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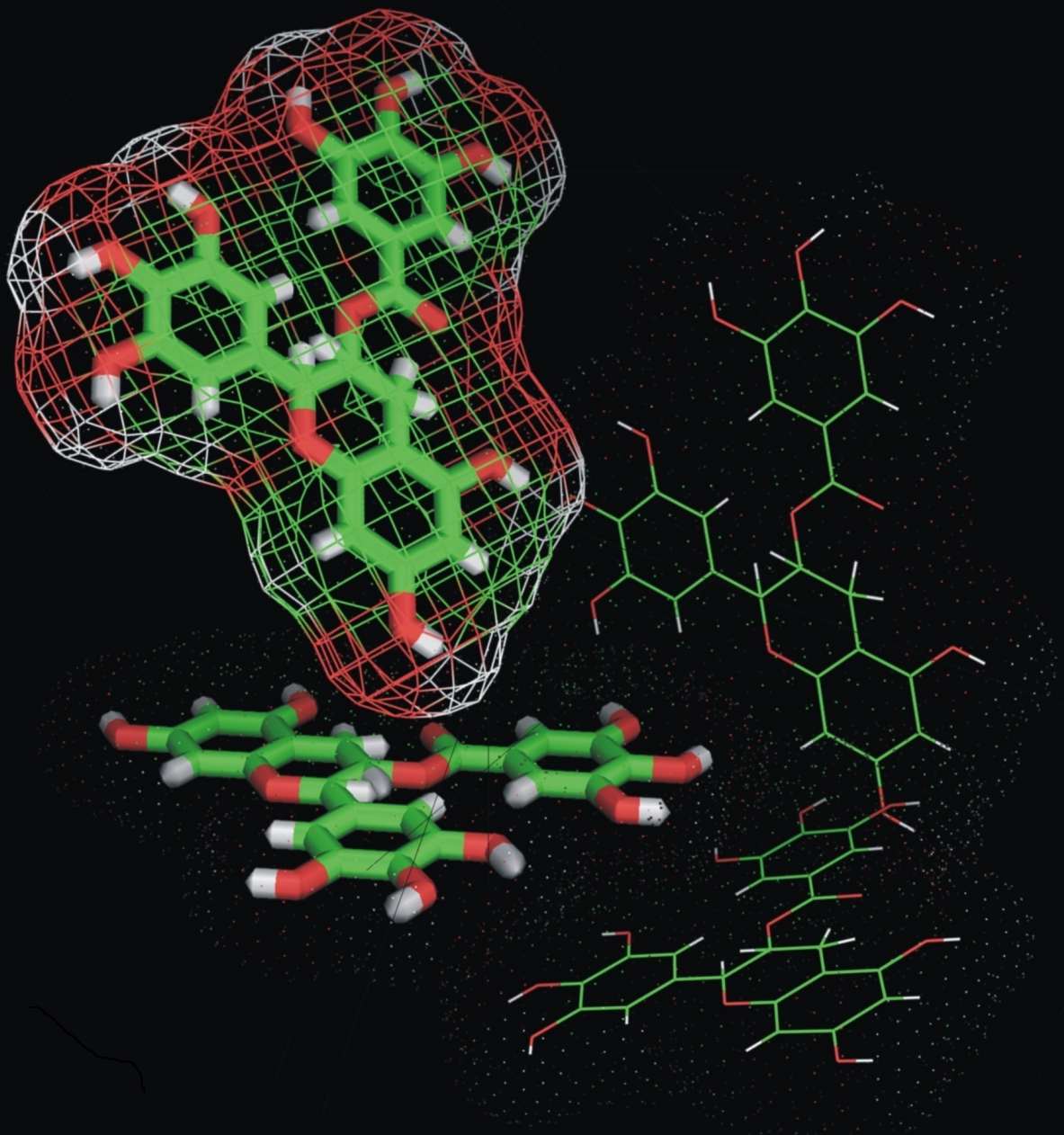

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Optimal Control on Model of SARS Disease Spread with Vaccination and Treatment

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Abstract

The spread of SARS (Severe Acute Respiratory Syndrome) disease in a human population is one of the phenomena that can be mathematically modeled. The exposed period of SARS disease underlies the formation of the SVEIR epidemic model which is a modification of the SVIR epidemic model by adding subpopulation E (exposed). In the SVEIR model, there are two control variables in the form of vaccination and treatment which aimed to minimize exposed subpopulation, infected subpopulation, and control implementation cost. The Pontryagin's minimum principle is used to obtain optimal control and system, thus minimizing objective functional as the objective to be achieved. Furthermore, the forward-backward sweep method is used for numerical simulation in order to determine the most appropriate control strategy in a finite time. The simulation results show that implementation of both vaccination and treatment is the most effective decision making to control the spread of SARS disease.

Keywords: optimal control, Pontryagin's minimum principle, SARS.

INTRODUCTION

Infectious diseases are caused by pathogens or biological agents such as virus, bacteria, fungi, and other microorganisms. Infectious diseases can be transmitted from one individual to another through a variety of intermediate such as body fluids, feces, and tools contamined by individual infected SARS virus [1]. Susceptible individual can be infected with the SARS virus as a result of making contact with infected individual so that it can lead to new infection that will spread the disease to other susceptible individual. This event is a sign of the spread of disease in a society or country that can increase mortality in a short time. It is reported by the WHO that infectious disease contribute about 1/6 of total deaths worldwide and second factor causes of death [2].

SARS is an infectious disease caused by virus. The clinical symptoms of SARS disease are fever, dry cough, shortness of breath, and other symptoms similar to pneumonia [3]. The spread of SARS disease is very rapid, progressive, and fatal. Most people with SARS disease are adults between 25-70 years, but in some cases SARS also affects children under 15 [4]. The spread of SARS disease was first detected in November 2002 in Guangdong province, China. Furthermore, SARS disease spread rapidly throughout the continent, especially in the Asia-Pacific region. In March 2003, WHO reported SARS disease caused by a virus called coronavirus or SARS-CoV [3]. That caused 774 people died and 8098 people infected SARS-coV virus [5]. The extermination of the SARS outbreak is estimated to have cost about $ 10-30 billion [6]. However, the amount of funds spent does not necessarily overcome the disease outbreaks optimally.

The impact of infectious diseases are very harmful in a country or population, it is important to understand the dynamics of disease progression and develop the control of disease spread and consider the costs associated with control implementation. In this case, a mathematical model is needed to illustrate future disease spread by involving information in the present. Mathematical modeling is an important tool in understanding the dynamics of disease spread and decision-making processes related to the control program of a disease spread. The branch of mathematics developed to find the optimal way of controlling dynamic systems is called optimal control theory. The application of optimal control theory aims to estimate the effectiveness of various policies, control measures, and their associated costs [7].

Vaccination is a very popular control policy. Implementation of vaccination program is estimated to prevent approximately 2-3 million

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deaths each year and 25 diseases with vaccination approved by WHO. In recent decades, vaccination and some control policies such as treatment, quarantine, isolation, screening, etc. are also applied to control the spread of infectious diseases [2].

