) at an average of 5%. Meanwhile, the highest motility percenteage among the experiment groups was found in the treatment A (20V) at the percentage of 3.67%, while the lowest one was found in the treatment C (40V) at 0.17%. When the motility of the control group compared to the motility of the treatment group, a decline in the values occurred as presented in Figure 1.

Table 2. Spermatozoa Motility (%)

Treatment	Repetition				
(%)	1	2	3	Total	Average
A	4	3	4	11	3.67
B	2	2	3	7	2,33
C	0.5	0	0	0.5	0.17
Total				18.5	
Control	5	5	5	15	5

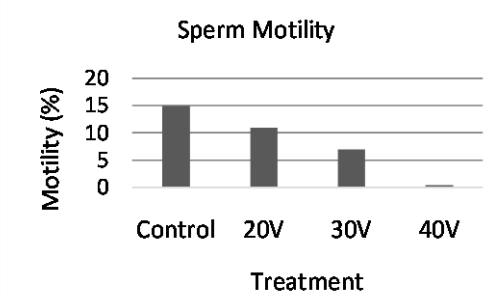

Figure 1. Sperm Motility Diagram

Figure 1 shows that the electroporation treatment using electric shock influence the motility of the fish' sperm. The higher the amount of the electric shock given, then lower the motility of the fish. The voltage and the shock frequency and the biological parameters of the sperm are able to influence the effectiveness, while the shock duration influences the efficiency of the gen transfer using certain DNA concentrate. The motility of the sperm tends to decline as the voltage increases and longer shock duration [14]. Previous study highlighted that the electric shock given to the muscle tissue plays two roles; changing the permeability structrure of the muscle tissue, while the electroporation helps the DNA transfer to pass through the membrane permeability. Membrane permeability of the spermatozoa is highly reated with the spermatozoa motility since the membrane permeability has a major role in the nutrition transportation in the cell metabolism process. The sperm cells tend to shirnk after the electroporation which explains the declining percentage of the sperm motility [8].

Based on the result of variance test presented in Table 3 significant differences were found. This shows that different voltage gives different influence toward the percentage of the sperm motility which implies that the H_1 is accepted and the H_0 is rejected. After that, Least Significant Difference test was administered which result is presented in Table 5.

Table 3. Result of Variance Test on the Sperm Motility Percentage

Source	df	SS	MS	F	F 5%	F 1%
Treatment	2	18.72	9.36	37.44*	5.14	10.92
Random	6	1.50	025			
Total	8					

Notes: * = F 5% < F value > F 1 %

Table 4. Least Significant DIfference Test

Treatment	Average	C	B	A	Notation
		0.17	2.33	3.67	
C	0.17	-			a
B	2.33	2.16	-		b
A	3.67	3.50	1.34	-	b

Notes: Similar notes shows no difference

The result of the Least Significant DIfference test shws that the treatment A (20V) has the best motility followed by the treatment B (30 V) and treatment C (40 V). The highest motility value was obtained by the treatment A for the sperm cells were in the most optimal condition compared to the sperm cells in treatment B and C which were treated with higher voltage, making the the motility value declined.

This is different from the results of Japanese Carp (*Cyprinus carpio var.Koi*) Luthfiyah *et al*. [15] which obtained the best results on 10 V treatment with motility score 4 and the lowest at 40 V with motility a score of 0.5 [15]. The treatment in this study without using level voltage (10V) because of based on the result from previous research in carp fish (*Cyprinus carpio*) by electroporation method on sperm with the best result treatment was voltage level (30V) [9]. Further, the motility and viability of Java barb fish sperm using treatment with voltages level among

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the previous treatment (20 V 30 V 40 V). Sperm motility after the electroporation process relies on the voltage, shock length, shock frequency, and the buffer ionic power [16].

Furthermore, the polynominal orthogonal test resulted to a linear equation y = -1.75x + 5.5556 with a correlation coefficient of 0.9815 which implies that different voltages and motility has a strong correlation with the treatment given as presented in Figure 2.

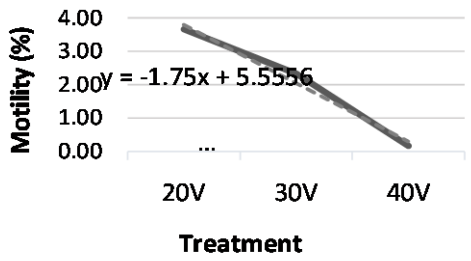

Figure 2. Relationship between Different Voltages and the Sperm Motility

Figure 3 shows that the treatment resulted to a decline on the sperm motility duration as the voltage got higher compared to the sperm in the control treatment.It implies that the the cell sperm which is given short electric shock stimulates the growth of temporary pores in the bilayer phospholipid of the cel membrane that allows the penetration of new DNA into the cell. After the electric shock, the cell membrane will get tightened again [6].

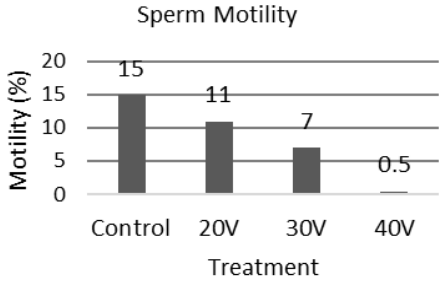

Figure 3. Motility Duration of the Sperm in the Control Group and after being given with Treatment

Based on this theory, food fluid exhance with outside the cells may occur for the metabolism of the sperm which results to the declining motality duration. This is due to the fact that the sperm need energy to make movement. Fructose fluid as the dissolver to the fish' spermatozoa are intended to supply energy and nutrition for the spermatozoa to use the energy in the form of ATP to increase and prolong the spermatozoa motility duration [17].

The condition of the fish' sperm determines the success of the fertilization. Strong sperms are able to fertilize the egg successfully. Changes that occur to the sperm will influence the quality of the sperm. The changes include the motility and the viability of the sperm. Good seminal fluid for the fertilization process should consist of lots of live sperms that move forward progressively. The percentage of the motile sperm should be more than 75%. For daily use, the sperm motility should not be less than 50% even some researchers suggest that it should be more than 60% [18].

Spermatozoa Viability

The result of the viability measurement of the Java Barb fish' sperm after the electroporation using different voltages can be seen in Table 5. Based on the observation, it is found that the highest viability percentage was found in the control group (without electric shock) at 85.66%. Meanwhile, the highest percentage among the treatment group was achieved by the treatment A (20 V) at 81.00% and the lowest one was fond in the treatment C (40 V) at 00.00%.

Table 5. Spermatozoa Viability

Treatment	Repetition			Total	%
	1	2	3		
A	81	83.5	78.5	243	81.00
B	43	88.50	74	205.5	68.50
C	0	0	0	0	0.00
Total				462.5	
Control	77	86	94	257	85.66

Figure 3 shoes that the percentage of the sperm viability declined in each treatment. Furthermore, the result of the variance test (Table 6) shos that different voltages give different influences toeward the fish' sperm viability. Therefore, the H_1 is accepted.

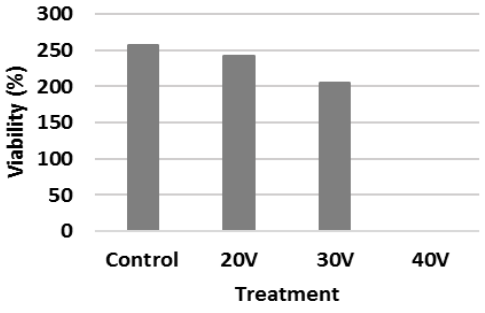

Figure 3. Sperm Viability in the Control Group after being given Electric Shock

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Table 6. The Result of Variance Test on Sperm Viability

Variance source	df	SS	MS	F	F 5%	F 1%
Treatment	2	11409.5	5704.75	31.31	5.14	10.92
Random	6	1093.00	182,16			
Total	8					

The result of the Least Significant Difference test shows that the treatment A (20 V) contributes the highest sperm viability value followed by the treatment B (30 V) and C (40 V). The highest viability value was found in the treatment A since the sperm cells were still in the best condition compared to the sperm in treatment A and treatment C for higher voltages decreases the motility value. This finding goes in line with Sin *et al.* (2000) who stated that the sperm motility is the indicator of the sperm viability after the electroporation, in which higher voltages and longer shock duration decrase the sperm motility [14].

Table 7. Least Significant Difference Test

Treatment	%	C	B	A	Note
		0.00	68.50	81.00	
20V	0.00	-			a
30V	68.50	68.50	-	-	b
40V	81.00	81.00	12.50	-	b

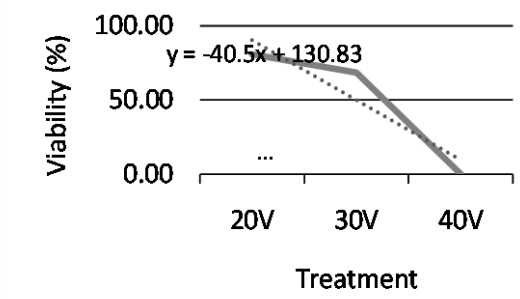

Figure 4. Relationship between Different Voltages and Sperm Viability

The result of the Least Significant Difference test shows that the treatment A (20 V) achieved the highest sperm viability value followed by the treatment B (30 V) and treatment C (40 V). In addition, based on the result of the polynomial orthogonal test, an equation has been obtained in which y = -40.5x +130.83 with correlational coefficient (r) at 0.8626 which implies that different voltages and sperm viability share with the treatment given to the sperm as illustrated in Figure 4. This is in accordance with the results of the study Luthfiyah *et al*. [15], based on the observation of motility and viability on post electroporation sperm, it is known that the increase of voltage (electric voltage) given at the time of sperm electroporation affects the decrease in the intensity of movement and ability of sperm to survive. It can be seen from the observation result E (40 $V.cm^{-1}$), where the value of motility score was only 0.5 and viability was 25.4%. That is because electroporation of sperm gives a very real effect on the biological and physiological conditions of sperm cells. This happens because an excessive electric shock can cause the sperm cell to lose its elastic properties. Therefore, in the activity of gene transfer using electroporation method, optimization is necessary to know the ability of sperm in becoming gene transfer vector [15].

The decrease on the sperm viability is mostly caused by the electric shock given to the sperm causes the pores to open way too wide, making the pores unable to get tightened like before. This causes the cells to break and triggers damages to the sperm membrane [19]. The membrane permeability of spermatozoa has a strong relationship with the spermatozoa biablity sicne membrane permeability has a major role in the nutrition transportation within the cell metabolism process [11].

It has been known that the percentage of the sperm viability determines the quality of the sperm. It means that higher amount of live sperm determines the success of the fertilization. The minimum percentage of spermatozoa cell within the sperm should not be less than 70%. The higher the amount of the sperm viability, the better the ability of the spermatozoa to pass through the micropile holes within the ovum [20].

CONCLUSION

The experiment on the use of electric shock using the electroporation method toward the Java Barb fish' sperm shows that the electric shock has a significant influence toward the motility and the viability of the sperm. To obtain the best effectiveness level of the motility and the viability of the sperm using the electroporation method, it is suggested that breeders use 20 Volt electric shock with 4 times of repetition in which each of the shock lasts for 1 ms. Further research is needed for sperm motility and viability of Java barb fish with electroporation method using 10 Voltage levels.

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A Solid Waste Pond Tiger Shrimp (*Peneaus monodon*) as Fertilizer for *Caulerpa lentillifera*

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Abstract

Farming the shrimp, fish and another comodity could produce large quantities of waste. Aquaculture waste can be formed as feces, residual feed and dead organism which are accumulated in a cultivation area. Generally, the waste is discharged directly into the water without filtration. Thus, one of problems that are often faced by farmer is the low quality of pure water due to the high content of nutrients. Moreover, suspended solid pollutant will be formed that lead to eutrophication, oxygen depletion, and precipitation. The aim of this research is to analyze the solid waste pond tiger shrimp as fertilizer for the growth of *Caulerpa lentiilifera*. Here, the Random Design complete (RAL) and three times in Deuteronomy are used in the experiment. The parameters of one control and three treatment doses are used 0, 2, 4, and 6 $g.L^{-1}$ respectively. The results showed that solid waste shrimp ponds can be used as fertilizer to meet the needs of *Caulerpa lentillifera* for growth and the results show the highest value is found in the dose of 6 $g.L^{-1}$ with NO_3^- (4.58 ppm), NH_4^+ (3.34 ppm), PO_4^{3-} (2.03 ppm) and the value of the rate of growth and the PH are obtained (3.64 $g.day^{-1}$) and (6,4-8), respectively.

Keywords: *Caulerpa lentillifera,* growth rate, Nitrification, solid waste, Tiger Shrimp.

INTRODUCTION

Aquaculture waste is waste generated from a shrimp or fish farming that can cause pollution in the aquatic environment if not immediately addressed. Cultivation of waste could be remnants of the digestion of fish or shrimp or leftover feed that settles on the bottom. The amount of waste goes to the water is linear to the production obtained. The waste could cause environmental problems, related to the magnitude of the amount of nitrogen (N) and phosphorus (P) which are dumped into waterways [1]. For instance, farmed shrimp in Australia estimate the amount of N and P produced was 290 and 16 $kg.ha^{-1}year^{-1}$. Whereas farmed shrimp in California estimate the amount of N and P produced amounted to 112 and 32 $kg.ha^{-1}year^{-1}$ [2]. While the amount in Indonesia, N and P produced from intensive and traditional ponds reached 399 and 37 $kg.ha^{-1}year^{-1}$ [3].

To overcome this problem, this research focused on making a fertilizer using the solid waste of shrimp. Since the solid waste ponds of shrimp contains 1.92% organic C, 0.54% N total, and 1.70% P [4]. Moreover, the solid waste can be used by plants to grow as organic fertilizer, since plants need NH_4^+ and NH_3^- amino acid in the formation. In his role as fertilizer, solid waste ponds shrimp will be used for cultivating *Caulerpa lentiilifera*.

Caulerpa lentiilifera is often called the sea grape is a type of seaweed green (*Chlorophyta*) that can survive in environments that have high pollution levels [5]. Cultivation of *C. lentillifera* has started developing in indonesia, because it can be eaten fresh and also it rich of nutrients that are good for the human body. This sea grape type can also be used as ingredients in cosmetics and pharmaceuticals. In addition, Sea grape types of *C. lentillifera* are highly favored by some countries such as Japan, Australia, and Fillipina [6].

The idea of this research is based on nitrification process. Nitrification is the process by which ammonia is converted to nitrites and then nitrates by the assistance of *Nitrosomonas* and *Nitrobacter* bacteria under aerobic conditions. The conversion process of organic material by heterotrophic bacteria in the laboratory has occurred within 1-2 days, while nitrification and De-nitrification process lasts for 2-6 days [7]. Microbes decompose organic matter in the system, causing an increase in the value of TAN (Total Ammonia Nitrogen) and nitrite, are both harmful for fish even at low concentrations [8]. TAN presence in the system can be changed to nitrite, nitrate and nitrogen

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gas [9]. Formation of nitrogen gas is considered negligible in pond aquaculture [10]. The bacteria present in the water and sediment to transform nitrogen through nitrification and denitrification [11]. Thus, this study aims to grow *C. lentillifera* by analyzing the content of nitrate, ammonium and phosphate in solid waste of tiger shrimp pond *(Peneaus monodon*) to be used as fertilizer.

MATERIALS AND METHODS

The research flow of this paper can be seen in the Figure 1.

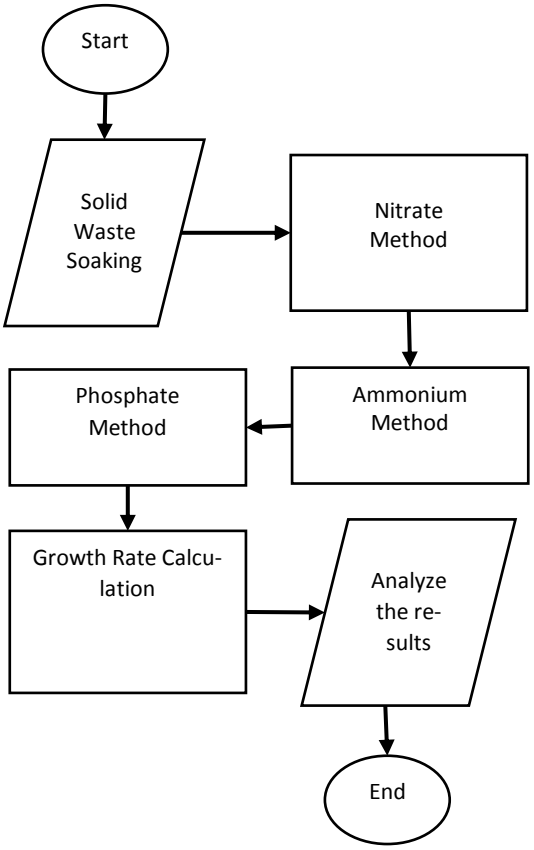

Figure 1. The research flow

Solid Waste Soaking

Solid wastes were obtained from BBPBAP (*Balai Besar Perikanan Budidaya Air Payau*) Jepara. The waste was taken at the time of recirculation process in the ponds by using Multi Cylone 16. Then, the waste was dried. After that, the dried solid waste was weighed according to the dosage that will be used in this research, which are 0 $g.L^{-1}$, 2 $g.L^{-1}$, 4 $g.L^{-1}$, and 6 $g.L^{-1}$. Administration of doses [12] has been modified.

Waste that had already been weighed would be soaked in the controlled tub (aquarium) that already contained sea water, then it was left for 48 hours to observe the content of nitrification [4].

Nitrate Method

The method of Nitrate analysis was appropriate [13], 12.5 mL water samples were taken and put in a porcelain cup. The sample waters were heated using hot plate until the crust appeared on the porcelain cup. Fenoldisulfonik acid solution of 1 ml were added to the crusted porcelain cup. After that, 2 ml of distilled waters were added into a porcelain cup and then the crust on a porcelain plate was scraped with a spatula. NH_4OH solution was added to the porcelain cup until the crust turned into stable yellow, next the distilled waters were added into the porcelain cup until volume reached 12.5 mL (initial volume). The samples were poured into cuvettes then and nitrate concentration was measured by UV Visible with a wavelength of 410 nm [14].

Ammonium Method

Methods of analysis in accordance with ammonium SNI 06-2479-1991 use the following steps. Water samples taken as much as 12.5 mL and put into a beaker glass 50 mL. Solution of 0.5 ml Nessler as much added to in a beaker glass, a beaker glass then is shaking in order to make the perfect solution and let sit for approximately 30 minutes. A sample cuvet is then inserted into the measure by using UV Visible Spectrophotometer with a wavelength of 425 nm [15].

Phosphate Method

Phosphate levels can be calculated in spectrophotometry, the steps undertaken in this analysis is the water sample taken approximately 50 mL and then inserted into a beaker glass. Added 1 drop indicator phenolftalin (in case of change of color to pink, then added H_2SO_4 drop by drop until the color is gone). Added 0.5 gram $K_2S_2O_8$ and boiled on top of the hot plate until the remaining volume ± 10 mL. Samples are cooled and diluted with aquades up to volume 30 ml. Next, added 1 drop indicator phenolftalin and neutralized with NaOH to pink color is shown. Again, melted H_2SO_4 until the pink color is vanished, then diluted to 100 mL. Furthremore, 50 mL of volume is takken into a measuring flask. Moreover, added 8 ml reactant combination, and then wait only for 15 minutes. Finally, the sample is inserted into its absorbance and measured with cuvet spectrophotometer with a wavelength of 880 nm [16].

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Growth Rate Calculation

Growth during the study calculated based on the difference between the weight at the beginning of the study with a weight at the end of the research. The growth of sea grapes daily for 1 month cultivation can be calculated using the following formula [17]:

$$g = \frac{Wt - W0}{t}$$

Description:
Wt = weight of seedlings at the end of the study (g)
W0= weight of seed research (g)
g = daily growth ($g.day^{-1}$)
t = the number of days the experiment (h)

Data analysis

Analyze the life data using variance (ANOVA). The analysis was used to examine the effect of treatment, followed by the smallest real difference test (BNT) at the 5% test level.

RESULT AND DISCUSSION

Nitrate (NO_3^-),Ammonium (NH_4^+) and Phosphate (PO_3^-) Content

From the soaking results, the higher waste dosage the higher Nitrate value. The detail can be seen in the Table 1 below.

Table 1. Nitrate, Ammonium and Phosphate Content (ppm) on different content

doses ($g.L^{-1}$)	NO_3^-	NH_4^+	PO_3^-
0	2.64	1.63	0.73
2	4.33	2.89	1.62
4	4.50	3.22	1.85
6	4.58	3.34	2.03

Based on Tabel 1, it was obtained that the Nitrate content was different in each treatment, where various values can be caused by several factors. The factors that may affect the decomposition of organic substances were C/N ratio, pH, temperature and DO [18]. Comparison of Nitrate content, Ammonium and Phosphate is the basis of water quality, where the highest Nitrate value reaches 4.58 ppm. Comparing with standard quality of water quality, it is classified as polluted, because the tolerance limit of nitrate value in pond is not more than 0.5 ppm [19]. Nitrogen in seawater consists of various compounds, but there are only three compounds containing toxic for fish and other organisms, namely ammonia (NH_3^-N), nitrite NO_2^-N and nitrate (NO_3^-N) [20]. However, for the crop, the high value is needed which will be a good nutrient for growth *C. lentillifera* [21].

Here, the value of ammonium with the largest value of 3.34 ppm is obtained. It is a very good value for growth Caulerpa lentillifera. Because ammonium is needed for the process of protein formation in plants, ammonium serves as a support when plants lack N elements in the water. This ammonium is classified as a form of ammonium nitrogen [22]. Absorption of nitrogen by plants can be almost entirely in the form of ammonium or nitrate. Ammonium is used to be the main source for the growth in agriculture and most of the natural environment.

Meanwhile, the value of phosphat with the highest value of 2.03 ppm is observed. This means water has a very high fertility rate, because waters with high fertility levels have phosphate levels of 0.51-1 ppm, medium fertility levels have levels of phosphate 0.2-0.5 ppm and a low fertility rate of 0-0.2 ppm. The main function of the phosphate element is to accelerate the growth of the root semia, accelerate and strengthen the growth of young plants into adulthood, accelerate flowering and ripening and increase seed production [23].

Growth Rate of *Caulerpa lentillifera*

The results obtained by the heavy growth of sea grapes are shown in Table 2. Table 2 is the results of the average daily growth, where this growth indicates the solid waste can be used as fertilizer for each treatment. The difference of growth is due to the absorption of nutrients in each treatment differently. In the C treatment where the nutrient (Table 1) produced is greater than the other treatments resulting in an average daily growth 3.64 $g.day^{-1}$. Whereas in the treatment K only 0.18 $g.day^{-1}$. Therefore, the greatest growth obtained in the treatment of C which is needed more than other treatments.

Table 2. Growth Rate of *C. lentillifera* During Cultivation

Doses Treatment ($g.L^{-1}$)	Average growth ($g.day^{-1}$)
K (0)	0.18
A (2)	0.63
B (4)	1.87
C (6)	3.64

The growth increases along with the increasing of the dose given (Fig. 2). The growth of Sea Grape can be accelerated by the addition of fertilizers in cultivation. Growth could be spurred by the addition of elemental Nitrogen (N) and Posphate (P), since both of these elements are the essential nutrients for algae [24].

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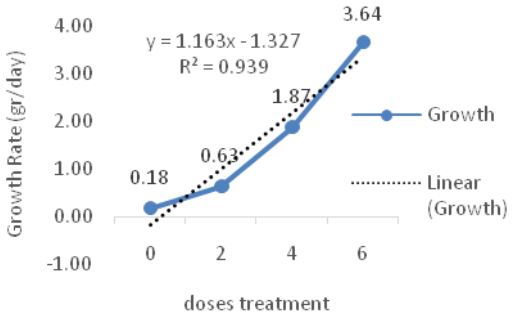

Figure 2. Growth Rate of *Caulerpa lentillifera*

Figure 2 shows the daily growth rate of *C. lentillifera* using various doses treatments. This can be proved that the difference of doses of solid waste ponds shrimp produces various growth rates of seedlings of sea grape (Fig. 2). Here, the higher dosage gives the growth rate increasing. Moreover, use the regression the function is obtained $y(x) = 1.1631x - 1.3274$, where $y(x)$ and x denote the growth rate functions and doses treatment respectively.

pH Water

Based on the observations that have been made during the study, the range of the pH is 6.4 – 8. The degree of acidity is good for the growth of algae is between 6-9 with optimal range 6.3-8.2 [25]. This condition describes that any algae have different torelansi towards a pH. According to [26] States that the increase in pH values will affect the lives of algae and aquatic tendencies have a high acidity level due to the entry of large quantities of organic waste. The number of nitrification is very closely related to the pH levels. The longer soaking nitrates will soften that also comes with a decrease in pH. PH changes depending on the process of amonifikasi and nitrification of nitrogen into ammonium and nitrate. The reaction of nitrate formation will free H^+ resulting pH in being dropped [27].

CONCLUSION

The results showed that solid waste shrimp ponds can be used as fertilizer to meet the needs of *C. lentillifera* for growth. Where nutrient obtained from the immersion of nitrate, ammonium and phosphate in the treatment with a doses of 6 $g.L^{-1}$ has a good value to grow *C. lentillifera*.

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Growth Parameter and Fecundity of Fringe Scale Sardine (*Sardinella fimbriata* Cuvier Valenciennes) in Alas Strait, East Lombok, West Nusa Tenggara

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Abstract

Fisheries in the Alas Strait, East Lombok, West Nusa Tenggara is one of important sea area for the activity of fishing, especially small scale fishermen. In addition, the Alas Strait have a good fishery for catching pelagic fish species of Fringe Scale Sardine (*Sardinella fimbriata*). The aim of this study was to asses the growth parameter and fecundity of Fringe Scale Sardine (*S. fimbriata*). The research was conducted on August to December 2016, at the Fish Landing Base Tanjung Luar, East Lombok, West Nusa Tenggara. Survey methods and stratification sampling method were used from commercial catch. The relation between weight and length of *S. fimbriata* males and females are the values of b range 2.62750-2.69449 and 2.63959-2.72040, respectively. The growth patterns of male and female fish showed negative allometric growth pattern. The growth parameters of male fish is L∞ 177.50 mm; K 0.51month^{-1}; t_0 -0.53month^{-1}, while for female fish L∞ 185.00 mm; K 0.67month^{-1}; t_0 -0.41month^{-1}. Fecundity ranges from 2801- 60 578 eggs and diameter size range of eggs 8-67 µm.

Keywords: fish growth, fecundity, *Sardinella fimbriata*.

INTRODUCTION

Alas Strait, East Lombok, West Nusa Tenggara is known as crucial area for the small scale activity of fishing. Alas Strait also a good fishery spot for catching pelagic fish species such as Fringe Scale Sardine (*Sardinella fimbriata*), or locally known as Tembang fish. People in Lombok know the fringe scale fish with other local name such as *teri ijo* and *onyok*. The fish is mostly utilize for food and livelihoods to the market price of Rp. 8,000 per kg accompanied by considerable development fisheries [1].

Fish resources are common property and has free rates of utilization. However, the higher exploitation of fish resources continuously followed by uncontrolled utilization, result in the significant decreased on the availability of fish stock in nature [2].

Potential of Tembang fish in the territorial waters of the Alas Strait, related to the future potential and opportunities, the high utilization rate and stock condition which tends to decrease, and the lack of information on the reproductive biology of Fringe scale sardine were feared to disrupt the sustainability of fish resources. Therefore, it is necessary to conduct anticipation act in the management through parameter information on the growth and reproduction of the fish. This study was aimed to assess the parameter of fish growth and fecundity of the Fringe Scale Sardine (*S. fimbriata*) in Alas Strait, East Lombok, West Nusa Tenggara.

MATERIALS AND METHODS

Research Site

The research was conducted on August to December 2016. *Sardinella fimbriata* fish collected during the study came from catches of fishermen in the waters of the Alas Strait at Tanjung Luar Fish Landing Base, East Lombok, West Nusa Tenggara (Fig. 1).

Methods of Fish Sampling

This study used stratification sampling of *S. fimbriata*, collected from a commercial catch. Collected fish were brought to the laboratory and thoroughly cleaned in Reproduction Laboratory analysis at the Faculty of Fisheries and Marine Sciences, University of Brawijaya, Malang.

Analysis of Data

Weight and Length of Fish

Equations for the relation of weight and length of fish almost follow the law of cubic, i.e. Weight of fish is the cubed number of its length.

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However, the relation on the fish was not cubed. The correct equation following the formula [3].

$$W = aL^b$$

Description:
W = weight (g)
L = length (mm), a and b = constants

Estimation of Growth Parameter

Walford plot is one of the simplest methods of predicting the growth parameters L∞ and K parameters used the von Bertalanffy equation with the same interval sampling [3].

$$Lt = L\infty\ (1\text{-}e^{[-K\,(t-t_0)]})$$

Description:
Lt = length of the fish at the age t
L∞ = theoretical maximum length (length asymptotic)
K = coefficient of growth (per unit time)
t_0 = theoretical age at the time of a length equal to zero

The results of calculations then analyzed further using the method of ELEFAN I (Electronic Length Frequencies Analysis) contained in FISAT II program. FISAT II Program is a method of analysis estimation of fish growth parameter based on the data of length frequency distribution which was obtained [4].

Maturity Stage of Gonads

Description analysis of maturity gonads based on the visual morphology and histology of gonads sample. The statement method used Tester and Takata, which explained by Effendie [3].

Fecundity

Egg fecundity is calculated on the maturity condition of gonad III and IV by combined method [3]. The measurement of eggs diameter used light microscope scale 4x 0.10 μm^{-1} [3].

$$F = \frac{G\ x\ V\ x\ X}{Q}$$

Description:
F = fecundity (eggs)
G = total gonad weight (g)
V = dilution volume (mL)
X = egg total in 10 cc
Q = gonad weight example (g)

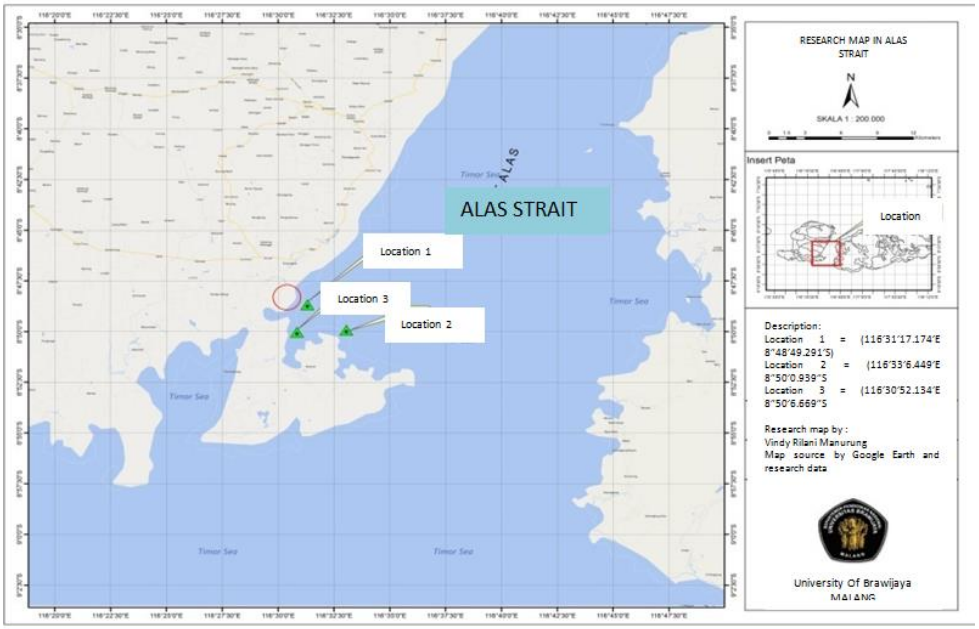

Figure 1. Research Location

RESULT AND DISCUSSION

Weight and Length of Fish

Analysis of relationship between weight and length of fish *S. fimbriata* obtained from 750 fishes compared to the total weight (gram) of fishes (Fig. 2).The relationship between weight and length of fish is 2.63959-2.72040 value b range for males, and females is 2.62750-2.69449 (also value of b). Otherwise, the growth patterns of male and female showed negative allometric growth pattern, indicated that the length (mm) of fish growth faster compared to the increase of weight (g) (Table 1). In the Aegean Sea, Mediterranean, the relationship equation of *S. fimbriata* weight with values range of b is 3.064

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in male and 3.084 for female, with positive values of allometric growth pattern [5].

Other study in the Sunda Strait found b value at 2.834 for males and 2.683 for females, also with negative allometric growth pattern [6]. *Sardinella fimbriata* fish tend to has different growth patterns to the each different location. This could be to the differences in the condition of the aquatic environment, the size of the fish samples taken and the amount of food [7].

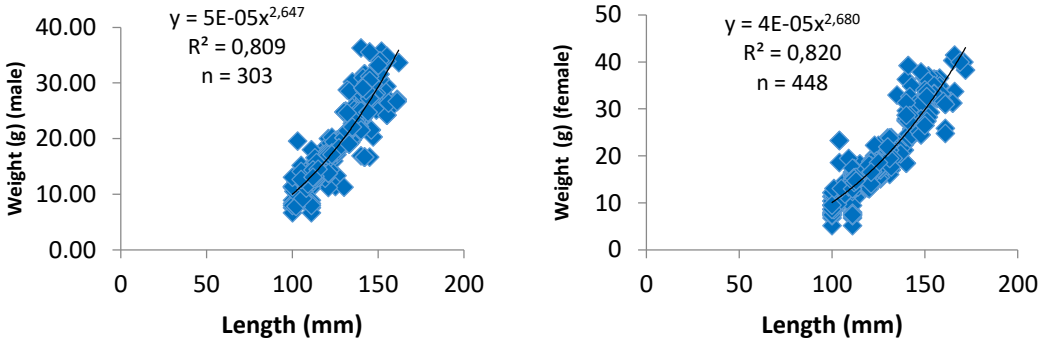

Figure 2. Correlation between Weight and Length of *S. fimbriata* Male and Females

Table 1. Weight and Length of *S. fimbriata*

Gender	R^2	Value prediction b(α=0.05)	Growth after test T (α=0.05)
Male	0.80	2.63959-2.72040	Allometric negative
Female	0.81	2.62750-2.69449	Allometric negative

Estimation of Growth Parameter

Analysis of fish growth parameter show that female fish growth rate higher than the growth rate of male fish (Table 2). This suggests that the length growth of female fish (L∞ (mm)) will be faster than male. The faster pace of growth, the fish achieve the asymptotic length faster and will quickly experience death. If the growth coefficient of a species is lower, then the longer time required by the species to achieve the asymptotic length [8].

Table 2. Growth parameter fringe scale fish (*S. fimbriata*)

Gender	L ∞ (mm)	K (month $^{-1}$)	t_0(month)
Male	177.50	0.51	-0.53
Female	185.00	0.67	-0.41

Maturity Stage of Gonads

The observation of gonads morphology and histology can be seen in Figure 3. The proportion of the maturity stage of gonads dominated by observation month of gonad maturity stage I, while the maturity stage of gonads IV are less found. The total amount obtained from 750 fishes, total of male stage IV found is 28 fishes and total stage IV female found is 41 fishes. We assumed that the spawning season which occurs before August thus certain fish found in stage III and IV that can be used as an indicator of fish spawning in waters.The peak spawning season of *S. fimbriata* in the waters of Karwar is January and April. While, *Sardinella* spawning season in the south and west coast of India is August to February with individual spawning only once in a season [9].

Other study found the fish spawning season in the sea of the Indian took place in May-June [10]. Meanwhile in the sea of the Mediterranean in Egypt, spawning of *S. aurita* (family Clupeidae) from the period of June to September, with a peak in late July and early August; when the annual water temperature reaches its maximum [11].

Based on the percentage of stage IV fish, we concluded that the fish spawning season that occurs throughout the year. Differences in fish spawning season caused by the fluctuation of the annual rainy season, geographical location, and condition of the fish [12].

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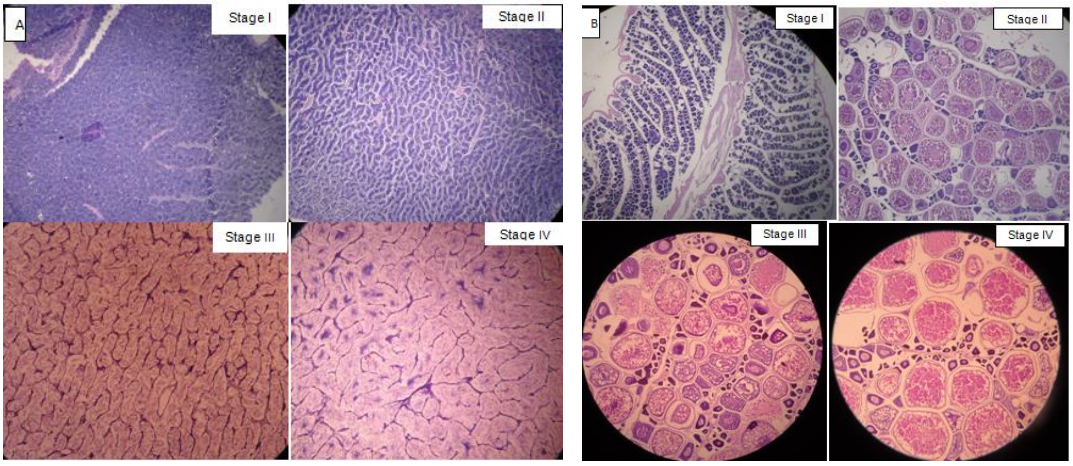

Figure 3. Gonads Histology *S. fimbriata*, male (a) and females (b)

Fecundity

The analysis and calculations showed the value of fish fecundity female of stage IV ranges from 2801- 60 578 eggs. Previous research in the Indian sea found *Sardinella* female fecundity from 5500 to 41700 eggs [13]. Other study of *S. fimbriata* fish in the waters of Karwar, India estimated from 14508-25485 fecundity [9]. Diameter size range of fish eggs is 8-67 µm (Fig.4). Type of fish spawning can be seen from the distribution of the diameter of the eggs in the gonad maturity stage IV. Distribution of the highest grade fish eggs diameter is at interval of 38-42µm.

Figure 4. Diameter Size Range of Fish Eggs

These data indicate that this type of fringescale is a partial spawning fish. Essentially, female pelagic fish have fecundity and has a very high spawning periods are often protracted, since all eggs are rarely released in one spawning periode. However, the shortest spawning period is found in small pelagic fish that live in the middle zone, because the optimal conditions for the survival of embryos and larvae tend to depend on the period of the season [14]. The size of the diameter of the eggs is influenced by the amount of food (food supply) on the female fish metabolic processes [15].

CONCLUSION

The growth pattern of *Sardinella fimbria-ta*male and female fish showed the pattern of negative allometric growth. The value of the growth parameters of male fish is L∞ 177.50 mm, K0.51month^{-1}, t_0-0.53 month^{-1}, while for female fish L∞ 185.00 mm, K 0.67month^{-1} and t_0 -0.41month^{-1}. The proportion of the maturity stage of gonads dominated by gonad maturity stage I, while the maturity stage of gonads IV are less. The fish fecundity of femalestage IV ranges from 2801- 60 578 eggs with diameter size range of fish eggs is 8-67 µm. For further research, we suggest to focus on the biological aspects of fish represent all seasons to represent the time series data, thus the trend of each year can be seen.

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Influence of Different Pulse Length towards Motility and Viability of Ornamental Japanese Carp (*Cyprinus carpio* Var. Koi) Sperm through Electroporation Method

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Abstract

Successful studies about implementation of electroporation method which sperm becomes gene transfer medium for fish transgenesis have reported. Motility and viability are two major factors in successful of electroporation. The objective of the study was to determine optimal pulse length of electroporation towards the motility and viability of Koi fish sperm. The study was conducted at the Central Laboratory of Biological Sciences and Reproduction of Brawijaya University. The data analysis was conducted using completely randomized design. The electroporation method was carried out using voltage of 30 volt with pulse number of 4 times. The pulse length of the electroporation (based on treatment) was 0.5, 1 and 1.5 ms. The result showed the highest percentage of motility was 3.67% in treatment A (pulse length 0.5 ms) with coefficient correlation R^2 = 0.9643 and the best percentage of viability was 79.67% in treatment A (pulse length 0.5 ms) with coefficient correlation R^2 = 1. Correlation between the pulse length treatment (electroporation) toward motility and viability of Koi fish sperm, in which longer duration of pulse length treatment (electroporation) would decrease the motility and viability percentage of the fish sperm.

Keywords: Electroporation, Motility, Pulse length, Sperm, Viability.

INTRODUCTION

Electroporation is one of the effective and efficient gen transfer method widely applied in aquaculture besides microinjection. The principle of electroporation is to form reparable-holes on cell membrane with help of electrical current and the cells are suspended in DNA solution which will then enter the cell through the already formed hole [1].

Electroporation method is mostly conducted using sperm as vector that carries a foreign gene to be introduced (sperm-mediated gene transfer). Based on some previous studies, it has been found that sperm has unique ability as natural vector for carrying foreign gene [2]. The sperm electroporation method is simpler and massive, in which large quantities of sperm can be inserted in transgenes [3].