In 2017, Kumar and Srivastava added control variables to the SVIR (*Susceptible Vaccinated Infected Recovered*) epidemic model in order to control the spread of the disease. In addition to vaccinations, treatment is also needed to prevent the spread of disease. Therefore, vaccination and treatment control strategies are used. On the economic side, the implementation of a control strategy within a country requires substantial cost. Limited funding by health or government institutions is a matter to be considered, so Kumar and Srivastava also consider the costs in their research [2].

Some infectious diseases such as tuberculosis, influenza, measles, etc. have exposed period in the natural world. In the exposed period the susceptible individu does not show symptom of the disease and does not transmit the disease because the immune system is in good condition. This individu will stay in the exposed class during the exposed period. After the exposed period end or the immune decrease, the individu is in the class of infection and shows symptoms and then transmit the disease [8].

Epidemic models with latent stage and vaccination were investigated, expressed in the form of SVEIR namely *Susceptible Vaccinated Exposed Infected Recovered* [9]. Before the study, the SVEIR model has been studied to describe the spread of SARS disease [10]. However, there is no effort to control the spread of disease from those researchs. Previous research aimed to assess the potential impact of an anti-SARS vaccine is not perfect through mathematical modeling [10]. Further result show that an imperfect anti-SARS vaccine successfully reduces the spread of SARS in the community on the condition that the vaccine must have efficacy or effectiveness of at least 75% [10].

This study modifies the SVIR epidemic model into SVEIR by adding exposed subpopulation (E). This is due to the exposed period of SARS for 3-5 days [1] and the subpopulation model E is based on the study [9]. In addition to adding subpopulation E, it is assumed that individual infected with SARS cannot recover naturally by model [1]. This modification has a goal to make the model more real. Furthermore, two control functions that is vaccination control and treatment control are added to the SVEIR model. Vaccination is used to control the spread of SARS disease, where susceptible individual is given anti-SARS vaccine to build up immunity against SARS virus [10], and apply control policies in the form of treatment given to individuals infected with SARS. The existence of control function in this study aims to minimize exposed subpopulation and infected subpopulation so that the spread of SARS disease is not widespread and minimize the costs associated with the implementation of control. Optimal control issues resolved with the Pontryagin's minimum principle. At the end, a numerical simulation is done using the forward-backward sweep method. Furthermore, the simulation results are analyzed to determine the most effective control strategies in controlling the spread of SARS disease.

MATERIALS AND METHODS

In this study, SARS disease spread model assumed that the individual who became the object of research is human, interaction between individu occur in one population without any individu entering or leaving the population, the birth rate and the death rate are constant. The spread of SARS disease in this study focused on one population divided into 5 subpopulations, namely susceptible subpopulation S, vaccinated subpopulation V, exposed subpopulation E, infected subpopulation I, and recovered subpopulation R. Furthermore, several research methods are used to achieve the objectives.

Literature Study

Literature study related to the research process, such as the literature discussing the spread of disease, optimal control theory, the Pontryagin principle, and forward-backward sweep method. We also used other supporting references in problem solving in this study.

Model Formulation

Constructed spread model of infectious diseases SARS with type SVEIR. The SVEIR model is obtained from the SVIR model which added subpopulation E (exposed). The subpopulation model E is based on the research of Li et al [9], where the susceptible individual infected with the disease due to contact with the infected individual will enter into the exposed (E) subpopulation during the exposed period. After the exposed period end, the individual begins to show clinical symptoms and has the ability to transmit the disease, so that the individual is included in the

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infected subpopulation (I). Furthermore, an assumption is added to the SVEIR model that individual infected with SARS cannot recover naturally, it's based on the model of Huang's research [1]. The addition of these assumption causes the natural recovery rate of infected individual is zero.

Optimal Control Simulation

Formulated optimal control problem and resolved with the Pontryagin's minimum principle. Based on these principle required a necessary condition that must be met. The necessary condition can be generated with the Hamiltonian H function. The H function must be optimal in order to obtain the optimal system. If the optimal system then achieved the optimal goal. The system can achieve optimal if the H function satisfies state equations, costate equations, and stationer condition.

Numerical simulation is performed for optimal control problem. The simulation includes simulating the spread of SARS disease when not given vaccination and treatment controls, when only vaccination control is given, when only treatment control is given, and when combination control of vaccination and treatment are given. The numerical simulation is solved using the forward-backward sweep method with Matlab software. The initial parameter values used in the simulations will be mentioned in the results and discussion. The initial parameter values are used to analyze and emphasize the simulated results obtained.

Analyzing the results of numerical simulation to find out the most effective or appropriate control strategies, thus minimize exposed subpopulation, infected subpopulation, and costs associated with the implementation of control (vaccination and treatment).

RESULT AND DISCUSSION

SARS Epidemic Model

In this section, the SVEIR epidemic model is formulated to illustrate the spread of SARS in a population divided into 5 subpopulations. Individu in each subpopulation interact with each other. This interaction causes the movement of individu from one subpopulation to another. The flow of individu movement from one subpopulation to another subpopulation is illustrated in Figure 1. The arrows entered in a subpopulation indicate the presence of incoming individual, so the number of individual in the subpopulation increases. The outbound arrows of a subpopulation indicate the presence of an outgoing individual, so the number of individuals in the subpopulation is reduced.

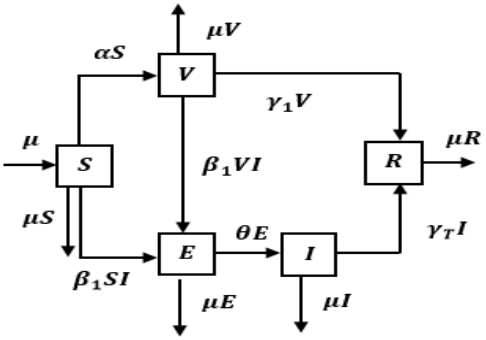

Figure 1. Compartment diagram of the SVEIR epidemic model

The number of individuals in each subpopulation may vary from time to time due to the influence of natural factors such as birth and death as well as the presence of susceptible individual into infected individual and so on, so that the number of individuals each time can be expressed $S(t), V(t), E(t), I(t), R(t)$.

The rate of susceptible subpopulation change between time t_0 and T is

$$\frac{dS}{dt} = \lim_{\Delta t \to 0} \frac{\Delta S(t)}{\Delta t}.$$

The rate of subpopulation change also applies to the rate of change of vaccinated, exposed, infected, and recovered subpopulation. Based on Figure 1, the SARS disease spread model is expressed in the system of differential equations as follows:

$$\begin{aligned}
\frac{dS}{dt} &= \mu - \beta SI - \alpha S - \mu S, \\
\frac{dV}{dt} &= \alpha S - \beta_1 VI - \gamma_1 V - \mu V, \\
\frac{dE}{dt} &= \beta SI + \beta_1 VI - \theta E - \mu E, \\
\frac{dI}{dt} &= \theta E - \gamma_T I - \mu I - \sigma I, \\
\frac{dR}{dt} &= \gamma_1 V + \gamma_T I - \mu R,
\end{aligned} \tag{1}$$

with initial density
$S(0) \geq 0, V(0) \geq 0, E(0) \geq 0, I(0) \geq 0, R(0) \geq 0.$

In the SVEIR model expressed in the system of equations (1), all values of the parameters are assumed to be non-negative constants. Parameter μ represents birth and mortality

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natural rate, β is the transmission rate of disease when infected individual make contact with susceptible individual. β_1 represent the transmission rate of disease from individual infected with SARS against vaccinated individual who have not been immune to the disease, α is constant vaccination rate in susceptible subpopulation, γ_1 represent immunity rate of vaccinated individual so that individual become recovered individual during or after the vaccination process, γ_T is treatment rate of infected individual, dan θ represent rate of exposed individual become infected individual.

Vaccination and treatment are selected as control policies because vaccination are easy obtained and applied in practice and supported by WHO [2]. Therefore, in this study, the SARS disease spread model expressed in model system (1) is modified into a model system (2) with vaccination and treatment as a control strategy to be discussed in the next chapter.

Optimal Control Problem

The SARS disease spread model with vaccination and treatment control strategies is obtained based on the system of equation (1) and the following statements.

i. Susceptible subpopulation are given anti-SARS vaccine with the aim of providing immunity to susceptible individual from SARS disease, thereby preventing transmission of the disease to susceptible individual. In some diseases, constant vaccination may not be a good choice in the economic context for any country. In addition, vaccine administration for large population need substantial cost and difficult to give vaccine for all susceptible individual in the population. Therefore, it is important to know the exact level and timing of the vaccine so that maximize the vaccinated individual with minimum vaccination cost over a finite time period. According to Kumar and Srivastava [2], it can be done by changing the constant vaccination rate α on model (1) become $u_1(t)$ function as vaccination control. Due to limited fund and time for health agencies in implementing vaccination policies, then the policies should be limited in accordance with established goal. Thus, it is assumed that vaccination control is limited, i.e. $0 \le u_1(t) \le 1$ [2].
ii. Infected subpopulation are given treatment to reduce the burden of disease and control the spread of infection. Treatment programs include diagnosis, drug administration, hospitalization, and other medical services. Similar to vaccination programs, treatment programs for infected individuals also require a large cost and must be minimized. Therefore, treatment rate γ_T on model (1) become $u_2(t)$ as treatment control and assumed that $0 \le u_2(t) \le 1$ [2].

Thus, SARS disease spread model with vaccination and treatment control is as follows:

$$\begin{aligned}
\frac{dS}{dt} &= \mu - \beta SI - u_1(t)S - \mu S,\\
\frac{dV}{dt} &= u_1(t)S - \beta_1 VI - \gamma_1 V - \mu V,\\
\frac{dE}{dt} &= \beta SI + \beta_1 VI - \theta E - \mu E,\\
\frac{dI}{dt} &= \theta E - u_2(t)I - \mu I - \sigma I,\\
\frac{dR}{dt} &= \gamma_1 V + u_2(t)I - \mu R,
\end{aligned} \tag{2}$$

with a set of control function U , i.e.

$$U = \{u_1(t), u_2(t) \mid 0 \le u_i(t) \le 1, i = 1,2, t \in [0,T]\},$$

t represent time and T represent final time for control strategy SARS disease. The final time for each disease is varied and the implementation of the control strategy depend on the objective of the control policy. Implementation of control will be stopped when the objective have been achieved.

Application of optimal control theory in case of SARS disease spread with vaccination and treatment aims to minimize exposed subpopulation, infected subpopulation, and costs associated with control implementation. This objective is expressed in an objective function J, i.e.

$$J[u_1(t), u_2(t)] = \int_0^T [E + I + w_1 u_1^2(t) + w_2 u_2^2(t)]dt, \tag{3}$$

with positive constant w_1 represent the weight of vaccination and w_2 represent the weight of treatment should be minimized. The objectives expressed in equation (3) have the constraints expressed in the model systems (2). These constraints illustrate the pattern or model of the spread of SARS disease in a population where the

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initial value of each subpopulation is known i.e. $S(0) \geq 0, V(0) \geq 0, E(0) \geq 0, I(0) \geq 0, R(0) \geq 0$.

The initial value is used to determine future value after vaccination and treatment control are given, so it can be seen the effectiveness of the methods to prevent the spread of SARS disease. If the functional value in equation (3) has a minimum functional value, then the control strategy is more effective.

The functional value of J can be minimized by applying the Pontryagin's minimum principle. On these principle, a necessary condition must be met. These necessary condition may be generated by the Hamiltonian function (denoted H) defined as follows:

$$H\left(t, \vec{x}, \vec{u}, \vec{\lambda}\right) = f(t, \vec{x}, \vec{u}) + \sum_{i=1}^{n} \lambda_i(t) g_i(t, \vec{x}, \vec{u}),$$

Where f is the integrand of the objective function J in equation (3), g_i is the right-hand side of the equations in system 2, and λ_i is the adjoint functions dependent on the number of state variables. The state variable in this research are 5 (S, V, E, I, R). Furthermore, H function must be optimal to obtain the optimal J value, that is state equation, costate equation, and stationary condition must be satisfied.

The state equation is satisfied when the partial derivative of H function to adjoint function λ yields equations in system (2). The costate equation is satisfied when the partial derivative of the H function for each state variable is negative. The stationary condition is obtained when the partial derivative of the H function to the control variable $u_1(t)$ and $u_2(t)$ is zero, so that the optimal control of $u_1^*(t)$ dan $u_2^*(t)$, that is

$$u_1^*(t) = \min\left\{\max\left\{0, \frac{(\lambda_1 - \lambda_2)S}{2w_1}\right\}, 1\right\}, \text{and}$$

$$u_2^*(t) = \min\left\{\max\left\{0, \frac{(\lambda_4 - \lambda_5)I}{2w_2}\right\}, 1\right\},$$

with $\lambda_1, \lambda_2, \lambda_4$, and λ_5 are *adjoint* functions. If optimal control $u_1^*(t)$ and $u_2^*(t)$ are substituted in the state and costate equations then the optimal system is obtained which can optimize the objective function J.

Numerical Simulation

In this section, numerical simulation results are used to analyze the impact of control policy strategies on disease dynamic and related costs of control implementation. Numerical simulations of systems (2) and (3) use forward-backward sweep method in the MATLAB software. Initial values and parameter values are used for numerical simulations are presented in table 1.

Table 1. Initial Values and Parameter Values

$S(0)$	$V(0)$	$E(0)$	$I(0)$	$R(0)$
0.8	0.04	0.08	0.04	0.04
μ	γ_1	β	β_1	θ
0.00005	0.04	0.3	0.01	0.02

In order to analyze the utility of control policy, three numerical control strategies are performed, i.e.

strategy A: implementation of a single control policy that only applies vaccination to prevent the spread of SARS disease,

strategy B: implementation of a combination control policy: vaccination and treatment to prevent the spread of SARS disease, and

strategy C: implementation of a single control policy that only applies treatment to prevent the spread of SARS disease.