The success of gene transfer method by electroporation method using sperm as the gene transfer media depends on the voltage level and the concentration of DNA vector during electroporation [4]. The integration of DNA into the sperm depends on the electric voltage (Vcm^{-1}), pulse number and concentration of DNA, where as the transfer efficiency of DNA with sperm-media electroporation is strongly affected by electric voltage and pulse length [5].

The main factor of success in fish sperm transgenesis is to succeed in maintaining the quality of sperm which includes motility and viability for fertilization. Previous research has shown that Chinook Salmon (*Oncorhynchus tshawytscha*) sperm activity decreased from 82% to 2% post electroporation [5], it is important to find out how to use electroporation method optimally. The study of optimum pulse length in Ornamental Japanese carp (*Cyprinus carpio* Var. Koi) fish transgenesis using electroporation method has not been studied and therefore it is necessary to conduct experiment on influence of different pulse length towards motility and viability of Ornamental Japanese carp fish sperm to get optimum result. The purpose of this research is to describe the influence of electroporation method using voltage level (30 volt) and different pulse length towards motility and viability of Ornamental Japanese carp fish (*Cyprinus carpio* Var.Koi) sperm.

MATERIALS AND METHODS

This experiment was conducted in May 2017 at Fish Breeding and Reproduction Laboratory, Faculty of Fisheries and Marine Science and Central Laboratory of Life Sciences of Brawijaya

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University, Malang. The materials used in this research were Ornamental Japanese carp fish sperm, physiological solution, eosin negrosin, tissue, aluminum foil, and aquades. The equipments were a set of BIO-RAD Gene Pulser Xcell™ including electrophorator, shock pod and cuvet, inverted Olympus BX 51 microscope, binoculars Olympus CX 21 microscope, micropipet, appendorf, blue tip, yellow tip, white type, digital camera, glass object, petri disk, and handtally counter.

The experimental design used in this study was Completely Randomized Design. There were 3 treatments and 1 control; each was repeated 3 times. The treatments were as follow:

Treatment A = Pulse Length for 0.5 ms.
Treatment B = Pulse Length for 1.0 ms.
Treatment C = Pulse Length for 1.5 ms.
Treatment K = No treatment

Collection of Sperm

Fish sperm was obtained from male fish of which gonad had been mature by stripping. The sperm resulting from the stripping was inserted into 1 mL syringe and Na-physiologically was added as a diluent with a ratio of 1: 1 and then, these were put into the appendorf [6].

Electroporation Process

1 set of Gene Pulser Xcell™ Electroporation System Biorad was used during electroporation process. 25 µL of sperm was inserted into the electrophorator cuvet (0.2 mm) [7]. Next, it was pulsed with 30 volt electric level [8], pulse length was according to treatment and the pulse number was 4 times. Post electroporation, 275 physiological NaCL (total of 300 µL solution) was added into the sperm [9]. The following step was to observe the motility and viability of the post electroporation sperm.

DATA COLLECTION

Sperm Viability

Staining was the method used for observing sperm viability or survival of the sperm. 5 µL sperm was taken and 1 µL was dripped on the object glass. The next step was to add eosin negrosin dye and polish it with cover glass. The following step is to wait until it was dry and observe it under the microscope. Having finished, the researchers calculated the percentage of living sperm of which color was clear and dead sperm of which color was reddish using handtally counter. Susilowati [10]'s formula was adapted for calculation of the sperm viability:

$$\text{Viability} = \frac{\Sigma \text{ Living Sperm}}{\Sigma \text{ Dead Sperm}} \; x \; 100 \; \%$$

Sperm Motility

To observe sperm motility, the researchers took 1 µL of the post electroporation sperm and put it on the glass object. Then added water, and covered it with cover glass and observed sperm motility under the inverted microscope for 7 seconds. Sperm percentage was observed using the scoring criterion adapted from Dewi [11]. Table 1 described the scoring criteria.

Table 1. Motility Scoring

Percentage	Criteria	Score
>70%	Spermtozoa moved forward and rapidly with various tail movement	5.0
55 – 70%	Spermtozoa moved forward and showed rapid movement	4.0
40 – 55%	Spermtozoa moved forward and some of them showed rapid movement	3.0
25 – 40%	Spermtozoa moved forward	2.0
10 – 25 %	Spermtozoa was moving	1.0
1 – 10 %	Most of the spermtozoa did not move	0.5
<10 %	No movement	0.0

Data Analysis

The data analysis method was variance analysis (ANOVA) to match the design of the study, Completely Randomized Design. When there was significant difference, the researchers continued the experiment using Least Significance Different (LSD) test. Regression analysis was used to describe the correlation between the treatment and the result used; the objective of regression analysis is to determine the nature of the regression function which gives information about the effect of the best treatment towards the response.

RESULT AND DISCUSSION

Motility of the Post Electroporation Sperm

Based on the result, each of the treatments after electroporation method with different influence of pulse length towards motility percentage of the Ornamental Japanes Carp fish sperm; the percentage was compared to the control (no treatment). Table 2 described percentage of the sperm motility.

The table 2 showed that treatment A (pulse length for 0.5 ms) resulted in the highest motility percentage (3.67%) whereas treatment C (pulse

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length for 1.5 ms) resulted in the lowest percentage of motility (0.67%).

Table 2. Sperm Motility (%)

Treatment	Repetetion			Total	Average
	1	2	3		
A	4	3	4	11	3.67
B	2	3	3	8	2.67
C	0.5	1	0.5	2	0.67
Total				21	
Control	5	4	5	14	4.6

The motility showed the decreasing sperm motility during each of the treatments. The control (no treatment) had the highest percentage of sperm sperm motility (4.6%) compared to the treatment where the pulse length was applied. It showed the higher pulse length caused decrease in the percentage of post electroporation sperm motility. According to Tsong [12], the pulse length (milliseconds) and electrical voltage level (volts) may cause change in pore size of the sperm cell membranes and will be decreasing quality of the sperm.

Based on the result of the variance analysis in Table 3, the shock duration of the treatment had significant influence towards the motility of the Ornamental Japanese Carp fish sperm after electroporation treatment. It was indicated by the comparison between F-ratio, F-table 5% and F-table 1%.

Table 3. Variance Analysis

Source of Variance	Df	SS	MS	F	F 5%	F 1%
Treatment	2	14	7			
Random	6	1.5	0.25	28	5.14	10.92
Total	8					

The polynomial testing (Fig. 1) resulted in the following linear equation, $Y = -1.5x + 5.3333$ where correlation coefficient between the pulse length and the sperm motility of $R^2 = 0.9643$. In other words, there was very significant correlation between the pulse length during the electroporation method and the motility of Ornamental Japanes carp fish sperm.

The analysis showed that the longer of pulse length causes more decreasing percentage of sperm motility. The highest motility was the result of treatment A (0.5 ms) in which the percentage was 3.67%. It is important to describe the quality of the fish sperm because it will determine the success of the fertilization process using egg as the medium. If the quality of sperm decreased, it would affect the work of sperm. Percentages of sperm motility should not be lower than 50% [13].

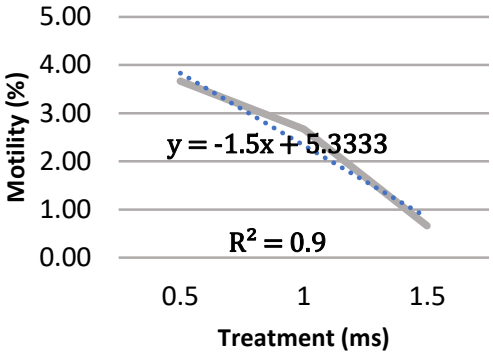

Figure 1. Correlation between Different pulse length and Sperm Motility Chart

Motility of fish sperm representing the sperm survival would decrease with increasing voltage level and pulse length [14]. The shape of sperm plasma membrane was related to the motility and viability of spermtozoa. If the plasma membrane was damaged, the enzymes that played a role in energy metabolism would be lessen or even be lost. This may lead to a decrease in energy sources so that sperm movement will slow down, and would certainly decrease the life and power of fertilization [15].

Viability of Post Electroporation Sperm

Table 4 described the average viability of the treatment that analyzed the influence of different pulse length during the electroporation towards Ornamental Japanes carp fish sperm. Based on Table 4, treatment A (0.5 ms) resulted in the highest percentage of viability (79.67%) while treatment C (1.5 ms) resulted the lowest percentage of viability (63.00%). The viability of the control (no treatment) was 90.66% or higher than that of the treatments. Increasing of pulse length lead to decreasing percentage of sperm viability. Furthermore, each treatment had different viability result.

Table 4. Sperm Viability (%)

Treatment	Repetition			Total	Average
	1	2	3		
A	78	82	79	239	79.67
B	77	69	68	214	71.33
C	61	59	69	189	63.00
Total				642	
Control	92	90	90	272	90.66

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The variance analysis (Table 5) explained that the F-ratio was higher than both the 5% and 1% F-Table. In other words, the treatment (pulse length during the electroporation) had significant influence towards the viability of Ornamental Japanese carp fish sperm. The polynomial test (Fig. 2) resulted in a linear equation, which was $Y = -8.3333x + 88$ and the correlation coefficient between the pulse length and the sperm viability was $R^2 = 1$ meaning there was strong correlation between pulse length treatment pasca electroporation and the viability of Ornamental Japanese carp fish sperm.

Table 5. Variance Analysis

Source of Variance	df	SS	MS	F	F 5%	F 1%
Treatment	2	416	208			
Random	6	113	18	11	5.14	10.92
Total	8					

Based on the data analysis, the percentage of the sperm viability plummeted as the pulse length during the electroporation was getting longer. It was then concluded that pulse length duration may decrease the viability of Ornamental Japanese carp fish sperm, similar to their motility. Normally, sperm viability is between 1 and 2 minutes after stripping [16]. Changes in infrastructure of the plasma membrane, as well as the loss of some mitochondrial matrices and decreased electron density of the mitochondrial matrix may result in loss of spermtozoa viability [17]. Treatment A (0.5 ms) resulted in the highest percentage of post treatment viability. It was the lowest pulse length treatment.

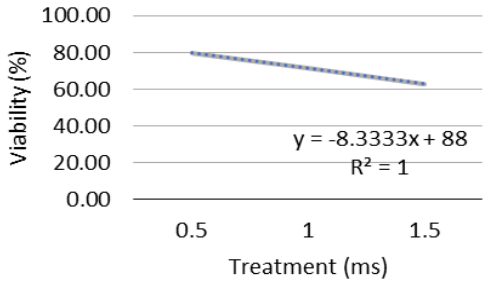

Figure 2. Correlation between Different pulse length and Sperm Viability Chart

Electroporation in sperm may stretch sperm cells as high-intensity electric fields temporarily destabilized cell membranes and during that period, membranes were highly permeable with exogenous molecules (DNA or RNA) located around the cell medium. DNA then moved into the cell (internalization process) through this permeable hole. When the electric field stopped (turn off), the inner membrane holes closed and the exogenous DNA entered the cell [17].

Therefore, the 30-volt electric level and pulse length applied during the treatment may be used as electroponic method where sperm became medium to produced transgenic Koi fish. Maintining motility and viability of sperm was important in conducting transgenesis technique where sperm became gene carrier vector.

CONCLUSION

Based on the result of the experiments that was giving different pulse length during electroporation towards the Ornamental Japanese carp (*Cyprinus carpio* Var. Koi) fish sperm, it was revealed that pulse length has significant influence toward motility and viability of fish sperm. 30 volt electric volatage level and 0.5 milisecond of pulse length results in the highest percentage of the motility and viability of Ornamental Japanese carp (*Cyprinus carpio* Var. Koi) fish sperm.

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The Role of Local Hydromacrophytes in Leachate Phytoremediation Performed Using Constructed Wetland System

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Abstract

The purpose of this research is to analyze the effectiveness of using local hydromacrophytes for performing leachate phytoremediation in constructed wetland (CW) system. It was an ex situ experiment carried out in a glass house by means of free water surface (FWS) CW reactors containing sand and gravel substrates in 3:5 ratio. The reactor was waterlogged by 9 L leachate. The phytoremediation tests were performed in two-factor treatment design involving hydromacrophyte species and hydraulic retention time. Species of local hydromacrophyte used in this research included *Alternanthera sessilis*, *Commelina nudiflora*, *Paspalum conjugatum*, *Typha angustifolia* and the polyculture of the four species. The improvements in leachate qualities were identified through decreases of physico-chemical parameters. Among the mean values of the percentages indicating the decreases of physico-chemical leachate parameters, the greatest ones were resulted from the treatments using *T. angustifolia* and *A. sessilis* on the 30th day. It has proven in the experiment that these two species of hydromacrophyte perform the most effective process of improving leachate quality.

Keywords: constructed wetland, leachate, local hydromacrophytes, phytoremediation.

INTRODUCTION

Waste management in final dump site that use sanitary landfill and controlled landfill systems produces methane gas and leachate as its by-products. Leachate is characterized as the external water or liquid that is percolated into heaps of solid waste and leached out the dissolved constituents including some organics materials from biological decomposition. To prevent leachate from contaminating the land and/or groundwater in its environment, it is important to have an effective leachate treatment. Sanitary landfill system in solid waste management involves the process of gathering leachate on the bottom of the landfill site by relying on gravity and channeling it into Leachate Treatment Plant [3,7]. Conventional Leachate Treatment Plant (LTP) has become a common facility in landfills in Indonesia for waste management purpose. The conventional method involves physical and chemical processing that is carried out in a stabilization pool (primary, secondary and tertiary treatment tanks). The resulted leachate is then disposed to the surrounding ground or recirculated to be the *starter* for the decomposition process of another waste mass. Unfortunately, this method can only manage less than 40% of the leachate organic contents [7]. In Indonesia, the standard of leachate quality acceptable for a release to the environment is ruled by the Regulation of The Minister of Environment and Forestry, Number P.59/Menlhk/Setjen/Kum.1/7/2016 on standard leachate quality for waste control in final disposal facility.

Phytoremediation refers to the use of certain plants to remove or reduce pollutants from the ground, sediments, surface water, groundwater and liquid waste [4]. A constructed wetland (CW) with selected plants grown on it is one of methods to adopt in treating wastewater with phytoremediation. Hydromacrophyte is a type of vegetation that can be grown in CW to reduce the amount of pollutant substances in its environment. Different species of hydro-macrophyte perform different ways of pollutant reduction including absorbing, degrading, extracting, accumulating or stabilizing pollutant elements. The effectiveness of phytoremediation as an alternative technique in controlling wastewater has been known in many countries worldwide. Wastewater remediation in CW is a combination of physical, chemical and biological processes. The effectiveness of CW system in reducing the amount of organic, inorganic and heavy metal pollutants has been studied and tested. It is

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proven easier to apply in improving the quality of various kinds of contaminated water such as domestic wastewater, industrial wastewater, leachate and wastewater from mining industry [1,4]. So far there are four types of CW that have been developed for different functions or specific purposes of remediation:

1. *Free water surface CW*. This type resembles the natural wetland which has free water surface areas on it.
2. *Subsurface water flow or vegetated submerge bed CW*. In this type of CW, the water surface isn't higher than the substrate and it is only the roots of the plants that come in direct contact with the wastewater;
3. *Vertical flow CW*. In this type, wastewater is distributed vertically from the substrate downward along the root zone because of gravitational force;
4. *Sludge dewatering beds* (*reed beds*) *CW*. This type of CW applies evapotranspiration from plants to remove water from mud waste deposit [11].

Waste management in the Final Waste Disposal Site of Talangagung is carried out using sanitary landfill system and the LTP leached out the leachate that often exceeded the standard of leachate quality effluent. Because of that, the leachate from the tertiary tank in LTP is not discharged into the surrounding ground. Instead, it is re-circulated or introduced into the new batches of waste and is reused as a compost starter. Re-circulation process could results in several adverse impacts such as the increase of methane production, leachate overflowing, and the accumulation of volatile organic acid compound together with some hazardous heavy metal elements which are not easily degraded [6,10]. The research presented in this article aimed to analyze the effectiveness of using local hydromacrophytes for performing leachate phytoremediation in constructed wetland system.

MATERIALS AND METHODS

This research was an experiment that conducted in a glass house of Department of Biology, Faculty of Mathematics and Natural Sciences, University of Brawijaya. The applied procedure was factorial experiment in completely randomized design with two treatment factors (6 x 6). The first factor was hydromacrophyte species and the second was hydraulic retention time (HRT). HRT is the length of time that a compound (e.g. water) remains in a storage unit. There were four species of local hydromacrophyte and their polyculture planted in CW reactors with four replications. Each of the reactors was made from plastic tank 40 cm in diameter and 24 cm in height. The reactor was filled with sand and gravel substrates in 3:5 ratio and a local hydromacrophyte was planted on it. The hydromacrophytes had previously been acclimatized in the glass house before the 9 L of leachate was poured into it. Four species of local hydromacrophyte used in this research were *Alternanthera sessilis*, *Commelina nudiflora*, *Paspalum conjugatum* and *Typha angustifolia*.

Data Collection

The measurement of physico-chemical parameters of leachate was performed at 6 HRT (Hydraulic Retention Time) which were plotted on the 0^{th}, 5^{th}, 10^{th}, 15^{th}, 21^{st}, dan 30^{th} days in the Microbiology Laboratory and Ecology-Animal Diversity Laboratory in the Department of Biology, Faculty of Mathematics and Natural Sciences, University of Brawijaya. The leachate quality were observed in 6 parameters (Conductivity, Turbidity, TDS, BOD, Nitrate, Ortophospate). The observations of the physical conditions of the plants in the reactors were recorded and the results were documented to provide secondary data regarding local hydromacrophytes's tolerance to toxic environment.

The resulting data were tabulated using an SPSS (Statistics software program) and then analyzed by one-way ANOVA test for those data which were identified as normal and homogenous based on *Levene* test, and by *Brown-Forsythe* test for those which were identified as normal but not homogenous. The results from one-way ANOVA test and *Brown Forsythe* test that showed significant differences were further analyzed to observe the specific values of the differences using Tukey-HSD test (as the follow-up of ANOVA) and *Games-Howell* test (as the follow-up of *Brown-Forsythe*). Afterwards, biplot graph were applied to group and to describe the overall results of measurement/ analyses for all observed physico-chemical parameters. The percentage of decreasing leachate quality parameters also tabulated using *Ms. Excel*.

RESULT AND DISCUSSION

Physico-Chemical Parameters of the Leachate Qualities

The data that represent physico-chemical parameters of the leachate qualities in this research are normally distributed with mean values as

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presented in Supplementary 1. The biplot in Figure 1 illustrates the whole conditions of the leachate qualities throughout the allotted retention times whereas the biplot in Figure 2 illustrates those for all selections of hydromacrophyte species.

Figure 1 also shows the three groups of leachate quality measurements. The first group is represented on the right-hand quadrant of the figure and comprises the measurements for all the observed parameters on the 0th day. The group indicates relatively high mean values for all parameters of the leachate qualities. The second group consists of the leachate quality measurements from the 5th and 10th days. The data obtained from the two points of retention time show insignificant differences and are therefore categorized into the same group. They indicate lower values for all parameters of leachate quality except for BOD which is still relatively high, but the values in this group has created wide gap with the first group. The rest of leachate quality data, which are obtained from the 15th, 21st and 30th days, belong to the third group.

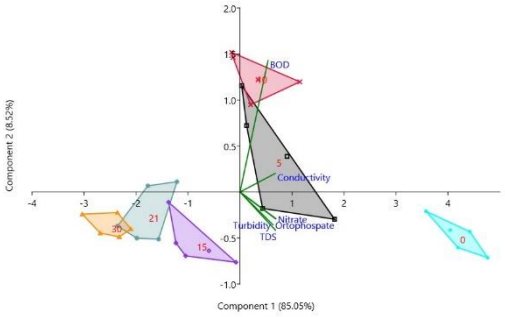

Figure 1. Biplot Graph of leachate quality parameters through out points of hydraulic retention time

It is shown in the figure that they converge on the same quadrant because their values are close to each other. The quadrant indicates a much lower measurements for all leachate quality parameters in this group compared to those in the other two groups. Furthermore, the biplot analysis of the differences in the leachate qualities in daily monitoring based on hydromacrophyte species factor is shown in Figure 2 (the 5th day), Figure 3 (the 15th day), and Figure 4 (the 30th day).

The values of decreases for each physicochemical parameter of the leachate qualities are presented in detail in Supplementary 2. Which species of hydromacrophyte that had resulted in the highest value of decrease is indicated in the table.