The discussion of this paper includes numerical simulation by implementing control strategies A, B, C, and without vaccination or treatment control. The numerical simulation result in Figure 2 show the changes in behavior of exposed subpopulation (E) when applied control strategy A, B, C and the weight of costs $w_1 = w_2 = 20$. The number of exposed subpopulation has increased each time when no vaccination or treatment control are provided. After control strategy A is applied, the number of exposed subpopulation decreases faster than without control. The number of exposed subpopulation also decreased when applied control strategy B, i.e. the combination control strategy of vaccination and treatment are applied.

When strategy C is applied, the number of subpopulation E shows no difference when it is given only treatment control and no control. This means, the implementation of the treatment control strategy only (strategy C) is ineffective to

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reduce the number of exposed subpopulation. Thus, the implementation of vaccination control only can minimize the number of exposed subpopulation. However, it is better to be given a combination of controls i.e. vaccination and treatment to suppress the number of exposed subpopulation.

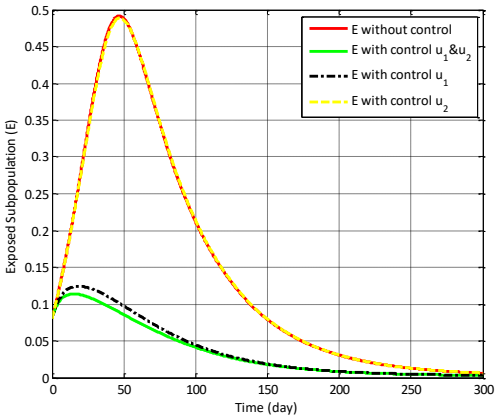

Figure 2. Density of Exposed Subpopulation (E) with Control Strategy A, B, C, and Without Control

The change in the number of infected subpopulations (I) at any time in Figure 3 is same as the number of exposed subpopulation. The number of subpopulation I decreased significantly when the combination control of vaccination and treatment are given. The strategy is more effective than either vaccination control strategy only or treatment control strategy only.

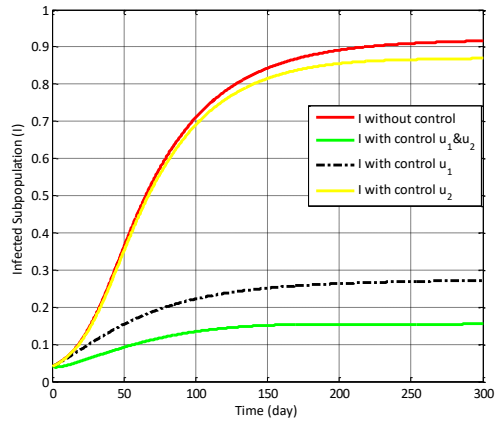

Figure 3. Density of Infected Subpopulation (I) with Control Strategy A, B, C, and Without Control

From the Figures 2 and 3, it can seen that the number of exposed (E) and infected (I) subpopulations reach the least value when combination control of vaccination and treatment is given. In other words, control strategy B is more effective than control strategy A and C. Level of vaccination and treatment are given every time (day), it can be seen in Figure 4. At first, vaccination control is given equal to 0.16 and treatment control is 0.07. When the number of exposed and infected subpopulations decrease, vaccination and treatment control are lowered to zero. That means, control of vaccination and treatment are stopped.

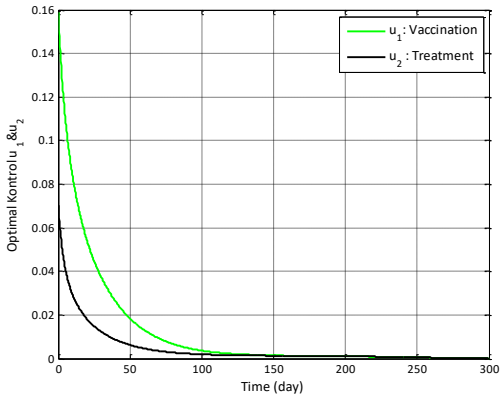

Figure 4. Vaccination control u_1 and treatment control u_2 are given every time (day)

Figure 5 shows the effect of the weight of vaccination cost w_1 and the weight of treatment cost w_2 on the number of exposed subpopulation (E), $w_1 = w_2 = 2000$. When the weight of vaccination and treatment costs is enlarged a hundred times from the initial weight, the number of exposed subpopulation is not much different from the number of exposed subpopulation when no control is given. This is due to very small vaccination and treatment controls that is equal to 8.391×10^{-3} and 2.96×10^{-3}, as shown in Figure 7.

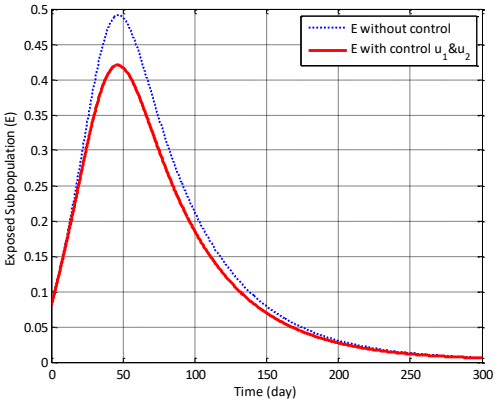

Figure 5. Density of Exposed Subpopulation (E) with Strategy Control B and Without Control when $w_1 = w_2 = 2000$

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The small levels of vaccination control and treatment resulted in the number of infected subpopulations (I) not minimal, as the simulation results in Figure 6. That means, the population is still in an endemic condition where the spread of the disease occurs continuously.

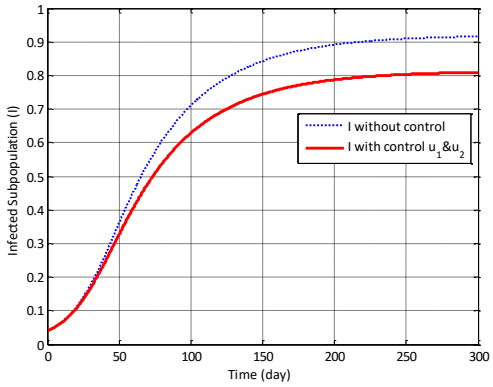

Figure 6. Density of Infected Subpopulation (I) with Strategy Control B and Without Control when $w_1 = w_2 = 2000$

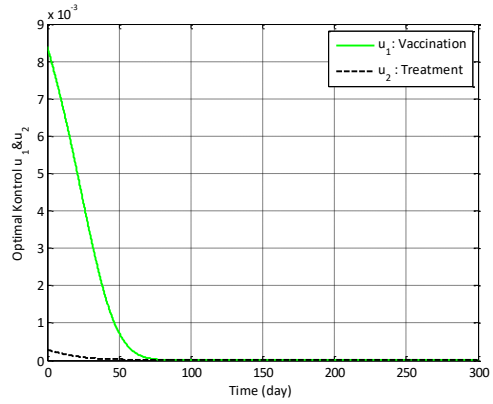

Figure 7. Vaccination control u_1 and treatment control u_2 are given every time (day) when $w_1 = w_2 = 2000$

Furthermore, it is shown the effect of the weight of vaccination and treatment costs when it is reduced to ten times, i.e. equal to $w_1 = w_2 = 2$. The simulation results in Figure 8 show that the number of exposed subpopulation is more minimum than the number of exposed subpopulation on other simulation results. This is similar to the change in the number of infected subpopulation in Figure 9. This is because the control level of vaccination and treatment is greater than the control level of other simulations, as shown in Figure 10. The vaccination control is given equal to 0.4322 and the treatment control is 0.3 at first. After the number of exposed and infected subpopulations decrease, the control level of vaccination and treatment is reduced every time until it reaches zero value where the control is discontinued.

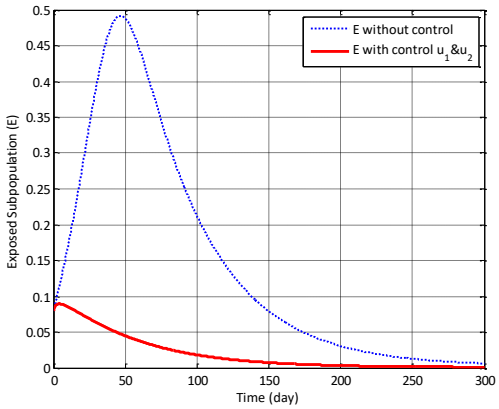

Figure 8. Density of Exposed Subpopulation (E) with Strategy Control B and Without Control when $w_1 = w_2 = 2$

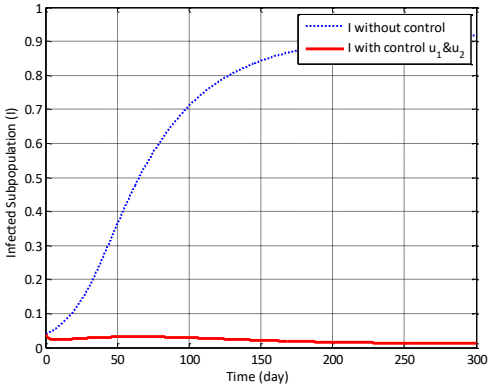

Figure 9. Density of Infected Subpopulation (I) with Strategy Control B and Without Control when $w_1 = w_2 = 2$

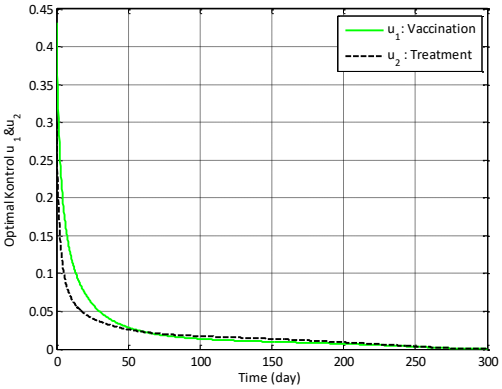

Figure 10. Vaccination control u_1 and treatment control u_2 are given every time (day) when $w_1 = w_2 = 2$

The purpose of applying optimal control in this research is to minimize the objective functional J in equation (3). The objective functional value is influenced by the increase and decrease of the number of exposed subpopulation, infected subpopulation, and controls.

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From Table 2, the minimum objective functional value is equal to 17.0064. If the objective functional value is smaller then the control strategy applied in controlling the spread of SARS disease is more effective. Thus, it can be concluded that control combination strategy of vaccination and treatment with small cost weight is the most effective strategy.

Table 2. The objective functional value of each simulation for $t = 300$

Strategy	Objective Functional
without control $(u_1 = u_2 = 0)$	419.5444
with control u_1 $(w_1 = 20)$	141.8792
with control u_2 $(w_2 = 20)$	403.3616
with control u_1 and u_2 $(w_1 = w_2 = 20)$	88.3218
with control u_1 and u_2 $(w_1 = w_2 = 2000)$	376.7691
with control u_1 and u_2 $(w_1 = w_2 = 2)$	17.0064

CONCLUSION

In this research, we conclude that the implementation of combination control strategy in the form of vaccination and treatment is more effective to reduce the number of exposed subpopulation (E) and infected subpopulation (I) than either the control strategy of vaccination only or the control strategy of treatment only. The weight of costs of vaccination and treatment controls can affect the levels of vaccination and treatment. If the weight of costs of vaccination and treatment are lower then the vaccination and treatment levels are greater. If the levels of vaccination control and treatment are getting bigger then the number of exposed and infected subpopulations are getting smaller. As a result, the objective functional value is getting smaller.

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Effect of Calcium Polysulfide Applications on Severity of Cocoa Pod Rot (*Phytophthora palmivora* Butl.) and Fungal Diversity

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Abstract

Indonesian government gives a high priority for the development and revitalization of cocoa production. In 2012/2013, production of cocoa in Indonesia reached 410,000 ton from 1,774,500 ha (about 231 kg ha^{-1}), lower than Ghana which is 835,000 ton from 1,600,300 ha (about 521 kg ha^{-1}). One of the constraints of cocoa production in Indonesia is pod rot disease caused by *Phytophthora palmivora*, which can reduce about 90% of production during wet season. Calcium polysulfide was suggested as one of the potential, cheap and save substance to control *P. palmivora*. The purpose of this study was to evaluate the calcium polysulfide potential to control the disease and its effect on the phyllosphere-fungal diversity. A positive correlation was found between concentration of calcium polysulfide and inhibition of fungal growth in the media. Otherwise, a negative correlation was found between concentration of calcium polysulfide and fungal biomass. Disease severity of cocoa treated by calcium polysulfide 80 mL L^{-1} every week was 11.67% significantly lower than control, i.e. 68.33%. Furthermore, it was also found that the index value of fungal diversity on the phyllosphere decreased by increasing concentration and interval of calcium polysulfide application.

Keywords: Calcium polysulfide, Cocoa, *Phytophthora palmivora*.

INTRODUCTION

Indonesia is one of the world's largest cocoa producers. In 2012/2013 Indonesia's cocoa production is in third position after Ivory Coast and Ghana. Indonesia cocoa production reached 410.000 tons, while Ivory Coast and Ghana are 1.449.000 and 835.000 tons respectively [1]. Cocoa cultivated area in Indonesia is 1,774,500 ha, not less than Ghana which is 1,600,300 ha [2]. However, Indonesia's cocoa production is still lower than Ghana.