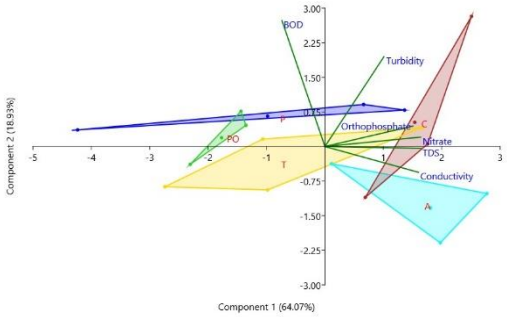

Figure 2. Biplot Graph of leachate quality parameters based on the hydromacrophyte species-treatments on the 5th day

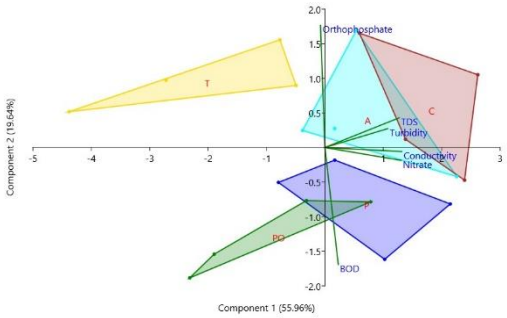

Figure 3. Biplot Graph of leachate quality parameters based on the hydromacrophyte species-treatments on the 15th day

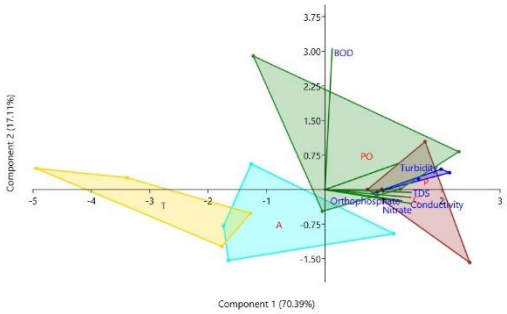

Figure 4. Biplot Graph of leachate quality parameters based on the hydromacrophyte species-treatments on the 30th day

The result of the biplot analysis indicates that the values representing conductivity, turbidity, TDS, nitrate and orthophosphate correlate to one another. Histograms in Figure 6 to Figure 10 represent statistically significant differences of mean values for all parameters of the leachate qualities with percentages of decreases shown above each column of the chart.

The levels of conductivity, as shown in Figure 5, declines significantly by 55.01% to 61.05% on the 5th day and keeps dropping until the 30th day

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when the decreases measure 79.56% to 87.88%. The levels of TDS, represented in Figure 6, generally show significant dropss ranging from 35.84% to 46.24% on the 5th day. On the last retention time (the 30th day), its decreases reach 55.24% to 74.04%. Likewise, the levels of turbidity also make substantial decreases from the 5th to the 30th days as illustrated in Figure 7. The range of the decline on the 5th day is 29.05% to 55.32% and it is 94.90% to 97.79% on the 30th day.

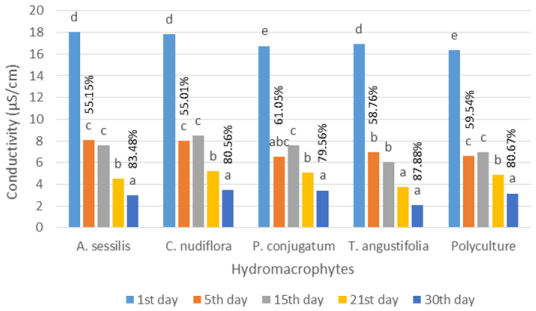

Figure 5. Mean value of conductivity measured based on HRT and hydromacrophyte species factors

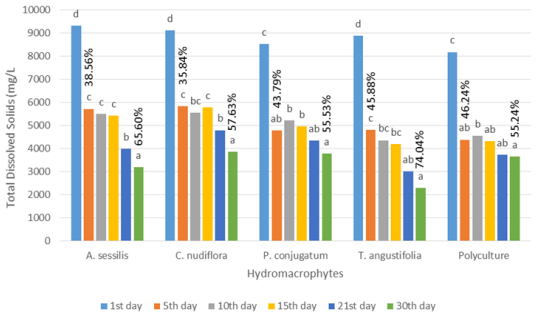

Figure 6. Mean value of TDS measured based on HRT and hydromacrophyte species factors

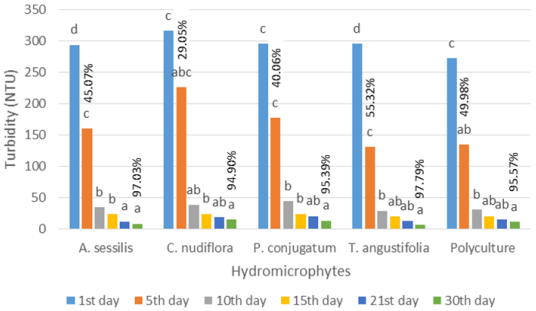

Figure 7. Mean value of turbidity measured based on HRT and hydromacrophyte species factors

The same case applies to the levels of nitrate and orthophosphate. Indicated in Figure 8, nitrate levels are dropping considerably from 57.25% to 64.33% on the 5th day to 76.00% to 89.19% on the 30th day. The overall of orthophosphate decreases, as indicated in Figure 9, are 42.17% to 51.94% on the 5th day and reaches 92.17% to 97.03% on the 30th day.

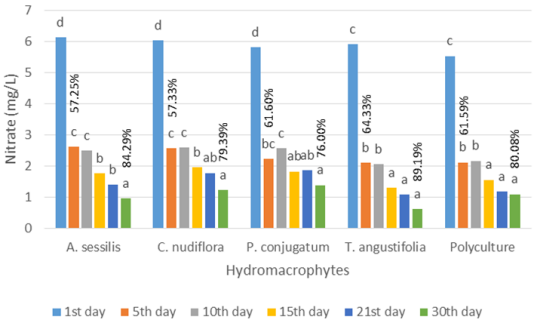

Figure 8. Mean value of nitrate measured based on HRT and hydromacrophyte species factors

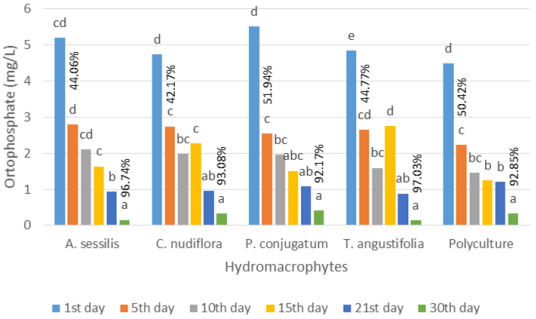

Figure 9. Mean value of ortophosphate measured based on HRT and hydromacrophyte species factors

Hydromacrophyte Species

Alternanthera sessilis has extensive distribution worldwide that encompasses tropical and subtropical regions. The natural habitats of this species of hydromacrophyte can be found in many parts of America, Africa, Australia and Asia where Indonesia is one of them. It is known as one of pioneer plants that can survive in wetland ecosystem that has suffered degradation and contamination. This weed normally grows on the edges of marshes, riparian zones, rice fields, the edges of irrigation channels and waterways, the edges of dams and roadside puddles. They can withstand an environment which is flooded by water up to 1 meter high [5]. In this research, *A. sessilis* grew very fast and were well adapted to the environment with high levels of organic contents. By the fifth day, the leaves went dry, but in the following days, the plant grew fast, forming a dense colony that covered almost the whole surface of the reactor. Several leaves were damaged by *Leptosia nina*, but this pest didn't hold up the plant's rapid growth. New buds were developing at the plant's nodes that grew under the water and there were no parts of the plant that died because of their submergence in the water. *A. sessilis* is an obligate wetland species that can grow well in FWS-CW in this experiment because of its tolerance to environmental stresses.

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Paspalum conjugatum can generally be found in many areas of tropical countries in American continent and it has been naturalized as far as Southeast Asia (including Indonesia) as well as several other tropical regions in the world. *P. conjugatum* is characterized by its sturdy and long stolon and its creeping growth pattern with roots at nodes, branching out and solid. They can grow well on high ground up to 1700 meters above sea level. They can also survive with or without shades but they are generally found in colony under the shade of other vegetation in riparian zones, roadsides and degraded zones [8]. In our experiment, *P. conjugatum* grew slower than expected since the leachate was poured into the reactor, from the first day up to the 30th day. Parts of the plant that were immersed in the water were decaying and the plant was dying. It is apparent that *P. conjugatum* is not an obligate wetland species. Evidently, this species could not survive in the kind of waterlogged environment such as FWS-CW.

The natural habitat of *Commelina nudiflora*, like *Alternanthera sessilis*, covers many Asian countries with tropical and sub-tropical climates including Indonesia (U.S. National Plant Germplasm System, 2007). This plant reproduces through its seeds and stolon and can grow rapidly with abundant supply of water. *Commelina nudiflora* commonly grow with upright stems in dense colonies. They proliferate in a very short time and compete well with other plants for spaces and available nutrition. They can adapt to the environment with pH 4 to 10. They are the kind of weed that can live in various levels of humidity and light intensity. They can also tolerate cutting [1]. *Commelina nudiflora* in our experiment experienced inhibition in its growth since a certain amount of leachate was poured into the reactor. Its leaves went dry as its endurance was slowly weakening because of the environmental stresses impacted by the leachate. Its condition was even more deteriorating due to the presence of *Bemisia tabacci* that was harmful to its vigor. On the 30th day, its biomass was nearly destroyed.

Typha angustifolia is an obligate wetland species that commonly grow in Europe but have also been naturalized in other areas in the world including Indonesia. They can survive low pH environment and anaerobic condition. This macrophyte grows through rhizomes and needs a lot of sunlight to survive. It is well adapted to free water surface and subsurface water flow constructed wetlands [10]. It has been widely known that *T. angustifolia* is capable of reducing toxic contents in the water [10]. Several species of endophytic bacteria in *T. Angustifolia*'s root system which are adept at adsorbing nitrogen and accelerating the plant's growth while some other species play important role in reducing nitrogen, phosphor and/or other toxic organic materials in eutrophic water [8]. *Typha spp*. has rhizodegradation/phytodegradation mechanism which is useful in reducing organic-aromatic and aliphatic materials, petroleum hydrocarbon contents as well as chlorinated solvent, TNT (trinitrotoluene) and pesticide [4]. The 30-day experiment in our research had proved that *T. angustifolia* was the species of hydromacrophyte with the highest performance in causing decreases in the leachate quality parameters and it adapted well to FWS type of CW.

Phytoremediation in Constructed Wetland System

Remediation in vegetated CW system is a complex activity that involves physical processes (sedimentation, filtration and adsorption), chemical processes (coagulation, oxidation-reduction and transformation) and biological processes (decomposition of organic matter into simpler elements by certain bacteria and plant uptake in various specific ways) [10,4]. The impacts of each process rely on the climate, the temperature, the amount of available bacteria, the substrates and the species of the plants [10]. The choice of vegetation plays crucial role in implementing constructed wetland system. The process of plant uptake is a very useful part in the attempts of removing heavy metal elements and of reducing petroleum hydrocarbon substances, synthetic hydrocarbon, nitrate and phosphate [10]. The treatments had resulted in different mean values of decreases in physico-chemical parameters for different species of hydromacrophyte as represented in Supplementary 2.

A liquid's capability in conducting electricity is referred to as conductivity and it is measured using conductivity meter [12]. The level of conductivity is contingent to the presence of soluble inorganic ions such as sodium, calcium, iron, nitrate, magnesium and the like. The decrease in conductivity in leachate is an indication of the drop in the amount of inorganic ions in it. Sedimentation process is the first physical process to occur as a result of the gravitational force. Afterwards, the chemical processes such as adsorption, precipitation and ion exchange take place at about the same time with biological processes

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such as microbial uptake and plant uptake. The research presented in this article resulted in the finding that *T. angustifolia* was the hydromacrophyte species with the most effective role in reducing conductivity in the leachate. The next species which showed its effectiveness in reducing conductivity is *A. sessilis* with 7% lower than *T. angustifolia*'s effectiveness in the same function for all points of HRT.

It was found that TDS positively is correlated to conductivity because the level of TDS signifies the amount of soluble solid present including inorganic matter such as those mentioned earlier. The decrease in conductivity will result in the decrease in TDS level as well. It is then evident that *T. angustifolia* played the most effective role in reducing TDS level in leachate. It was 7.32% to 10.96% more effective than *A. sessilis* which came in second for the same role.

Turbidity refers to the extent to which suspended solids occur in the water. The suspended solids consist of planktons and other microorganisms, fine sand, and organic and inorganic solids. The decrease in turbidity level is primarily caused by gravitational force that makes them go down to settle on and to be adsorbed by CW substrates or form biofilms on the surface of sand and gravel substrates. Biofilm is the mass of microorganism which is present in colonies and plays important role in the decomposition of organic matter in leachate. CW of FWS type is an effective method of reducing turbidity by up to 90%. It was found in the experiment that *T. angustifolia* is capable of reducing turbidity more effectively than *A. sessilis*. The difference between the two species in this particular performance was 10.25% on the 5^{th} day and it was declining throughout the following retention times well into the 30^{th} day.

Nitrate ($N0_3$) is the most stable form of nitrogen in the water and can be immediately taken in by plants. Nitrate content in the water can decline as the result of microbial uptake and plant uptake apart from sedimentation and adsorption on CW substrates. A research has been reported that the amount of nitrogen removed by plant uptake does not exceed 10% of the total removed nutrients (both nitrogen and phosphorous) even in optimum conditions [11]. As in the other cases previously mentioned, *T. angustifolia* was recorded as the most effective species in decreasing nitrate level in the leachate, 8% higher than *A. sessilis*'s effectiveness for the same performance.

Orthophosphate is one of inorganic phosphates in the water. It is a form of phosphor that can be absorbable by the hydromacrophytes and algae. The decrease in the amount of phosphate in the water was mainly resulted from sedimentation, precipitation, adsorption, microbial uptake (phytoplankton uptake) and plant uptake. The finding of our experiment showed that *T. angustifolia* was the hydromacrophyte species with the highest performance in decreasing the amount of orthophosphate in the leachate. Its uptake of phosphorus was 5% more effective than *A. sessilis'*s.

BOD (Biochemical Oxygen Demand) describes the amount of biodegradable organic matter in contaminated water. BOD is measured by the amount of oxygen needed by the bacteria to degrade the existing organic substance in the water. Leachate contains high level of BOD and it usually takes a long time to reduce it. The length of time needed in attaining significant decrease of BOD depends on the leachate's condition (anaerobic or aerobic). On the 5^{th} day of our experiment, we hadn't had significant decrease in BOD level in general, but in CW reactor vegetated with *A. sessilis*, there had been observable BOD decrease at 37.92%. The significant decrease was most apparent on the 30^{th} day which reached 76.28% to 90.87% in values. *A. sessilis* was recorded as the species with highest achievement in reducing the level of BOD in the leachate. The differences in the habitus possessed by *A. sessilis* and *T. angustifolia* led to a greater increase in BOD by *A. sessilis*. *A. sessilis* is an obligate wetland plant that is resistant to the exposure of leachate even to newly emerging shoots that is waterlogged by leachate. BOD will be decreasing when the amount of soluble oxygen in the water is increasing. The transfer of oxygen that takes place in every part of *A. sessilis* will supply the wastewater with sufficient oxygen to achieve the desired aerobic condition in *A. sessilis*-based leachate treatment. The aerobic condition is necessary for the bacteria to decompose the present organic matter easier and faster.

Based on the overall results of the research, it can be summarized that the most effective leachate phytoremediation was achieved when using *T. angustifolia* in FWS-CW. It had contributed to the decreases in the parameters of conductivity, turbidity, TDS, nitrate and orthophosphate by 87.87%, 97.79%, 74.04%, 89.19% and 97.03% respectively on the 30^{th} day. The second most effective leachate phytoremediation was attained when the FWS-CW was vegetated with *A.*

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sessilis. On the 30th day, it had contributed to the decreases in the parameters of conductivity, turbidity, TDS, BOD, nitrate and orthophosphate by 83.48%, 97.03%, 65.60%, 90.87%, 84.29% and 96.74% respective-ly.

CONCLUSION

The effectiveness of leachate phytoremediation that makes use of local hydromacrophytes in constructed wetland system in this research has been proven. It through a series of experiments that involve treatments in CW reactors with four different species of vegetation and their polyculture and a number of hydraulic retention times. It has been found that the two of those local hydromacrophytes have higher levels of reduction in all physico-chemical parameters of leachate qualities than others. Those species are *Typha angustifolia* and *Alternanthera sessilis*. *T.angustifolia* and *A. sessilis* are made greatest decreases on each retention time. *T. angustifolia* attained 87.88%, 97.79%, 74.04%, 87.24%, 89.19%, and 97.03% reductions of conductivity, turbidity, TDS, BOD, nitrate and orthophosphate parameters respectively on the 30th day. *A. sessilis* caused 83.48%, 97,03%, 65.60%, 90.87%, 84.29%, and 96.74% reductions of conductivity, turbidity, TDS, BOD, nitrate and orthophosphate parameters respectively on the 30th day.

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Dynamical Analysis of Fractional-Order Hastings-Powell Food Chain Model with Alternative Food

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Abstract

In this paper, a fractional-order Hastings-Powell food chain model is discussed. It is assumed that the top-predator population is supported by alternative food. Existence and local stability of equilibrium points of fractional-order system are investigated. Numerical simulations are conducted to illustrate analysis results. The analysis results show that alternative food can give a positive impact for top-predator population.

Keywords: Alternative food, Fractional-order, Grunwald-Letnikov approximation, Hasting-Powell model, Stability.

INTRODUCTION

Nowadays, fractional calculus becomes the main focus for the researchers. Many problems of science and engineering can be modeled by using fractional derivatives. The process of developing a differential system of integer order into fractional-order becomes popular in dynamic systems [1]. Basically, a biological mathematical model in predicting the future, not only depends on the current but also the memory or the previous condition. In fractional derivatives, at some certain conditions contain information of previous condition, therefore fractional derivatives can be used to explain more realistic natural phenomena.

Interactions between populations can be described in a food chain model. One of the interactions in the food chain is predation process. Mathematical model used to describe interactions between predator and prey is called the predator-prey model. Furthermore, many interesting fenomena in ecology can be described by mathematical model through predator-prey models such as harvesting in predator population [1], supplying alternative food in a predator population [2], refuging prey population [3], spreading disease in ecosystem [4], and the effect of the present an omnivore [5]. In predator prey model [2], it is assumed that prey populations do not always exist, they also experience migration to find new habitats due to climate change factors and low food reserves. Therefore, predators need additional food or alternative prey to survive.

In this paper, a food chain model of three-species fractional-order with alternative food is introduced. Examples of three-species ecosystems in this model: vegetation-hare-lynx, mouse-snake-owl and worm-robin-falcon. Moreover, predator-prey food chains have been studied in structured populations in [5,6]. In this paper, Model is a modification of model [7]. Then the conditions of existence and stability of the equilibrium points of the fractional-order are examined in the result and discussion. Numerical simulations are illustrated by the Grunwald-Letnikov approximation [8].

MATERIALS AND METHODS

Model Formulation

In this research, the predatory-prey model [7] is the main object of the study. The model construction is done by modifying the model of Sahoo and Poria [2] by changing the integer order into the fractional-order.

Determination of the Equilibrium Point

In dynamical analysis, the first step is to determine the equilibrium points. The equilibrium point is obtained when the population rate of the system is unchanged or zero. From this condition, the existence properties of each equilibrium points is also obtained.

Stability of the Equilibrium Point

In this paper, the local stability of equilibrium points is analyzed. The discussion of local stability is begun by linearizing the model by using Taylor series. The linearization around its equilibrium

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point is done to change the nonlinear model into linear form. Approximation of linear system using Taylors series will be in the form of Jacobian matrix. From the Jacobian matrix, it is determined the roots of the characteristic equation or eigenvalue. The determination of local stability can be obtained from the absolute of its eigen value argument.

Numerical Simulation

The behavior of the system [3] is described by numerical simulation. The numerical simulation approach uses the Grunwald-Letnikov scheme. The numerical simulations are conducted by using MATLAB software. An important step in this stage is to determine parameters that match the condition of existence and the stability of the equilibrium points. The behavior of local stability is visualized by graphic based on kinds of parameter values pointed. The last step at this stage is do the interpretation results of numerical simulation.

RESULT AND DISCUSSION

Model Formulation

In this model, definition of Caputo fractional derivative is used. In [9] Caputo's definition of fractional derivatives can be written as follows

$$D_t^\alpha f(t) = I_t^{n-\alpha} D_t^n f(t), \qquad n = 1,2,\dots$$
$$= \frac{1}{\Gamma(n-\alpha)} \int_a^t (t-s)^{n-1-\alpha} f^{(n)}(s) ds,$$

with $n-1 < \alpha < n$ and Γ is a Gamma function and $D_t = \frac{d}{dt}$.

Hastings and Powell [10] has discussed the food chain model of three species. The three species are prey population (X), intermediate-predator (Y) and top-predator (Z). The prey population is hunted by the intermediate-predator population and the top-predator hunts intermediate-predators. Both predation processes use the Holling Type II response function. R_0 and K_0 express the rate of growth and carrying capacity of the prey population. C_1 and C_2 are the interaction rates between prey populations, intermediate-predators with predators, top-predators. B_1 and B_2 are the rate of environmental protection to the prey and the intermediate-predator populations. A_1 and A_2 are the maximal predation rate of intermediate-predator and top-predator populations. D_1 and D_2 are the natural death rates of intermediate-predator and top-predator populations respectively. Mataouk *et al.* [10] modified the Hastings-Powell model [8] by changing the integer order into the fractional-order as follows:

$$\begin{aligned} D_t^\alpha X &= R_0 X\left(1 - \frac{X}{K_0}\right) - \frac{C_1 A_1 XY}{B_1 + X}, \\ D_t^\alpha Y &= \frac{A_1 XY}{B_1 + X} - \frac{A_2 YZ}{B_2 + Y} - D_1 Y, \\ D_t^\alpha Z &= \frac{C_2 A_2 YZ}{B_2 + Y} - D_2 Z, \end{aligned} \tag{1}$$

with $0 < \alpha < 1$.

Model (1) explains that top-predator food sources only depend on intermediate-predators. However, alternative prey (supplementary feeding) for top-predators can reduce predation rates in intermediate-predators [2], then to give this effect, model (1) can be modified to

$$\begin{aligned} D_t^\alpha X &= R_0 X\left(1 - \frac{X}{K_0}\right) - \frac{C_1 A_1 XY}{B_1 + X} \\ D_t^\alpha Y &= \frac{A_1 XY}{B_1 + X} - \frac{AA_2 YZ}{B_2 + Y} - D_1 Y \\ D_t^\alpha Z &= C_2 A_2 Z\left(\frac{AY}{B_2 + Y} + (1 - A)\right) - D_2 Z \end{aligned} \tag{2}$$

where A is a time independent constant to get the alternative resource $(0 < A < 1)$. To make easier the model analysis, variables and some parameter are selected to be

$x = \frac{X}{K_0}, y = \frac{C_1 Y}{K_0}, z = \frac{C_1 Z}{C_2 K_0}, \quad t = R_0 T, \; a_1 = \frac{A_1 K_0}{R_0 B_1}, \; a_2 = \frac{A_2 K_0 C_2}{B_2 R_0 C_1}, b_1 = \frac{K_0}{B_1}, \; b_2 = \frac{K_0}{B_2 C_1}, \; c = \frac{C_1 B_2}{K_0}, \; d_1 = \frac{D_1}{R_0}, \; d_2 = \frac{D_2}{R_0}$, where $a_1 = \frac{A_1 K_0}{R_0 B_1} > b_1 d_1 = \frac{K_0}{B_1}\frac{D_1}{R_0}$ or $A_1 > D_1$ and $a_2 = \frac{A_2 K_0 C_2}{B_2 R_0 C_1} > b_2 d_2 = \frac{K_0}{B_2 C_1}\frac{D_2}{R_0}$ or $A_2 C_2 >$,

and the non-dimensional version of model (2) is

$$\begin{aligned} D_t^\alpha x &= x(1-x) - \frac{a_1 xy}{1 + b_1 x} \\ D_t^\alpha y &= \frac{a_1 xy}{1 + b_1 x} - \frac{a_2 Azy}{1 + b_2 y} - d_1 y \\ D_t^\alpha z &= \frac{a_2 Azy}{1 + b_2 y} + (1 - A) a_2 cz - d_2 z \end{aligned} \tag{3}$$

and initial condition is $x(0) = x_0, y(0) = y_0, z(0) = z_0$.

Stability of Equilibrium Points

To determine the equilibrium points of differential equation (3) , let

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$$D_t^\alpha x = D_t^\alpha y = D_t^\alpha z = 0,$$

then the equilibrium points are

$E_1 = (0,0,0)$, $E_2 = (1,0,0)$, $E_3 = (\bar{x}, \bar{y}, 0)$, and $E_4 = (\hat{x}, \hat{y}, \hat{z})$ where
$\bar{y} = \frac{a_1-(b_1+1)d_1}{(a_1-b_1d_1)^2}$, $\bar{x} = \frac{d_1}{a_1-b_1d_1}$,
$\hat{x} = \frac{-(1-b_1)+\sqrt{(1-b_1)^2-4b_1\left(\frac{(a_1+b_2)(d_2-a_2c(1-A))-a_2A}{a_2A-(d_2-a_2c(1-A))b_2}\right)}}{2b_1}$,
$\hat{y} = \frac{-a_2c(1-A)+d_2}{a_2A-(-a_2c(1-A)+d_2)b_2}$,
$\hat{z} = \left(\frac{a_1\hat{x}}{1+b_1\hat{x}} - d_1\right)\left(\frac{1}{a_2A-(a_2c(1-A)+d_2)b_2}\right)$.

The Jacobian matrix of system (3) at the equilibrium point (x^*, y^*, z^*) is given by

$$J(x^*, y^*, z^*) = \begin{pmatrix} a_{11} & a_{12} & 0 \\ a_{21} & a_{22} & a_{23} \\ 0 & a_{32} & a_{33} \end{pmatrix}, \quad (4)$$

where $a_{11} = 1 - 2x^* - \frac{a_1y^*}{(1+b_1x^*)^2}$, $a_{12} = -\frac{a_1x^*}{1+b_1x^*}$, $a_{21} = \frac{a_1y^*}{(1+b_1x^*)^2}$, $a_{22} = \frac{a_1x^*}{1+b_1x^*} - \frac{a_2Az^*}{(1+b_2y^*)^2} - d_1$, $a_{23} = \frac{a_2Ay^*}{1+b_2x^*}$, $a_{32} = \frac{a_2Az^*}{(1+b_2y^*)^2}$, and $a_{33} = \frac{a_2Ay^*}{1+b_2y^*} + ca_2(1-A) - d_2$.

Theorem 1. The equilibrium E_1 of system (3) is always a saddle point.

Proof. The Jacobian matrix at E_1 is given by

$$J(E_1) = \begin{pmatrix} 1 & 0 & 0 \\ 0 & -d_1 & 0 \\ 0 & 0 & ca_2(1-A) - d_2 \end{pmatrix}.$$

Eigenvalues of matrix $J(E_1)$ are obtained by solving the characteristic equation

$$P(\lambda) = \det(J(E_1) - I\lambda) = (1-\lambda)(d_1-\lambda)(ca_2(1-A) - d_2 - \lambda) = 0.$$

The eigenvalues corresponding to the equilibrium E_1 are $\lambda_1 = 1 > 0$, $\lambda_2 = -d_1$, and $\lambda_3 = ca_2(1-A) - d_2$. Thus $|\arg(\lambda_1)| = 0 < \frac{\alpha\pi}{2}$, $|\arg(\lambda_2)| = \pi > \frac{\alpha\pi}{2}$, if $d_2 > ca_2(1-A)$ then $|\arg(\lambda_3)| = \pi > \frac{\alpha\pi}{2}$. Since $|\arg(\lambda_1)| = 0 < \frac{\alpha\pi}{2}$,

it follows from convergence of Mittag-Leffler function [9] that the equilibrium E_1 is always a saddle point.

Theorem 2. The equilibrium E_2 of system (3) is locally asymptotically stable if $\frac{a_1}{1+b_1} < d_1$ and $ca_2(1-A) < d_2$.

Proof. The Jacobian matrix of E_2 is given by

$$J(1,0,0) = \begin{pmatrix} -1 & -\frac{a_1}{1+b_1} & 0 \\ 0 & \frac{a_1}{1+b_1} - d_1 & 0 \\ 0 & 0 & ca_2(1-A) - d_2 \end{pmatrix}.$$

Eigenvalues of matrix $J(E_2)$ are obtained by solving the characteristic equation

$$P(\lambda) = \det(J(E_2) - I\lambda) = 0$$
$$= (-1-\lambda)\left(\frac{a_1}{1+b_1} - d_1 - \lambda\right)$$
$$(ca_2(1-A) - d_2 - \lambda) = 0.$$

The eigenvalues corresponding to the equilibrium E_2 are $\lambda_1 = -1 < 0$, $\lambda_2 = \frac{a_1}{1+b_1} - d_1$, and $\lambda_3 = ca_2(1-A) - d_2$. Thus $|\arg(\lambda_1)| = \pi > \frac{\alpha\pi}{2}$, if $\frac{a_1}{1+b_1} < d_1$ then $|\arg(\lambda_2)| = \pi > \frac{\alpha\pi}{2}$, if $d_2 > ca_2(1-A)$ then $|\arg(\lambda_3)| = \pi > \frac{\alpha\pi}{2}$.

It follows from convergence of Mittag-Leffler function [9] that the equilibrium E_2 of system (3) is locally asymptotically stable.

Furthermore, the equilibrium points E_3 and E_4 are discussed as follows. The Jacobian matrix at E_3 is given by

$$J(\bar{x}, \bar{y}, 0) = \begin{pmatrix} b_{11} & b_{12} & 0 \\ b_{21} & b_{22} & b_{23} \\ 0 & 0 & b_{33} \end{pmatrix}.$$

where $b_{11} = 1 - 2\bar{x} - \frac{a_1\bar{y}}{(1+b_1\bar{x})^2}$, $b_{12} = -\frac{a_1\bar{x}}{1+b_1\bar{x}}$, $b_{21} = \frac{a_1\bar{y}}{(1+b_1\bar{x})^2}$, $b_{22} = \frac{a_1\bar{x}}{1+b_1\bar{x}} - d_1$, $b_{23} = \frac{a_2A\bar{y}}{1+b_2\bar{y}}$, and $b_{33} = \frac{a_2A\bar{y}}{1+b_2\bar{y}} + ca_2(1-A) - d_2$. Eigenvalues of matrix $J(E_3)$ are $\lambda_1 = b_{33} = \frac{a_2A\bar{y}}{1+b_2\bar{y}} + ca_2(1-A) - d_2$ and the other λ_2, λ_3 are got by solving the characteristic equation

$$P(\lambda) = \lambda^2 - \omega_1\lambda + \omega_2 = 0$$

where $\omega_1 = 1 - 2\bar{x} - d_1 + \frac{a_1\bar{x}}{1+b_1\bar{x}} - \frac{a_1\bar{y}}{(1+b_1\bar{x})^2}$, $\omega_2 = \left(1 - 2\bar{x} - \frac{a_1\bar{y}}{(1+b_1\bar{x})^2}\right)\left(\frac{a_1\bar{x}}{1+b_1\bar{x}} - d_1\right) + \frac{{a_1}^2\bar{x}\bar{y}}{(1+b_1\bar{x})^3}$.

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The eigen values corresponding to the equation $P(\lambda)$ are $\lambda_2 = \frac{1}{2}(\omega_1 + \sqrt{\psi})$, $\lambda_3 = \frac{1}{2}(\omega_1 - \sqrt{\psi})$, where $\psi = (-\omega_1)^2 - 4\omega_2$. Thus, E_3 is locally asymptotically stable if it satisfies $|arg(\lambda_1)| = \pi > \frac{\alpha\pi}{2}$ by $\frac{a_2 A\bar{y}}{1+b_2\bar{y}} + ca_2(1-A) < d_2$, and λ_2, λ_3 are following one of the conditions

1. if $\psi = 0$ and $\omega_1 < 0$ then $\lambda_2, \lambda_3 < 0$ such that $\left|arg(\lambda_{2,3})\right| = \pi > \frac{\alpha\pi}{2}$,
2. if $\psi > 0$, $\omega_1 < 0, \omega_2 > 0$ and $\sqrt{\psi} < |\omega_1|$ then $\lambda_2, \lambda_3 < 0$ such that $\left|arg(\lambda_{2,3})\right| = \pi > \frac{\alpha\pi}{2}$,
3. if $\psi < 0$ then $\left|arg(\lambda_{2,3})\right| > \frac{\alpha\pi}{2}$.

To analyze the stability of equilibrium point E_4, first the Jacobian matrix at E_4 is evaluated by

$$J(\hat{x}, \hat{y}, \hat{z}) = \begin{pmatrix} a_{11} & a_{12} & 0 \\ a_{21} & a_{22} & a_{23} \\ 0 & a_{32} & a_{33} \end{pmatrix},$$

.

where $a_{11} = 1 - 2\hat{x} - \frac{a_1\hat{y}}{(1+b_1\hat{x})^2}$, $a_{12} = -\frac{a_1\hat{x}}{1+b_1\hat{x}}$, $a_{21} = \frac{a_1\hat{y}}{(1+b_1\hat{x})^2}$, $a_{22} = \frac{a_1\hat{x}}{1+b_1\hat{x}} - \frac{a_2 A\hat{z}}{(1+b_2\hat{y})^2} - d_1$, $a_{23} = -\frac{a_2 A\hat{y}}{1+b_2\hat{y}}$, $a_{32} = \frac{a_2 A\hat{z}}{(1+b_2\hat{y})^2}$, and $a_{33} = \frac{a_2 A\hat{y}}{1+b_2\hat{y}} + ca_2(1-A) - d_2$. Eigenvalues of matrix $J(E_4)$ are got by solving the characteristic equation

$$P(\lambda) = \det(J(E_4) - I\lambda) = \lambda^3 + K_1\lambda^2 + K_2\lambda + K_3 = 0$$

where

$$K_1 = -(a_{11} + a_{22} + a_{33}),$$
$$K_2 = a_{22}a_{33} + a_{11}a_{33} + a_{11}a_{22} - a_{32}a_{23} - a_{21}a_{12},$$
$$K_3 = a_{32}a_{23}a_{11} + a_{12}a_{23}a_{32} + a_{11}a_{22}a_{33}.$$

Let $D(P)$ is the discriminant of a polynomial $P(\lambda)$, it can be written

$$D(P) = -\begin{vmatrix} 1 & K_1 & K_2 & K_3 & 0 \\ 0 & 1 & K_1 & K_2 & K_3 \\ 3 & 2K_1 & K_2 & 0 & 0 \\ 0 & 3 & 2K_1 & K_2 & 0 \\ 0 & 0 & 3 & 2K_1 & K_2 \end{vmatrix},$$

$$D(P) = 18K_1K_2K_3 + (K_1K_2)^2 - 4K_3{K_1}^2 - 4{K_2}^3 - 27{K_3}^2.$$

Proposition

Let the equilibrium E_4 in $\mathbb{R}^3_+$. Then the equilibrium E_4 of system (3) is asymptotically stable if one of the following conditions [11] are satisfied

1. $D(P) > 0$, $K_1 > 0$, $K_3 > 0$,and $K_1K_2 > K_3$.
2. $D(P) < 0, K_1 \geq 0$, $K_2 \geq 0$, $K_3 > 0$,and $\alpha < \frac{2}{3}$.
3. $D(P) < 0, K_1 > 0$, $K_2 > 0$, $K_1K_2 = K_3$, and for all $\alpha \in (0,1)$.

Numerical Method and Simulations

Numerical method which is introduced by Grunwald and Letnikov [8] is used to solve nonlinear fractional differential equation [3]. As described in [8,12], by using the Grunwald-Letnikov approximation method, it is obtained the following nonstandard explicit scheme for system [3].

The parameters chosen in the first simulation are $a_1 = 2, a_2 = 0.1, b_1 = 2.5, b_2 = 2, d_1 = 0.6$, $d_2 = 0.02, c = 0.45, A = 0.8$, and $h = 0.1$. The aim of this simulation is to show that E_2 is stable. Figure 1 indicates the different values of α can decide the speed of convergence of solutions. The graph moves from the initial

$$\begin{aligned} x_{n+1} &= h^\alpha f(x_n, y_n, z_n) - \sum_{j=0}^{m+1} (-1)^j \binom{\alpha}{j} x_{m+1-j} - \frac{\left((m+1)h\right)^{-\alpha}}{\Gamma(1-\alpha)} x_0 \\ &= h^\alpha \left(x_n(1-x_n) - \frac{a_1 x_n y_n}{1+b_1 x_n}\right) - \sum_{j=0}^{m+1} (-1)^j \binom{\alpha}{j} x_{m+1-j} - \frac{\left((m+1)h\right)^{-\alpha}}{\Gamma(1-\alpha)} x_0 \\ y_{n+1} &= h^\alpha g(x_n, y_n, z_n) - \sum_{j=0}^{m+1} (-1)^j \binom{\alpha}{j} y_{m+1-j} - \frac{\left((m+1)h\right)^{-\alpha}}{\Gamma(1-\alpha)} y_0 \\ &= h^\alpha \left(\frac{a_1 x_n y_n}{1+b_1 x_n} - \frac{a_2 A z_n y_n}{1+b_2 y_n} - d_1 y_n\right) - \sum_{j=0}^{m+1} (-1)^j \binom{\alpha}{j} y_{m+1-j} - \frac{\left((m+1)h\right)^{-\alpha}}{\Gamma(1-\alpha)} y_0 \\ z_{n+1} &= h^\alpha h(x_n, y_n, z_n) - \sum_{j=0}^{m+1} (-1)^j \binom{\alpha}{j} z_{m+1-j} - \frac{\left((m+1)h\right)^{-\alpha}}{\Gamma(1-\alpha)} z_0 \\ &= h^\alpha \left(\frac{a_2 A z_n y_n}{1+b_2 y_n} + a_2 z_n c(1-A) - d_2 z_n\right) - \sum_{j=0}^{m+1} (-1)^j \binom{\alpha}{j} z_{m+1-j} - \frac{\left((m+1)h\right)^{-\alpha}}{\Gamma(1-\alpha)} z_0 \end{aligned}$$

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condition $(0.5, 1.0, 0.75)$), then the solution convergent to equilibrium point $\mathrm{E}_2(1, 0, 0)$. If the value of α is approaching to one then the convergence of the rate of change of the three populations is faster and vice versa.

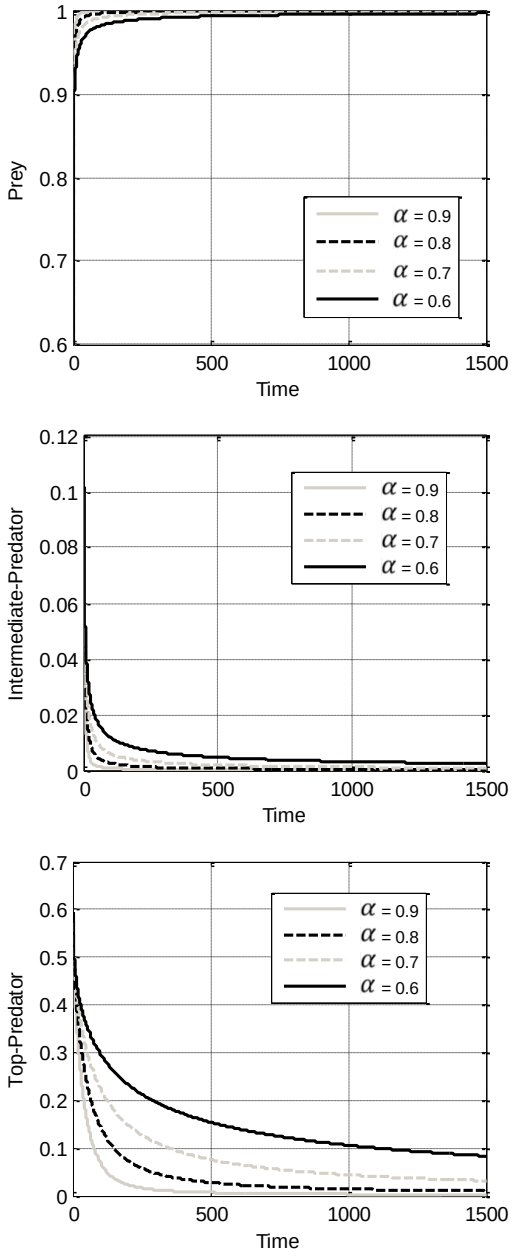

Figure 1 Stability of the equilibrium E_1 for $\alpha = 0.6, \alpha = 0.7, \alpha = 0.8$ and $\alpha = 0.9$

Parameters used in Figure 2 are $a_1 = 5, a_2 = 0.9, b_1 = 3, b_2 = 2.5, d_1 = 0.6, d_2 = 0.3, c = 0.45, A = 0.6$ and $h = 0.1$. According Matignon's condition [13], stability of the equilibrium E_3 is stabilized by $\alpha^* = 0.94$. The initial condition of Figure 2 is $(1, 0.8, 0.5)$ and the solution is stable at point $(0.461, 0.414, 0)$ for $\alpha = 0.9$, and it is unstable for $\alpha = 0.95$. From this simulation, it can be concluded that the stability of the equilibrium point of the fractional-order model depends on the parameter of α if $\alpha^* > \alpha$ then the equilibrium E_3 is stable. Conversely if $\alpha^* < \alpha$ then the equilibrium E_3 is unstable.