One of the constraints of cocoa production in Indonesia is pod rot disease. Umayah and Purwantara collected twenty *Phytophthora palmivora* isolates from six provinces of cocoa producer area in Indonesia that cause cacao pod rot and stem cancer [3]. This disease can cause yield loss up to 90% especially in rainy or dry season on land with a large ants population [4]. The symptoms of cocoa pod rot can be found from the base, middle, tip, old fruit, young fruit, cherelle, fruit located below, in the middle, or at the top of tree. If the fruit is symptomatically affected by pod rot, it will split then flesh shown and seeds rotten, into brownish color. The advanced infection stage, cocoa beans wrinkled and color changed to black and mummified [5]. This condition cause yield loss and poor seeds quality [6].

Calcium polysulfide is one of the potential control for pod rot plant disease. Although classified as synthetic inorganic fungicide and possibly affect the microflora on cocoa surface, but calcium polysulfide is allowed in organic farming and relatively safe to the environment [7-12]. Calcium polysulfide is capable to kill spores by disrupting the electron transport [13] as well as penetrating the fungal mycelium so that the fungus dies [14]. Affects the respiratory complexes of mitochondria by disrupting the flow of electrons in the respiratory chain, causing multi-site toxicity on a broad spectrum [15,16]. Affects enzymes in mitochondria [17]. Calcium polysulfide toxicity against non-target organisms is between non-toxic to moderate toxicity [18-21]. The purpose of this study were to evaluate the calcium polysulfide potential to control the disease and its effect on the phyllosphere-fungal diversity.

MATERIALS AND METHODS

In Vitro

The used experimental method is poisoned food technique. The experiment was prepared based on a complete randomized design,

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consist of seven treatments and four replications. Treatment test include:

C : Vegetable 8 Juice Agar (V8JA);
CP_5 : V8JA + 5 ml L^{-1} of calcium polysulfide
CP_{10} : V8JA + 10 ml L^{-1} of calcium polysulfide
CP_{20} : V8JA + 20 ml L^{-1} of calcium polysulfide
CP_{40} : V8JA + 40 ml L^{-1} of calcium polysulfide
CP_{80} : V8JA + 80 ml L^{-1} of calcium polysulfide
M_2 : V8JA + 2 g L^{-1} of Mancozeb.

Relative Growth Inhibition and P. palmivora Biomass

Colonies diameter of *P. palmivora* for each replication was calculated from four quadrants, i.e. two transverse diameters and two longitudinal diameters. The influence of treatment on pathogen growth is showed in (%) value of relative growth inhibition (RGI) against control [22]:

$$\frac{colony\ diameter\ of\ control - colony\ diameter\ of\ treated\ fungi}{colony\ diamter\ of\ control} \times 100\%$$

Biomass observation of *P. palmivora*, colonies on the surface of V8JA medium were washed with 10 mL HCl 4%. Filtered by No. 1 Whatman filter paper (filter paper weight has been known previously) and rinsed three times. Then dried in an oven with 70°C temperature for 3 days. After that the dry weight of *P. palmivora* was calculated from the dry weight difference between the empty filter paper and the filter paper contain *P. palmivora*.

In Vivo

The experiments were prepared based on factorial randomized block design, with 4 treatments and 3 replications. Each replication consists of 20 sample units (cocoa pod). Based on in-vitro experiment, obtained the best concentration of calcium polysulfide is 40 and 80 mL L^{-1} which used as in-vivo calcium polysulfide concentrations.

The first tested factor is concentration, consist of four levels: C: Control is sprayed with water; CP_{40}: 40 mL L^{-1} of calcium polysulfide; CP_{80}: 80 mL L^{-1} of calcium polysulfide; M_2: 2 g L^{-1} of mancozeb. The second factor is interval applications, which consists of three factors: once in a week, once in two weeks and once in four weeks.

Cocoa Pod Rot Attack Intensity

Observations on *P. palmivora* attack intensity were conducted once in a week and calculate using formula [23]:

$$I = \frac{\sum (ni \times Vi)}{N \times Z} \times 100\%$$

Description:
I = Attack intensity (%)
N = Number of pod observed
V = Value of damage scale
Z = The highest scale value
n = Number of damaged pod
Value of damage scale (V):
Score 0: healthy, no symptoms
Score 1: small, symptoms of pod infection <25%;
Score 3: moderate, symptoms of pod infection 25% ≤ x <50%;
Score 9: severe, symptoms of infection ≥ 50%

Level of Eficacy

Fungicide level of efficacy calculation conducted at last observation. Fungicide are effective when level of efficacy value > 50% and significantly different with control. Calculation using formula [24]:

$$\text{Level of Eficacy} = \frac{\text{Iac} - \text{Iat}}{\text{Iac}} \times 100\%$$

Description:
Iac = Pathogen intensity of attack on control
Iat = Pathogen intensity of attack on fungicide treatment

Fungal Diversity on the Phyllosphere

Cocoa pod sampling for fungal diversity were taken before application, 13th week and 21st week. To calculate fungal diversity using Shannon index formula (H') [25]. Species diversity index can be defined as high diversity if the value of H' > 3, medium diversity if 1 ≤ H' ≤ 3, low diversity if H' < 1 [26].

$$H' = \sum p_i Ln\ p_i, where\ Pi = \frac{n_i}{N}$$

Description:
n_i = the number of individuals (isolate) in each spesies
N = the total number of isolates

RESULT AND DISCUSSION

Relative Growth Inhibition (%)

Observation in two days after inoculation on 80 mL L^{-1} calcium polysulfide concentration was significantly different from 5, 10 and 20 mL L^{-1} calcium polysulfide concentrations and also the control (Fig. 2). Colonies diameter of *P. palmivora* on media treated with calcium polysulfide was smaller than control colony diameter. Likewise, enhancement of calcium polysulfide concentrations also causes decreasing *P. palmivora* colonies diameter (Fig. 1 and Fig. 2). One level increase of calcium polysulfide concentration causes an increase in 5.978% relative growth inhibition $y = -2.498 + 5.978x$ (Fig. 3).

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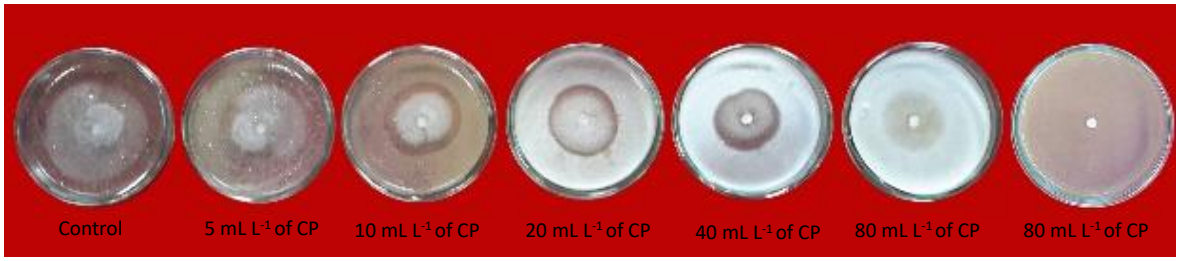