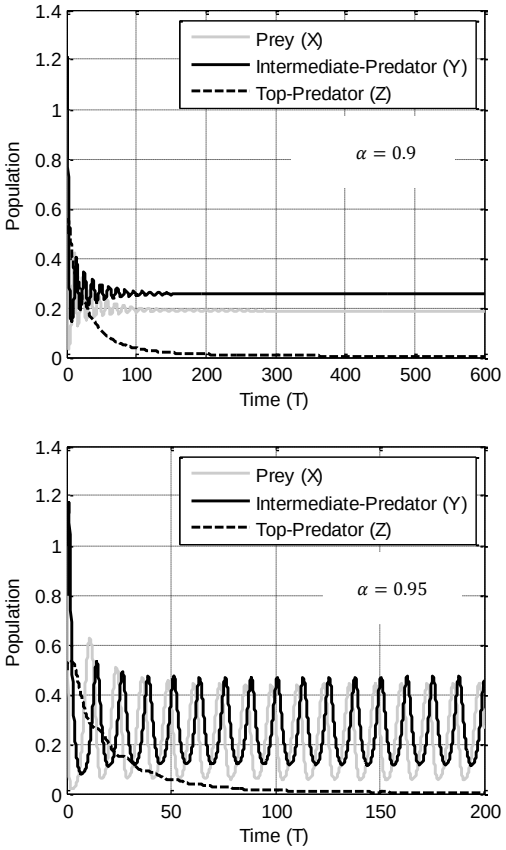

Figure 2. Stability of the equilibrium E_3 for $\alpha = 0.9$ and $\alpha = 0.95$

In Figure 3, some parameters are set as $a_1 = 3, a_2 = 0.1, b_1 = 2.5, b_2 = 2, d_1 = 0.6, d_2 = 0.02, c = 0.45$, and $h = 0.2$. With initial conditions $(1, 0.5, 1.5)$ and different value of $A = 1$, $A = 0.9$, and $A = 0.8$, then the solution convergent to $(0.6, 0.333, 2)$, $(0.71, 0.26, 2.89)$ and $(0.812, 0.189, 3.518)$ respectively. These simulations explain that, if there is no alternative food $(A = 1)$, the number of top-predator population decreases compared with the presence of alternative food $(0 < A < 1)$. When the value of $A = 1$ indicates that top-predator population doesn't perform activities to search for additional food and the food source of the top-predator population depends only on the intermediate-predator population. However, in this case the three populations still survive in a ecosystem. On the other hand, if top-predator eat only alternative food $(A = 0)$ then the top-

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predator can grow without bound (simulation not shown in this paper).

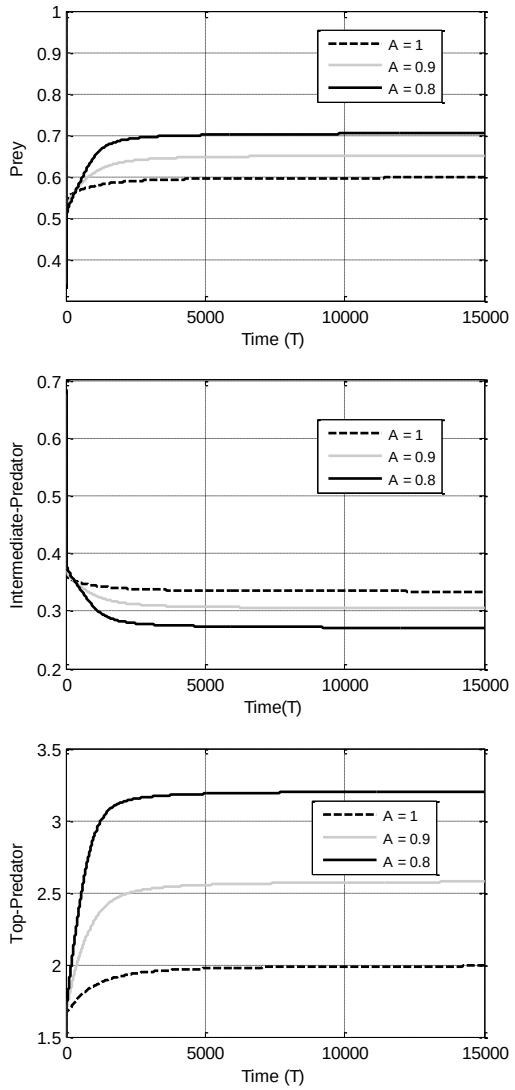

Figure 3. Stability of the equilibrium E_4 for $\alpha = 0.65$ and $A = 0.8, A = 0.9,\ A = 1$

CONCLUSION

In this work, the Hastings–Powell food chain model with alternative foods has been modified into a system of fractional-order. The local stability of all the equilibrium points of the fractional-order system is investigated. Numerical simulation results agree with the analytical result. It is also found that the fractional parameter α has effects on the stability of solution behavior. Furthermore, our analysis predicts that providing a suitable amount of alternative food has a positive impact for top-predator population.

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The Effect of Organic Stimulant and Inorganic Fertilizer on Two Rice Varieties (*Oryza sativa* L.)

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Abstract

Increased the yield of rice could be done by organic stimulant application and inorganic fertilizer, as well as the used of appropriate variety. The field experiment was aimed to study the interaction of fertilizer application and the using of variety on rice. The research was conducted at April until July 2016 in Sekarputih Hamlet, Pendem Village, Junrejo District, Batu City. This research used the Randomized Complete Block Design (RCBD) Factorial methods with 2 factors are Rice Variety (V) and Fertlizers Doses (P) with 3 replications. Cows Biourine application by dissolved 1 L biourine with 10 L water sprayed on soil and plants. EM-4 application was doing by dissolved 100 cc EM-4 with 10 L water sprayed on soil. The result of research showed that interaction of Mapan-P.05 hybrid variety (V_2) added with fertilizer doses 100 kg phonska+100 kg urea+cows biourine+EM-4 (P_6) can increase yield on rice in parameter 1000 grain weight to 15.29% against which added fertilizer doses 200 kg phonska+200 kg urea (P_1).

Keywords: Inorganic Fertilizer, Organic Stimulant, Rice, Variety.

INTRODUCTION

Rice is the crop plants which produced the rice as the staple food for Indonesian people. Population density increased 1.36% year by year assumed in 2020, total rice needed are 35.97 million ton with consume assumption is 137 kg capita^{-1}[1]. Two approached for increasing yield of rice are by used hybrid variety and improved cultivation [2]. Hybrid technology reached by heterosis utilization causing a more vigour F1 plant, which can increased to 1 ton ha^{-1}; higher than inbreed rice [3].

The balanced fertilization can stimulate the-optimization on the growth of plant [4]. Biourine are plant fertile organic matter made from cow urine and cow faeces with addition of nutrition which used microorganism [5]. Biourine application can enhanced plant height, number of leaves per plant, leaf area and leaf area index as 5.1%, 6.8%, 11.9% and 10.2% respectively, higher than without biourine application [6].

To enhance the yield and land productivity, EM-4 (*Effective Microorganisme*) can be used. It utilize microorganism technology to repair land fertility and soil physics characters [7]. Thus, this study aims to study the interaction between variety of rice and fertilizer to increased the growth and yield of rice.

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MATERIALS AND METHODS

The research was conducted in April to July 2016, in the Sekarputih Hamlet, Pendem Village, Junrejo District, Batu City. The area elevation is 600 m above sea level. Soil types is Andisol with N 0.13% (low), C Organic 1.22% (low), C/N ratio 10 (low), P 6.87 ppm (low), K 0.33 (low) and pH 5.7. Materials used in this study are the seeds of rice variety Ciherang and Mapan-P.05, cow biourine, EM-4, Urea (46% N), Phonska (15:15:15 NPK) and chemical pesticides. The method used was a Randomized Complete Block Design (RCBD) factorial with 2 factors repeated 3 times.

Table 1. Treatment Methods

Factor 1	V_1: Ciherangrice variety V_2: Mapan-P.05 rice variety
Factor 2	P_1: 200 kg Phonska+200 kg urea ha^{-1} P_2: Cow Biourine P_3: EM-4 P_4: 100 kg Phonska+100 kg urea ha^{-1} + Cow Biourine P_5: 100 kg Phonska+100 kg urea ha^{-1} + EM-4 P_6: 100 kg Phonska+100 kg urea ha^{-1} + Cow Biourine + EM-4

Land preparation was doing by ploughed with tractor. Land area was 295 m^2 made into 36 partition with 3 x 2 m^2 dimension. Manual planting with one seed per hole. Seedlings used rice aged 20 dap (day after planting) or has 3-4 leaves and stem was look bigger.

Inorganic Fertilizer Application

Fertilizer application was doing at 0 dap with doses of urea are ¼ doses from total doses. Next

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fertilizing of urea was doing at 20, 40, 60 dap with each doses is ¼ doses of the total dose. Phonska application was done at 10 dap.

Biourine Application

Biourine made from fresh urine and feces of cows in the morning. Cows urine were putted on the bottle with dosage 1 L urine, cows feces were putted on the receptacle with dosage about 5 kg feces and then added 30 L water, stired in earlier morning for 7 days. Biourine ready to be used when it scentless and colder than before, and there is a wax layer on the surface of biourine.

Biourine application was doing by dissolved 1 L biourine with 10 L water sprayed on soil and plants. Recommendation doses of cow's biourine are 2000 L ha $^{-1}$ for three times: at 20 dap as 400 L ha^{-1}, at 40 dap as 600 L ha^{-1}, and at 60 dap as 1000 L ha^{-1}.

EM-4 Application

EM-4 application was doing by dissolved 100 cc EM-4 with 10 L water sprayed on soil. Recommendation doses of EM-4 are 600 L ha $^{-1}$ giving by 34 times: at 14 dap as 100 L ha^{-1}, at 28 dap as 125 L ha^{-1}, at 42 dap as 175 L ha^{-1} and 56 dap as 200 L ha^{-1}.

Observations made on the growth and the yield of rice plants. For non-destructive observation variables include the high of the plant, number of leaves and number of tillers per plant. Destructive observations include leaf area, leaf area index, dry weight of total plant, crop growth rate and net assimilation rate. The observations were performed at 49, 63, 77 and 91 days after planting. Harvest observations include the number of panicles on the harvested crop, percentage of grain, 1000 grain weight, dry weight of total solar plants, grain weight harvest, and harvest index.

Data Analysis

Data obtained from observations then analyzed using analysis of variance (F test) with level of 5%. If there is a significant difference, then followed by HSD (Honest Significant Difference) test with a level of 5%.

RESULT AND DISCUSSION

Interaction Influences between Variety Treatment and Adding Fertilizer on Growth and Yield of Rice

There is an interaction on plant height result of Ciherang variety (V_1) respond to a higher plant height with adding fertilizer doses 100 kg phonska+100 kg urea+cow biourine+EM-4 (P_6) against cow biourine treatment (P_2). Mapan-P.05 variety (V_2) gave respons to fertilizer doses 100 kg phonska+100 kg urea+cowbiourine (P_4) and fertilizer doses 100 kg phonska+100 kg urea+cow biourine+EM-4 (P_6) by produced higher plant height than fertilizer doses 200 kg phonska+200 kg urea treatment (P_1) (Table 2). Organic matter combination and inorganic fertilizer caused plant growth and yield more higher to 91% for Ciherang variety and increased 78% for hybrid variety than control treatment [8]. Yield of Mapan-P.05 hybrid variety higher than Ciherang variety are no needed higher measurement and frequency, means higher fertilizer efficiency on hybrid variety [9].

There is an interaction on dry weight total plant result Mapan-P.05 variety (V_2) respons to fertilizer application with doses 100 kg phonska+100 kg urea+cow biourine+EM-4 (P_6) produce dry weight total plant higher than EM-4 treatment (P_3) (Table 3). Mapan-P.05 variety if giving organic stimulant and inorganic fertilizer, can produced higher dry weight total plant than just giving EM-4 only. Just Mapan-P.05 hybrid variety from 13 other hybrid variety resulted higher dry weight total plant consistently against which Ciherang variety on some locations at wet or dry season [9].

There is an interaction on 1000 grain weight parameter on Mapan-P.05 variety (V_2) giving respons to fertilization doses 100 kg phonska+100 kg urea+cow biourine+EM-4 (P_6) produced higher 1000 grain weight than fertilizer doses 200 kg phonska+200 kg urea (P_1) and 100 kg phonska+100 kg urea+EM-4 treatment (P_5) (Table 4). Total 1000 grain weight influences by environment factors, especially at grain maturity phase. Total 1000 grain weight is the number of biomass consist on grain [10]. Mapan-P.05 variety produce higher 1000 grain weight when added organic stimulant like cow biourine and EM-4, recommend to obtained higher yield of hybrid rice needed to combine inorganic fertilizer (75%) with organic fertilizer (25%) on spacing at 20 cm x 20 cm [11]. Combination of inorganic fertilizer and organic fertilizer can stablished the sustainability of growth, yield and nutrient uptake hybrid rice [12].

Effect of Variety Treatment to Growth and Yield of Rice

In the yield of rice, Mapan-P.05 variety produced grain harvest weight and harvest index higher than Ciherang variety (V_1) (Table 5). Grain harvest weight from this research on Ciherang variety reach 9.54 ton ha^{-1}, whereas on previous study, Ciherang variety resulted higher to 9.90

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ton ha^{-1}[9]. For Mapan-P.05 variety in this research, the grain harvest weight reached 13.30 ton ha^{-1}, while previous study for Mapan-P.05 hibryd variety just resulted 10.52 ton ha^{-1} [9]. Thus it can be said that result of this research were higher in hybrid variety Mapan-P.05 than previous study [9].

Effect of Organic Stimulant and Inorganic Fertilizer on Growth and Yield of Rice

Addition of doses fertilizer 100 kg phonska+100 kg urea+cows biourine (P_4) resulted higher amount of penicles than EM-4 treatment (P_3) (Table 5). Organic matter such as EM-4 can not produce maximal when apply without adding inorganic fertilizer. The organic matter in the area of study has very low nutrient soil thus less supported to the growth and yield of rice. However, we can repair the physical and biological characteristics of the soil properties [13].

Giving fertilizer with doses 100 kg phonska+100 kg urea+cows biourine (P_4) resulted higher grain harvest weight than cows biourine treatment (P_2) (Table 5). Combination of biourine concentration 1 L urine + 5 kg feces + 15 L water ha^{-1} and inorganic fertilizer with doses 50 kg N, 12.5 kg P_2O_5, 17.5 kg K_2O resulting amount of tuber on shallot increased to 27.33% than without biourine concentration and inorganic fertilizer doses [4]. Giving organic fertilizer 50% and inorganic fertilizer 50% produced optimal growth and yield on rice [14]. Organic fertilizer can reduce the inorganic fertilizer uses as 25% on rice field, by not decreasing the growth and yield on rice. It also can repairs the physical, biological and chemical soil properties [15].

Table 2. Average Plant Height per plant on rice result due to interaction Variety Different and Adding Fertilizer at 35 DAP

Rice Variety	Plant Height (cm)					
	P_1	P_2	P_3	P_4	P_5	P_6
Ciherang (V_1)	40.83 abcd	37.33 a	38.91 ab	38.91 ab	39.58 abc	44.92 bcde
Mapan-P.05 (V_2)	43.25 abcd	48 de	46.75 cde	51.25 e	47.67 de	50.83 e
HSD 5%	7.27					
CV (%)	5.56					

Notes: Numbers with same letters are not significantly different at 5% level using HSD test, CV= Coefficient of Variation

P_1 = 200 kg Phonska+200 kg urea
P_2 = Cow Biourine
P_3 = EM-4
P_4 = 100 kg Phonska+100 kg urea + Cow Biourine
P_5 = 100 kg Phonska+100 kg urea + EM-4
P_6 = 100 kg Phonska+100 kg urea + Cow Biourine + EM-4

Table 3. Average Dry Weight Total Plant per plant on rice result due to Variety Different and Adding Fertilizer at 91 DAP

Rice Variety	Dry Weight Total Plant (g)					
	P_1	P_2	P_3	P_4	P_5	P_6
Ciherang (V_1)	121.23 abc	102.78 a	108.51 ab	126.08 abcd	137.23 abcde	152.29 abcde
Mapan-P.05 (V_2)	133.08 abcde	177.44 de	116.38 abc	166.65 cde	154.57 bcde	179.81 e
HSD 5%	51.37					
CV (%)	12.38					

Notes: Numbers with same letters are not significantly different at 5% level using HSD test, CV= Coefficient of Variation

P_1 = 200 kg Phonska+200 kg urea
P_2 = Cow Biourine
P_3 = EM-4
P_4 = 100 kg Phonska+100 kg urea + Cow Biourine
P_5 = 100 kg Phonska+100 kg urea + EM-4
P_6 = 100 kg Phonska+100 kg urea + Cow Biourine + EM-4

Table 4. Average 1000 grain Weight per plant on rice result due to Variety Different and Adding Fertilizer at 115 DAP

Rice Variety	1000 Grain Weight (g)					
	P_1	P_2	P_3	P_4	P_5	P_6
Ciherang (V_1)	27.52 ab	27.14 a	26.68 abc	27.67 abc	29.12 abc	28.42 abc
Mapan-P.05 (V_2)	29.82 abcd	33.05 de	31.15 bcde	31.40 cde	29.58 abcd	34.38 e
HSD 5%	3.87					
CV (%)	4.39					

Notes: Numbers with same letters are not significantly different at 5% level using HSD test, CV= Coefficient of Variation

P_1 = 200 kg Phonska+200 kg urea
P_2 = Cow Biourine
P_3 = EM-4
P_4 = 100 kg Phonska+100 kg urea + Cow Biourine
P_5 = 100 kg Phonska+100 kg urea + EM-4
P_6 = 100 kg Phonska+100 kg urea + Cow Biourine + EM-4

Soil conditions

C-Organic and N-total increased at all fertilizer treatment (Table 6). Higher C-Organic available on treatment fertilizer doses 100 kg phonska+100 kg urea+cows biourine (P_4) increased to 37.70% compared with before treatment. Higher N-total available on treatment fertilizer doses 100 kg phonska+100 kg urea+cows biourine+EM-

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4 (P_6) increased 61.54% compared with before treatment. It is caused by the addition of N fertilizer with high dosages which provide higher N-total on soil [16].

Table 5. Average Yield of Rice for each treatment Variety Different and Adding Fertilizer

Treatments	Average Yield of Rice			
	Amount of Panicles	Grain Percentage (%)	Grain Harvest Weight (t ha^{-1})	Harvest Index (%)
Rice Variety				
Ciherang (V_1)	17.1	83.5	9.54 a	27.83 a
Mapan-P.05 (V_2)	17.08	79.8	13.30 b	33.17 b
HSD 5%	ns	ns	2.38	4.74
Adding Fertilizer (ha^{-1})				
200 kg Phonska+200 kg urea (P_1)	17.94 ab	85.31	12.51 ab	29.5
Cows Biourine (P_2)	15.44 ab	78.98	8.92 a	27.45
EM-4 (P_3)	14.36 a	83.13	10.01 ab	29.29
100 kg Phonska+100 kg urea + Cows Biourine (P_4)	19.81 b	83.76	13.12 b	32.84
100 kg Phonska+100 kg urea + EM-4 (P_5)	17.47 ab	75.11	12.46 ab	29.6
100 kg Phonska+100 kg urea + Cows Biourine + EM-4 (P_6)	17.53 ab	83.6	11.51 ab	34.33
HSD 5%	4.52	ns	4.12	tn
CV (%)	12.58	7.26	17.21	12.83

Note : Numeral within columns followed by same letters are not significantly different at 5% level using HSD test; ns:non significant; CV=Coeffiecient of Variation

Table 6. Soil Analysis Before and After Treatments

Parameters	pH	C-Organic (%)	N-Total (%)	C/N	P(mg kg^{-1})	K(me 100 g^{-1})
Before treatment	5.7	1.22	0.13	10	6.87	0.33
After treatment						
V_1P_1	5.3	1.35	0.17	8	3.03	0.23
V_1P_2	5.3	1.36	0.14	9	7.63	0.35
V_1P_3	5.4	1.36	0.15	9	1.53	0.42
V_1P_4	5.9	1.68	0.17	10	11.33	0.41
V_1P_5	5.5	1.27	0.15	8	3.81	0.29
V_1P_6	5.4	1.53	0.16	10	2.29	0.44
V_2P_1	5.6	1.28	0.15	8	1.53	0.22
V_2P_2	5.6	1.44	0.16	9	2.28	0.41
V_2P_3	5.9	1.34	0.17	8	1.51	0.43
V_2P_4	5.6	1.27	0.15	8	1.52	0.32
V_2P_5	6.0	1.51	0.17	9	2.27	0.51
V_2P_6	5.6	1.66	0.21	8	3.14	0.36

CONCLUSION

There is an interaction on Mapan-P.05 hybrid variety with fertilizer doses 100 kg phonska+100 kg urea+cows biourine+EM-4 on yield parameters are 1000 grain weight with percentage of increase as 15.29% compare with fertilizer doses 200 kg phonska+200 kg urea. Mapan-P.05 hybrid variety gives the significant influences on grain harvest weight and harvest index; each increase percentage as 39.41% and 27.83% compared with Ciherang variety. Fertilizer doses 100 kg phonska+100 kg urea+cows biourine increasing amount of panicles and grain harvest weight with increase percentage as 37.95% and 47.08% compare with EM-4 and cows biourine treatment.