Figure 1. *P. palmivora* colonies diameter (circled in red) at 6 day after inoculation
CP: Calcium polysulfide; M: Mancozeb

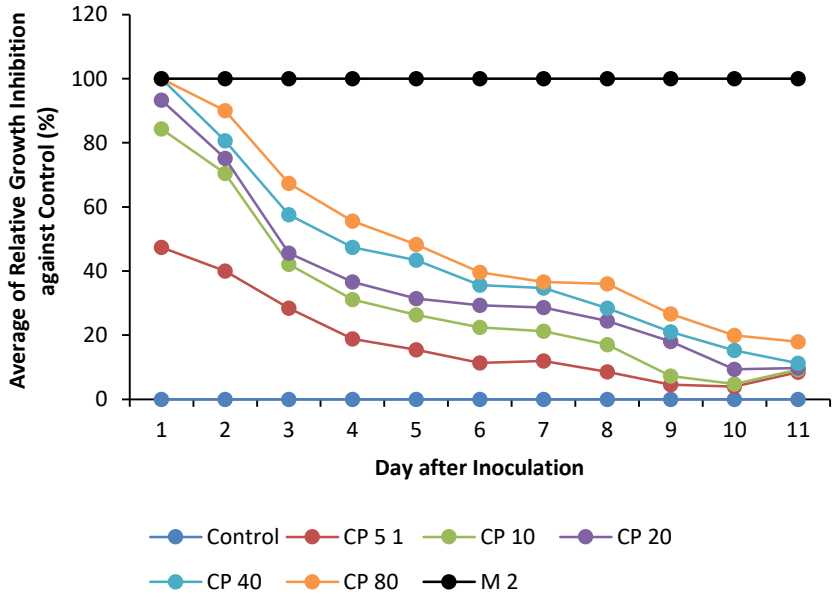

Figure 2. Effect of Calcium Polysulfide Concentration against Average of Relative Growth Inhibition against Control (%)
CP: Calcium polysulfide (mL L^{-1}); M: Mancozeb (g L^{-1})

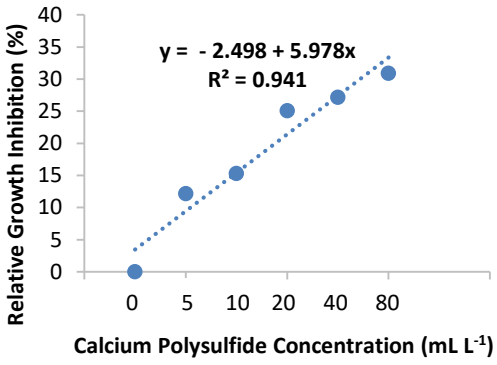

Figure 3. Regression between Calcium Polysulfide Concentration with Relative Growth Inhibition

Biomass of *P. palmivora*

Biomass is influenced by the fungi component such as mycelia that is formed. The colony diameter on calcium polysulfide treatment is smaller and thinner than the control. Similarly, comparison between calcium polysulfide concentrations also shows differences in *P. palmivora* diameter colonies and its thickness. The high calcium polysulfide concentration causes colony diameter become small and thin (Fig. 1). Its known that 80 mL L^{-1} calcium polysulfide treatment is significantly different from the other treatments and is not significantly different from 40 mL L^{-1} calcium polysulfide treatment (Table 2). One level increase of calcium polysulfide concentration causes a decrease in 0.004 g of biomass $\mathbf{y = 0.031 - 0.004x}$ (Fig. 4).

Table 2. Effect of Calcium Polysulfide Concentration Against Average of *P. palmivora* Biomass

Treatments	Biomass (mg)
Control	24.2 b
CP 5 mL L^{-1}	20.7 b
CP 10 mL L^{-1}	20.2 b
CP 20 mL L^{-1}	18.9 b
CP 40 mL L^{-1}	8.1 ab
CP 80 mL L^{-1}	1.5 a
M 2 g L^{-1}	0.05 a

Description: Numbers with the same letter in the same column, based on the 5% DMRT show no significant difference; CP: Calcium polysulfide; M: Mancozeb.

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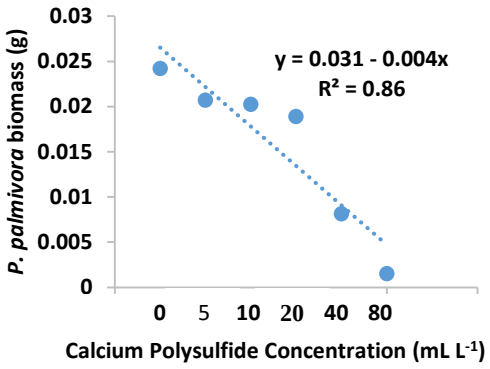

Figure 4. Regression between calcium polysulfide Concentration with *P. palmivora* biomass

Figure 5. Cocoa Pod Rot Attack Intensity (1: Healthy; 2: Small attack; 3: Moderate attack; 4: Severe attack)

Cocoa Pod Rot Attack Intensity

Observation and data analysis on cocoa pod rot (*P. palmivora*) intensity of attack was conducted every week until 21st week. There is interaction between treatments (4 levels) with applications interval (3 levels). The interaction occurred in the 2nd and 7th - 9th week observation (Table 3). This shows that the influence of each factor is not same for every other level of factor.

In general, observations at 7th – 9th weeks of 80 mL L^{-1} calcium polysulfide treatment showed a significant effect on intensity of cocoa pod rot attack mean than other treatments at each level of applications interval. Calcium polysulfide 40 mL L^{-1} and M 2 g L^{-1} treatment in general at every level of application interval showed that average cocoa pod rot attack intensity was not significantly different except with 80 mL L^{-1} calcium polysulfide and control treatment. Once in a week application interval of 80 mL L^{-1} calcium polysulfide was found as the best treatment to control the disease.

Fungicide Level of Eficacy

Level of efficacy value at once in a week application interval of 80 mL L^{-1} calcium polysulfide is 70% (Table 4). This is effective for controling cocoa pod rot in-vivo, because efficication rate value more than 50% and significantly different compared with control.

Table 4. Level of Eficacy (%) at the Last Observation

Treatments	Application Intervals (once in ... week)		
	1	2	4
Control	0.00 a	0.00 a	0.00 a
CP 40 mL L^{-1}	20.33 b	11.85 b	7.33 b
CP 80 mL L^{-1}	70.00 c	47.79 c	35.00 c
M 2 gL^{-1}	23.33 b	8.47 b	6.33 b

Description: Numbers with the same letter in the same column, based on 5% DMRT show no significant difference. Data is transformed to arcsin √x for statistical analysis. CP: Calsium polysulfide; M: Mancozeb.