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Phytochemical and Histochemical Screening of Toxic Plant Based on Knowledge of Tengger Tribe in Ngadiwono Village, Pasuruan

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Abstract

Around hundreds of plant's species have been confirmed to produce toxic that could endanger the life of human or domestic animals. Some of those plants have potentials to be developed as medicine. The objective of this study is to reveal the secondary metabolite content of toxic plants mentioned by Tengger tribe on Ngadiwono village through phytochemical and histochemical screening test. This study was conducted on Ngadiwono village, Tosari district, Pasuruan Regency. Informants were selected using snowball method by following the information of key person (n informant=14). Plants were selected and collected according to the local knowledge, then was analyzed qualitatively for its secondary metabolite content through phytochemical and histochemical screening test. The results showed that there are 8 plant species categorized as toxic by local people, including Bedor (*Girardinia palmata*), Kecubung Bunga Kuning (*Brugmansia suaveolens*), Kecubung Bunga Putih (*Brugmansia suaveolens*), Jarak (*Ricinus communis*), Terpasan Kuning (*Cestrum elegans*), Terpasan Merah (*Cestrum elegans*), Kudisan (*Euphorbia pulcerrima*), and Ciplukan (*Physalis peruviana*). The phytochemical result indicated that all toxic plants mentioned by the local people contain alkaloid substancess, while histochemical test showed that alkaloid substances were found in leaf trichomes, except in Terpasan Merah (*Cestrum elegans*).

Keywords: Fitochemical, Histochemical, Tengger Tribe, Toxic Plant.

INTRODUCTION

Most of the Tengger Tribe live by depending on the environment, including the utilization of plants and animals' biodiversity [1]. The knowledge of Tengger tribe about land and resource management is mainly affected by history, custom, and available resources [2]. The biological resources utilized by Tengger Tribe is including plants that have been utilized by human all over the world for a long time. Therefore, the interaction between human and plant is very important [3].

The knowledge of plants becomes very important recently along with the appearance of many kinds of diseases that threaten the human life. For example is the production of new medicines developed from coumpounds in plants. There are more than hundreds toxic plants, and some of them have potentials to be developed as medicine. Some species of toxic plants also utilized as pest control, such as nicotine in *Nicotiana* sp., seed extract of *Baringtonia* sp., and rotenoid in *Derris* spp. [4]. *Chondodendron tumentosum* contains of toxic alkaloid substancess called D-Tubocurarine. This toxic usually used by Indian society in Amazon to create poisoned arrows. Further, that substances is developed as muscle relaxant for surgery [5]. Surprisingly, all of those natural substancess become the basic for developing new medicine with a better quality. Reflecting the potency of secondary metabolite in toxic plants as revealed by previous study, therefore the aim of this study is to identify secondary metabolite in toxic plants according to the information from Tengger tribe in Ngadiwono village.

MATERIALS AND METHODS

Study Area

This study was performed on Ngadiwono village, Tosari district, Pasuruan regency, East Java, Indonesia (34°20'35.29" E - 35°09'27.04" E and latitudes 0°05'19.12"N - 0°53'53.81" N). Ngadiwono village is a buffer zone of Bromo Tengger Semeru National Park that comprised of 4 subdistricts: Ledoksari, Krajan, Ketuwon, and Banyu Meneng. Total area of the village is 639.03 ha. The distance between settlement area and forest is 2 km. The minimum temperature reached 10°C. Total number of male is 1097 individual, while female is 1474 individual, and the population density is 402 [6].

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Data Collection

Data of toxic plant species was collected using semi-structured and in-depth interview. Fourteen informants were determined using snowball method. The researchers were accompanied by local people during data and sample collection. Data collection was terminated if it already got saturated data [7]. Stems, leaves, seeds, and flowers were collected for each species. Secondary metabolite test was conducted by following the procedure described below.

Sample Preparation

Leaves were dried using oven in the temperature of 60° for 2 days. While seeds were dried using oven in the temperature of 60° for 3 days.

Alkaloid Test

Two grams of sample powder were extracted using small amount of chloroform. Sample was then added with 10 mL of chloroform-ammonia and was filtered. The collected filtrate was added with drops of H_2SO_4 2M, homogenized until it formed 2 layers. Acid layer (colorless) was moved into three new glasses reaction. Each solvent was tested using drops of Dragendorf, Mayer, and Wagner reagents. The results were categorized as positive if the solvent forming precipitate with color of orange (Dragendorf), yellowish white (Mayer), and brown (Wagner) [8].

Flavonoid Test

Samples were soaked in N-Hexane and filtered. The residue was added with N-Hexane and filtered. This procedure was conducted repeatedly until the filtrate color turned into colorless. Then, filtrate was added with methanol, filtered, added concentrated HCl and Mg powder. If the filtrate form red brick precipitate, then the plant positively contains flavonoid [8]

Terpenoid and Steroid Test

Samples were soaked in N-Hexane and filtered. Then, filtrate was evaporated until forming residue. Filtrate was then added with chloroform 0.5 mL, acetic acid anhydrous 0.5 mL, and concentrated H_2SO_4 1-2 mL. If it forms reddish purple precipitate, then the plant positively contains terpenoid. In contrary, green precipitation means that the plant positively contains steroid [9].

Tannin and Saponin Test

Samples were soaked in N-Hexane, filtered, and added with N-Hexane until its color turned into green. Then, filtrate was added methanol, filtered, added ethanol, and filtered again. Ethanol filtrate was divided into two tubes. The first tube was added with $FeCl_3$ 3. If the filtrate turns its color into blackish green, then the plant positively contains tannin. The second tube was heated, then shaken. If it produces foam, then the plant is positive to contain saponin [10].

Histochemical Test of Transverse Leaf Sections

Leaf samples were cut into transverse section about 20-25 µm using microtome. The section was examined using reagents and then observed under Olympus BX51 microscope. Alkaloid content was examined using Bauchardat reagent. The positive result of alkaloid is indicated by the presence of reddish brown or yellow [11].

RESULT AND DISCUSSION

Phytochemical Analysis

The result of this study revealed 8 toxic Plants that usually utilizes by local people. They are bedor (*Girardinia palmata*), terpasan merah (*Cestrum elegans*), terpasan kuning (*Cestrum elegans*), jarak (*Ricinus communis*), kecubung putih (*Brugmansia suaveolens*), kecubung kuning (*Brugmansia suaveolens*), ciplukan (*Physalis peruviana*), and Kembang kudis (*Euphorbia pulcherrima*). The toxic parts and the symptoms of poisoning of the plants mentioned above is presented in Table 1. The results of phytochemical screening on plants considered to be toxic by the Ngadiwono villagers are presented in Table 2.

Phytochemical tests performed are qualitative, so that the results only able to describe the group of substancess without more specific information about the type and concentration of secondary metabolite. Alkaloid test on jarak seeds (*Ricinus communis*) produces more alkaloid precipitate according to Gupta [12], risins, such as toxalbumin, phorbol, and cyanic acid. Kecubung (*Brugmansia suaveolens*) contains alkaloid scopolamine [13].

The results of phytochemical screening showed that all toxic plants mentioned by local people contained alkaloid substancess. Petersen [14] described several types of alkaloid substancess based on the structure of the molecular ring as well as 12000 alkaloid chemicals. Each alkaloid substancess will cause different symptoms of poisoning. The general symptoms of alkaloid poisoning were fever, anxiety, dilated pupils, reddened skin, dry skin, hallucinations and gastrointestinal symptoms. That symptoms could be occurred in both human and livestock. The screening results also found steroids and terpenoids in the toxic plants. Previous study stated that all those substancess are toxic to stomach [15].

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Table 1. List of Toxic Plant and Symptoms of Poisoning

Common Name	Scientific Name	Family	Toxic Parts	Symptoms of Poisoning
Bedor	*Girardinia palmata*	Urticaceae	Thorns at leaves and stems	Burning sensation in the skin.
Terapasan Merah	*Cestrum elegans*	Solanaceae	Stems, leaves, and flowers	Abdominal bloating and death in livestock.
Terpasan Kuning	*Cestrum elegans*	Solanaceae	Stems, leaves, and flowers	Abdominal bloating and death in livestock.
Jarak	*Ricinus communis*	Euphorbiaceae	Leaves and seeds	Abdominal bloating in livestock. Seeds cause hallucinations in human.
Kecubung putih bunga tidak rangkap/kecubung hitam	*Brugmansia Suaveolens*	Solanaceae	Leaves, seeds	Abdominal bloating in livestock. Seeds cause hallucinations in human.
Kecubung Kuning	*Brugmansia Suaveolens*	Solanaceae	Leaves, seeds	Abdominal bloating in livestock. Seeds cause hallucinations in human.
Ciplukan	*Physalis peruviana*	Solanaceae	Leaves	Abdominal bloating in livestock.
Kembang kudis	*Euphorbia pulcherrima*	Euphorbiaceae	Stem sap	Itching on exposed skin.

Table 2. Phytochemical Identification Result of Secondary Metabolite Substances in Toxic Plants at Ngadiwono village

Common Name	Secondary Metabolite Substances					
	Alkaloids	Flavonoids	Tannins	Steroids	Terpenoids	Saponins
Bedor	+	-	-	+	-	-
Terpasan Merah Leaves	+	-	-	+	+	-
Terpasan Kuning Leaves	+	+	-	+	-	-
Jarak Leaves	+	-	-	+	-	-
Jarak Seeds	++	-	-	-	+	-
Kecubung Putih Leaves	+	+	-	+	+	-
Kecubung Putih Seeds	++	-	-	+	+	-
Kecubung Kuning Leaves	+	-	-	-	+	-
Kecubung Kuning Seeds	++	-	-	-	+	-
Ciplukan Leaves	+	-	-	+	+	-
Kembang Kudis Leaves	+	-	-	-	+	-

Note: presence (+); plentiful (++); and absence (-)

Histochemical Analysis of Toxic Plant

The result of this study showed that several plants cause different poisoning symptoms. Bedor has thorn that causes itching if touched by the skin. Previous study by Hidayat also revealed that Urtica Family, such as Bedor, have trichomes specialized into hair that could induce itchiness [16]. Trichome are made up of long, broad-based cells that swell, narrowed, and pointed for its top (Figure 1). The tapered end wall contains silica, while the lower part contains calcium. If it is touched, the rounded part on the end of tapered part will break at the boundary are, and the pointed part will be easily cut through the skin and then the chemical substances (histamine and acetylcholine) will cause itchiness.

Histochemical identification of *Brugmansia suaveolens* leaves found alkaloid substances at the trichome of capitate gland. The trichome of capitate gland comprised of 1 to 2 head cells with varying stalks, from the shortest until the longest contained 1 to 3 cells. That results also found in various plant species, such as *Lavandula pinnata* L. [16]. Trichome cells at peltate and capitate glands in *T. quinquecostatus* secretes substances that is similar to alkaloids [17]. On the leaves of *Physalis peruviana*, trichomes of uniseriate gland was found to be containing alkaloid. Alkaloid substances is found at the cross-sectional of *Cestrum elegans* leaves (yellow flower) in the secretory epidermis. In contrary, *Cestrum elegans* leaves (red flower) did not have it.

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According to the result of phytochemical test, *Cestrum elegans* leaves contained alkaloid substances. In contrary, histochemical result did not detect any alkaloid compond in the plant's tissue. The presentage of metabolite concentra-tion is predicted to be increasing along with the growth of the plants. However, adult organs have higher concentration compared to old organs that is experiencing degradation on its secretion structure [18]. Leaves samples used in this study did not collected based on the leaves age, so it affected the substances concentration in each leaves tissue. Besides, phytochemical test used the whole leaves, while histochemical test only observed the secretory structure of leaves tissue.

Basically, all plants experiencing secretion. Secretion is the event of separation of a number of substances from protoplasm or isolation inside several protoplasm. The secreted substances could be in from of excessive ion that is separated in a form of salt. Excessive assimilation could be issued as sugar or substances in the cell wall, such as lignin, suberin, and chitin.

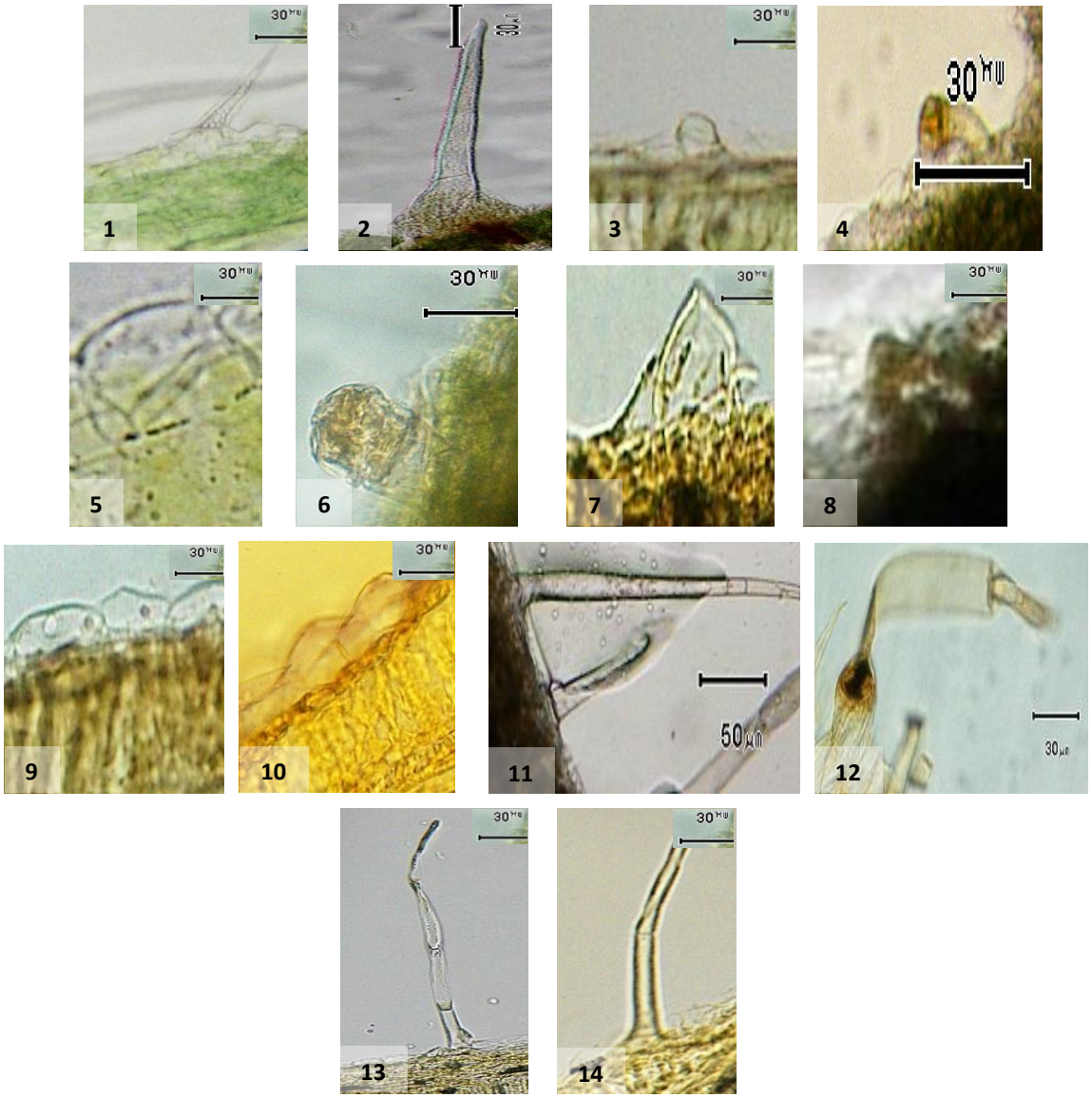

Figure 1. Histochemical Screening on the Leaves Trichome

Description:
(1) Control and (2) Alkaloid test, *Girardinia palmata*
(3) Control and (4) Alkaloid test, *B. suaveolens* (white)
(5) Control and (6) Alkaloid test, *B. suaveolens* (white)
(7) Control and (8) Alkaloid test, *C. elegans* (yellow)
(9) Control and (10) Alkaloid test, *C. elegans* (red)
(11) Control and (12) Alkaloid test, *P. peruviana*
(13) Control and (14) Alkaloid test, *E. pulcherrima*

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In addition, secretions also included substances that are the final product of metabolism or not the final product, but can not be used or only half of it that can be used physiologically (alkaloids, tannins terpen, harsa, and various crystals), or substances that physiologically functionate after secretion (enzyme, hormone). Secretion includes the release of material from the cell (either the surface of the cell or the space in the plants), or the accumulation of secretions in one part of the cell. Secretion in plants is usually produced in hair glands, tubes, and latisifer (sap cells, latex cells) [16].

CONCLUSION

Phytochemical screening test resulted that all toxic plants in this study contained alkaloid. While steroids found in *G. palmata* leaves, *C. eleagans* (yellow) leaves, *C. elegans* (white) leaves, *R. communis* leaves, *B. suaveolens* (white) leaves and seeds, and *P. peruviana* leaves. Terpenoid substances found in *C. elegans* leaves, *R. communis* seeds, *B. suaveolens* (white) leaves and seeds, *B. suaveolens* (yellow) leaves and seeds, and *E. pulcherrima* leaves. Flavonoids only found in terpasan kuning and kecubung putih leaves. According to histochemical test, trichome of toxic plant contained alkaloid, except for terpasan merah leaves (*C. elegans*).

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The Impact of Dissolved Nitrate and Phosphate on Maximum Growth Rate and Carrying Capacity of *Oscillatoria* in Intensive Shrimp (*Litopenaeus vannamei*) Farming Pond Situbondo, East Java, Indonesia

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Abstract

The aims of study are to analyze the effect of dissolved nitrate and phosphate content of the intensive shrimp farming pond Situbondo to maximum growth rate and carrying capacity of *Oscillatoria* population density in the laboratory. This is an experimental research method using completely randomized design with three replications. The treatment were variation of nitrate and phosphate concentration (N0; N6; N12; N24; N48; P0,2; P0,4; P0.8, P1.6 $mg.L^{-1}$). Experiment was done using a pure *Oscillatoria* culture in condition 25 watt lamp; 12 hours a day. The initial amount of *Oscillatoria* cells used for the treatment is $8 – 15 \times 10^4$ $cell.mL^{-1}$. During the incubation process, chemical parameters were also observed including nitrate, phosphate, DO, and pH at the beginning of the incubation period. *Oscillatoria* cell was count every day until stationary phase for 30 days. Furthermore, a different test between treatments was conducted to determine levels of nitrate and phosphate in triggering the blooming of *Oscillatoria* using Oneway ANOVA analysis with SPSS Program. The results showed that the intensive shrimp pond waters of Situbondo already contain dissolved phosphate between 0.4 - 0.5 $mg.L^{-1}$. *Oscillatoria* growth is strongly influenced by dissolved phosphate content in waters, phosphate levels of 0.2 - 0.4 or equivalent to the actual level of 0.6 - 0.7 $mg.L^{-1}$ can cause the highest abundance of *Oscillatoria* that could endanger the ecosystem.

Keywords: dissolved phosphate, intensive shrimp farming, Nitrate, *Oscillatoria*.

INTRODUCTION

Nitrates and phosphates are essential components that determine the primary productivity of the water. Generally, the utilization of nitrates by organisms aims for the formation of biomass, as a component of amino acids, and various proteins from the synthesis [1]. While phosphate is a component of nucleic acid that regulates protein synthesis and transformation of adenosine phosphate as an energy source in intracellular transport [2]. In the intensive shrimp farming pond system, both compounds have been fulfilled on the content of artificial feed that was applied during the cultivation takes place.

Provision of artificial feed to the maximum and continuously expected to spur high shrimp productivity. But over the long period of shrimp cultivation cycle, there has been an increased content of organic matter in the waters. The content of organic materials in the pond ecosystems comprised of dissolved nitrate and phosphate, which is generally derived from shrimp feed residues that were applied during cultivation process [3]. In addition, dirt, shrimp, and plankton dead bodies, can also increase the load of organic matter in the water. The high content of organic matter in the waters can trigger a population explosion of harmful algae [4]. Based on the results of weekly monitoring during the four cultivation cycles in intensive shrimp (*Litopenaeus vannamei*) farming ponds Situbondo, it is known that *Oscillatoria* has a high density and susceptible population explosion [5].

Oscillatoria belongs to the non-heterocys Cyanobacteria filamentous group [6]. Generally the group is categorized in harmful algae because it is capable of producing natural biotoxins that negatively affect human and animal health [7,8]. The toxic compounds produced by *Oscillatoria* are microcystins (MCYs), anatoxin-a, anatoxin-a (S), and aplysiatoxins, which attack liver, nerve, and skin tissues [9]. In addition density of *Oscillatoria* along with Actinomycetes bacteria can produce geosmine and methylisoborneol (MIB) compounds that cause off-flavor odor on shrimp or other aquaculture organisms [10]. The genus is categorized as one type of organic pollutant bioindicator at moderate to high level of organic nutrient [11].

A wide variety of studies suggest a blooming of Cyanobacteria groups in aquatic ecosystems

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may affect ecosystem services, including in pond ecosystems [2,3]. Thus, to keep the water quality of shrimp intensive farms remain in good performance and guarantee a stable shrimp production, control of *Oscillatoria* population density needs to be done. This study aims to analyze the effect of dissolved nitrate and phosphate content of intensive shrimp farming ponds Situbondo to the maximum growth rate and maximum abundance of *Oscillatoria* popula-tion density in the laboratory.

MATERIALS AND METHODS

Sampling and Cultivation of Culture

Test on nitrate and phosphate concentration was done in Ecology and Microbiology Laboratory of Biology Department, Faculty of Mathematics and Natural Sciences, Brawijaya University, Malang, Indonesia. Pure *Oscillatoria* culture was obtained from Limnology LIPI (Indonesian Institute of Science), Cibinong.

The research method used laboratory experiment using complete factorial randomized design with three replications to find out the effect of nitrate and phosphate concentration toward the growth rate and carrying capacity of *Oscillatoria*. The treatments were conduted in the natural media of intensive shrimp farming ponds. The treatments include variations in N (0, 6, 12, 24, 48 mgL^{-1}) and P (0.2, 0.4, 0.8, 1.6 mgL^{-1}) concentrations. Natural media used in this experiment were the combination of pond water and shrimp ponds derived from intensive shrimp (*Litopenaeus vannamei*) farming ponds Klatakan Village, Kendit District, Situbondo Regency, to reach salinity 10 ppt. Prior to the application of nitrate and phosphate variations, the natural sterilized growth media was filtered using paper filter. Each treatment of nitrate and phosphate requires natural media of 2 L pond water, placed on a glass jar size 3 L. The next step is the incubation process in the laboratory using 25 watt lamps, 12 hours a day. Next, the number of *Oscillatoria* cells were observed every day until the stationary phase for 30 days.

Observation of Chemical Parameter on *Oscillatoria* Natural Growth Medium

Chemical parameter observation was performed on *Oscillatoria* growth media. Measurements were made on natural growth medium with various variations of nitrate and phosphate concentration with three times repetitions. Water quality parameters observa-tion included nitrate content using Brusin-colorimetric method, phosphate using Stannous chloride-colorimetric method, DO using titri-metric method, and pH media using potentio-meter method [12].

Number of *Oscillatoria* Cells

The number of *Oscillatoria* cells was determined by sampling procedure 2 mL aseptically and added methylene blue 0.1% by two drops and formalin 10% by one drop into the test tube. The sample then be piped and inserted into the haemocytometer. Observation was done in a microscope with 400 × magnification and calculated the number of *Oscillatoria* cells contained in a large volume box of 1 x 10^4 cm^3. Furthermore, the calculated *Oscillatoria* cell density is entered into the formula below [14].

$$\frac{\textbf{Number of Cell}}{\textbf{mL}} = \frac{\textbf{Number of Counted Cells} \times \textbf{dillution factor}}{\mathbf{1 \times 10^4\ cm^3}}$$

Data Analysis

Data analysis was conducted to know the effect of nitrate and phosphate concentration variation on growth rate of *Oscillatoria* in the natural media of pond water. Furthermore, growth data of *Oscillatoria* were analyzed to determine the intrinsic growth rate (r) and environmental carrying capacity (K) that affected the growth of *Oscillatoria* [14]. From the information then a growth curve were made in order to note the value of nitrate and phosphate concentration that can trigger blooming of *Oscillatoria* in pond waters. The following formula was used to find the intrinsic growth rate (r) and environmental carrying capacity (K). Further calculation results are tested between treatments to determine levels of nitrate and phosphate which trigger the blooming of *Oscillatoria* using Oneway Anova analysis with SPSS application for windows Ver.16 [13].

$$N_t = \frac{K}{1 + e^{a-rt}}$$

Note:

N_t = population density at a given t time of population growth
K = carrying capacity of the environment
e = natural logarithms (2.71828)
r = inrinsic growth rate
t = time incubation

RESULT AND DISCUSSION

Media Quality

The observation of chemical parameters on *Oscillatoria* growth media showed varying values. At this stage, we observed actual content of nitrate and dissolved phosphate, and the level of DO and pH growth media at the beginning of the incubation period (Table 1). It is known that the

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pond waters of intensive shrimp pond culture of Situbondo contained dissolved phosphate with levels between 0.4 - 0.5 mgL^{-1}. While the actual nitrate content is ranged from 0.07 - 0.42 mgL^{-1}. The nutrient content of the waters including nitrates and phosphates is generally derived from the residual feed or decomposition process of dirt, shrimp bodies, plankton and bacteria that died during the cultivation. High levels of dissolved phosphate in the waters can lead to population blooms of various types of harmful algae groups [1]. Figure 1 showed that Oscillatoria growth pattern in the treatment of phosphate variation was higher than in nitrate treatment. If it is allowed continously, it will disrupt the balance of the pond ecosystem and affect the productivity and sustainability of the shrimp farming process.

DO concentration of *Oscillatoria* growth media ranged from 4 - 5 mgL^{-1}. In general, the dissolved oxygen content in the water depends on the rate of phytoplankton photosynthesis [15]. The fluctuation of DO value is strongly associated with *Oscillatoria* growth. It is known that DO levels in phosphate treatment are lower compared to nitrate treatment. This can be happened because the level of oxygen consumption in the waters depends on the biomass load of the organisms that make up the ecosystem [16]. The higher biomass number of organism in an ecosystem making up higher oxygen demand.

The pH value of the media both in nitrate or phosphate treatment is known to have the same trend, i.e. in the range value of 8. The stability of the water pH is strongly influenced by the growth of phytoplankton, in this case related to the utilization of CO_2 as material for photosynthesis process. Increased pH of the waters may be due to CO_2 consumption by microalgae characterized by increased levels of DO and chlorophyll- *a* [16].

Oscillatoria Growth Pattern

Based on the observation of *Oscillatoria* growth pattern, it is known that the addition of varied nitrate and phosphate in natural media has been able to support *Oscillatoria* growth (Fig. 1). In general, it can be seen that the addition of variations in nitrate and phosphate levels showed different responses. The addition of nitrate to natural growth medium is known to cause a slower response to *Oscillatoria* growth compared to phosphate addition treatment.

The addition of phosphate in the medium is capable of supporting the *Oscillatoria* growth, but the addition of nitrate without the addition of phosphate is not capable on supporting the maximum growth of *Oscillatoria* as in Figure 1a. The addition of 0 - 0.4 mgL^{-1} phosphate levels has been able to cause the highest abundance of *Oscillatoria*. In addition it is known that the intensive shrimp farming pond Situbondo already contained dissolved phosphate (Table 1). Thus the treatment of nitrate or phosphate can lead to differences in growth patterns of *Oscillatoria*.

Table 1. Chemical Parameters Observed at the Beginning of the Incubation Period in Natural Media of *Oscillatoria* Growth

Addition treatment	Actual levels of nitrate (mgL^{-1})	Actual levels of dissolved phosphate (mgL^{-1})	DO (mgL^{-1})	pH
N0				
P0	0.07 ± 0.07	0.50 ± 0.07	4.82 ± 0.30	8.19 ± 0.02
P0.2	0 ± 0	0.61 ± 0.04	4.89 ± 0.17	8.11 ± 0.01
P0.4	0 ± 0	0.71 ± 0.04	4.36 ± 0.66	8.12 ± 0.07
P0.8	0 ± 0	0.92 ± 0.10	4.64 ± 0.79	8.16 ± 0.05
P1.6	0.42 ± 0.72	1.54 ± 0.05	4.46 ± 0.68	8.16 ± 0.04
P0				
N0	0.07 ± 0.07	0.50 ± 0.07	4.82 ± 0.30	8.19 ± 0.02
N6	6.79 ± 0.64	0.48 ± 0.03	5.14 ± 0.08	8.20 ± 0.02
N12	10.77 ± 2.64	0.44 ± 0.09	5.01 ± 0.52	8.15 ± 0.02
N24	30.57 ± 2.15	0.53 ± 0.05	5.28 ± 0.23	8.15 ± 0.03
N48	59.35 ± 8.98	0.51 ± 0.04	4.11 ± 0.57	7.99 ± 0.08

Note: Data is average ± SD (n=3)

The presence of nutrients in water bodies especially phosphates can lead to changes in the structure of the phytoplankton community and is usually dominated by the Cyanobacteria group [6,7,17]. Phosphate is a major essential component in plankton life [1]. Plankton utilizes phosphate content as a nucleic acid component that regulates protein synthesis and transforma-tion of adenosine phosphate as an energy source in intracellular transport [2]. Another reason that causes phosphate content in water bodies to be a control in the process of ploriferation on differ-

ent types of Cyanobacteria groups is the ability of this group in fixing free nitrogen in the air [5]. Algae population explosion usually occurs in warm waters, which can be a distinct advantage for the group [6,7,8]. The concentration level of phosphate absorption by *Oscillatoria agardhi* capable of supporting maximum growth in waters ranged from 0.2 to 0.3 µmol PL^{-1} [1].

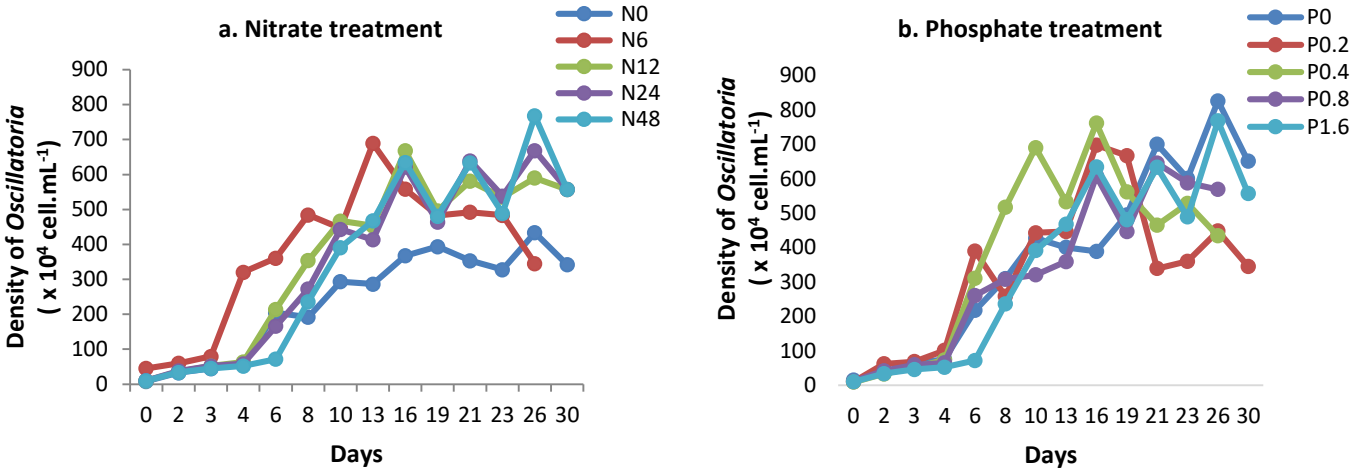

Figure 1. *Oscillatoria* Growth Pattern on the Treatment Variation of (a) nitrate and (b) phosphate
Note: Actual level of nitrate and phosphate was performed in Table 1.

***Oscillatoria* Carrying Capacity and Growth Rate**

Based on the calculation of *Oscillatoria* abundance cells for 30 days, then we calculated the intrinsic growth rate (r) and maximum abundance or carrying capacity that can be supported by media (K) at each treatment. The average calculation result of both values can be seen in Figure 2.

Anova test results indicated the growth of *Oscillatoria* is significantly influenced by phosphate level compared to nitrate. It is reflected from the value of growth rate and maximum abundance that supported by media (Fig 2). Maximum abundance of *Oscillatoria* has a higher value in the treatments dissolved phosphate levels of 0.2 and 0.4 mgL^{-1} with values between 700 - 1000 (x 10^4 cell.mL^{-1}). It is increasingly asserted that *Oscillatoria* is strongly influenced by the phosphate element. Phosphate is an essential component of ATP that plays a role in various biochemical processes within the cell of organism [18]. In addition, phosphate elements have an important role in cell development and DNA formation [19].

While the variation of nitrate addition on the maximum abundance value of *Oscillatoria* is in addition of nitrate 0 mgL^{-1} with value 700 x 10^4 cell.mL^{-1}. It is due to the diazotrophic nature of the organism. The nature of diazotroph is the organism's ability to block free N_2 from air [5]. Most members of Cyanobacteria filamentous non-heterocys including *Oscillatoria* are diazotroph, thus causing the organism to survive as long as there is still an environmental phosphate element [5,11]. It is also related to the actual dissolved phosphate content of the media which has reached 0.5 mgL^{-1}, so that the nitrate content of the media can be enriched directly through free N_2 fixation from air. The low maximum abundance value on the treatment of other nitrate variations (6, 12, 24, and 48 mgL^{-1}) corresponds to the optimal N:P ratio that is capable of being supported for the microalgae group growth. The optimal N:P ratio that can cause maximum microalgae growth is 1:15 [20, 21].

The calculation results of the maximum growth rate of *Oscillatoria* in phosphate and nitrate addition showed relatively similar fluctuations with result of carrying capacity. In general, variations in the addition of phosphate levels of 0.2 - 0.4 mgL^{-1} can produce high growth rate values, while the addition of phosphate levels more than that value can not support the increase in maximum *Oscillatoria* growth rate in the media. The results are consistent with previous studies which reveal the production of *O. agardhi* toxin characterized by addition of biomass depending on low phosphate levels (0.1-0.4 mgL^{-1}) and elevated phosphate levels do not provide additional effects because they are not supported with sufficient dissolved nitrate content [17].

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Furthermore, the treatment of nitrate addition 48 mgL^{-1} capable of causing the highest growth rate value. The N element is one of the essential components of a protein that acts as a biomass-forming cell and plant tissue [5,21]. Another important function of the N element is as an essential biochemical agent such as chlorophyll formation and its role in the process of photosynthesis, formation of various enzymes used for biochemical processes and assimilation of nutrients by organisms, as well as nucleic acid components such as DNA and RNA [1].

The *Oscillatoria* group is categorized as one type of organic pollutant bioindicator at moderate to high level nutrient organic [10]. The existence of *Oscillatoria* in pond ecosystems can gradually initiate the growth of other CyanoHABs groups such as *Microcystis* and *Anabaena* [2,22]. If it is left continuously it can cause more severe ecosystem damage. So that through the control of *Oscillatoria* in the pond is expected to become a breakthrough of environmentally friendly habitat management as an early warning system in an effort to maintain water quality during the cultivation process in order to produce a continuous increase in production.

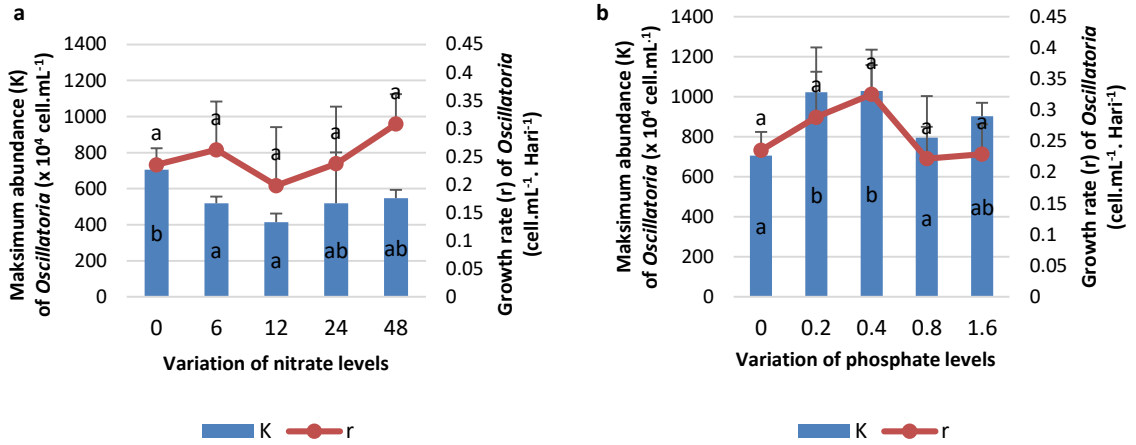

Figure 2. Maximum abundance (K) and growth rate (r) *Oscillatoria* which can be supported by the treatment of (a) nitrate and (b) phosphate addition in natural media

Note: Notation of treatment of variation of P on parameter K showed no significant difference based on Tukey test. N treatment notation on N parameter shows no significant difference based on Games-howel test.

CONCLUSION

Intensive shrimp farming pond Situbondo waters are known to contain nitrate with levels ranged from 0.07 - 0.42 mgL^{-1} and dissolved phosphate ranged from 0.4 to 0.5 mgL^{-1}. The addition of nitrate content in natural media is not able to support the growth of *Oscillatoria* to its full potential. While the addition of soluble phosphate as much as 0.2 - 0.4 or equivalent with the actual content of phosphate 0.6 - 0.7 mgL^{-1} in natural media able to support *Oscillatoria* growth maximally.

The results show that maximum abundance or carrying capacity of *Oscillatoria* is strongly influenced by dissolved phosphate in the water bodies. Thus controlling the population of *Oscillatoria* in pond waters can be done through the manipulation of habitat by controlling the phosphate content of waters that is not exceeding 0.2 or equivalent to the actual content of phosphate 0.6 mgL^{-1}.

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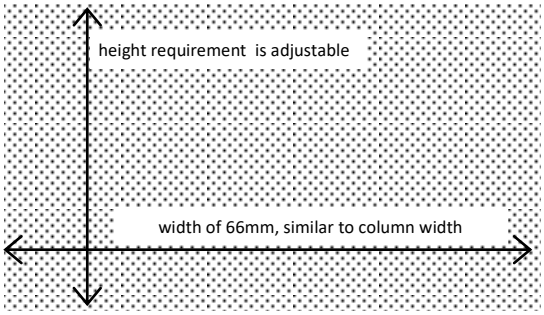

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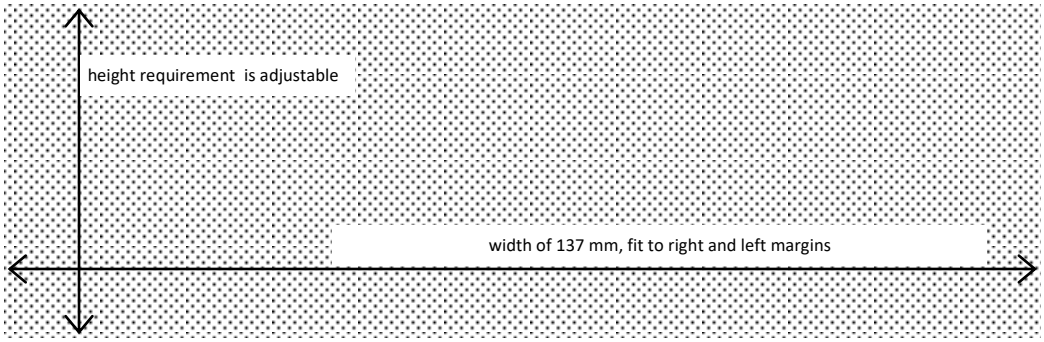

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