Table 3. Effect of Calcium Polysulfide Concentration against Average of Cocoa Pod Rot (*P. palmivora*) Intensity of Attack (%)

Treatments	Application Intervals (…once a week)	Observations							
		2nd Week		7th Week		8th Week		9th Week	
Control	1	0.33	ab	62.67	gh	62.67	e	68.33	ef
	2	2.00	b	65.67	h	71.00	f	85.00	g
	4	5.33	c	88.33	i	88.33	g	89.00	g
CP 40 mL L^{-1}	4	0.33	ab	37.33	d	51.67	d	58.33	d
	2	2.67	b	45.00	e	50.67	d	64.67	def
	1	0.67	ab	49.33	e	58.38	de	72.00	f
CP 80 mL L^{-1}	4	0.33	ab	10.00	a	11.00	a	11.67	a
	2	0.00	a	17.00	b	17.67	b	18.00	b
	1	1.00	ab	30.67	c	35.33	c	42.67	c
M 2 gL^{-1}	4	2.67	b	52.00	ef	52.67	d	59.33	d
	2	1.00	ab	57.33	fg	61.67	e	62.00	de
	1	0.00	a	63.67	gh	64.00	e	72.33	f

Description: Numbers with the same letter in the same column, based on 5% DMRT show no significant difference. Data is transformed to √x (2nd week); arcsin √x (7th – 9th week) for statistical analysis. CP: Calcium polysulfide; M: Mancozeb

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Fungi Diversity on Cocoa Pod Surface

In general, the fungi diversity index has decreased, where the highest decrease occurred in once a week application interval of 80 mL L^{-1} calcium polysulfide treatment (Fig. 6). But from the third time cocoa pod sampling from the field, according to Fachrul [26] fungi diversity index is still categorized as medium (1.08-1.93).

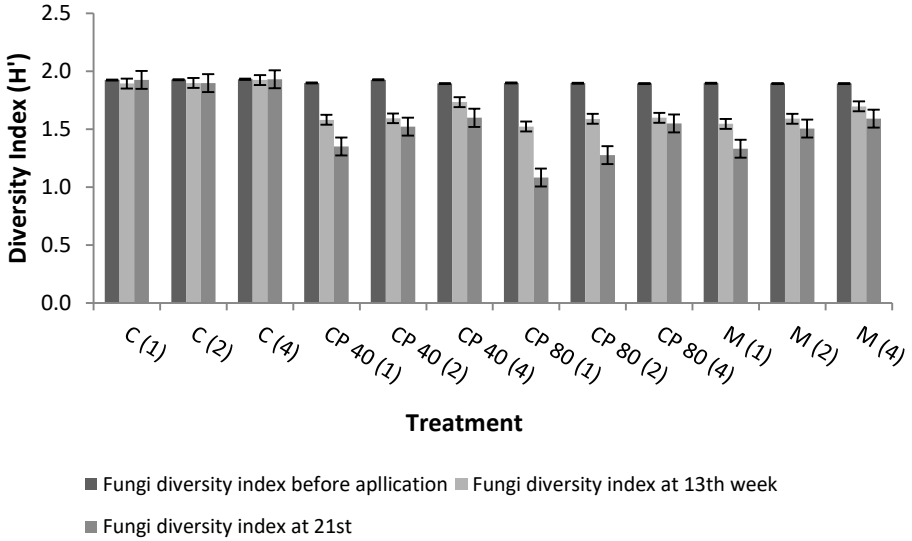

Figure 6. Fungi Diversity Index on Cocoa Pod Surface
C: Control: CP 40: 40 mL L^{-1} of calcium polysulfide; CP 80: 80 mL L^{-1} of calcium polysulfide; M: 2 g L^{-1} of Mankozeb; 1, 2, 4: Applications Interval (once in ... week)

Discussion

Calcium polysulfide has ability to inhibit growth, affect the biomass, affect the *P. palmivora* intensity of attack and affect the fungal diversity index. Because sulfur content in calcium polysulfide can inhibit fungal enzymes [27]. Sulfur affects fungal enzymes that can prevent mycelial growth [28]. Indicates the presence of polyphenols oxidation inhibition [27,29].

Calcium polysulfide is able to penetrate fungi mycelium so that the fungus dies [20]. Sulfur affects complex mitochondrial respiration by interrupting flow of electrons in respiratory chain, leading to multi-site toxicity [15,16,30].

Calcium polysulfide kills germinated spores [31]. This mode of action involves the interception of electrons in effective respiration chain by reducing element of sulfur to hydrogen sulfide, which is also toxic to cell proteins [16,32].

Sulfur as an effective inhibitor of conidia formation and germination and may affect multiple target sites in fungal cells. Among others, by inhibiting respiration by receiving electrons in the cytochrome c region that can interfere with proteins and bind heavy metals in cells [28,33,34].

At conidia, calcium polysulfide is able to prevent citrate production from exogenous acetate, and causes increased succinate, indicating enzymes blockage in pathway between acetate and citrate and inhibiting the succinoxidase system in Krebs cycle [35]. Sulfur as a hydrogen acceptor and provides interference in dehydrogenation reaction [34]. Free radicals from sulfur are formed in the sulfur-polysulfide-H_2S conversion and these are highly reactive free radicals, which are toxic [35].

Some enzymes that can be disturbed by calcium polysulfide i.e. tricarboxylic acid enzymes, hexokinase enzymes, triosphosphate dehydrogenase enzymes, aldolase enzymes, cocarboxylase enzymes, thiamin pyrophosphate enzymes, keto acid dehydrogenase enzymes, polyphenol oxidase enzymes, citric acid synthetase enzymes, akonitase enzymes, isositrate dehydrogenase enzymes, α-ketoglutarate dehydrogenase enzymes, succinate dehydrogenase enzymes, malate dehydrogenase enzymes and glycolytic enzymes (hexokinase, phosphohexose isomerase, phosphofructokinase, aldolase, triosa phosphate isomerase, glyceraldehyde 3-phosphate dehydrogenase, phosphoglycerate kinase, phosphoglycerate mutase, enolase and pyruvate kinase) [17].

CONCLUSION

It was found a positive correlation between concentration of calcium polysulfide and

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inhibition of fungal growth in the media. Growth inhibition of fungi in the agar medium contained calcium polysulfide 80 and 40 mL L^{-1} was 17.921% and 11.295% respectively, significantly higher than control. It was found a negative correlation between concentration of calcium polysulfide and fungal biomass. Fungal biomass contained calcium polysulfide 80 and 40 mL L^{-1} was 1.5 mg and 8.1 mg respectively, significantly lower than control, i.e. 24.2 mg. Disease severity of cocoa treated by calcium polysulfide 80 mL L^{-1} every weeks was 11.67% significantly lower than control, i.e. 68.33%. Furthermore, it was also found that the index value of fungal diversity on the phyllosphere decrease by increasing concentration and interval of application calcium polysulfide.

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Phylogenetic Patterns of Genus *Megophrys* (Anura: Megophryidae) from Java and Sumatra

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Abstract

The emergence of Java and Sumatra islands at the end of Miocene, has impact to the diversity of fauna that living in the islands. Amphibia, as one of a genetic information series, can be used as a good phylogenetic comparison. Genus *Megophrys* (Anura: Megophryidae), is a member of amphibians that live isolated at an altitude of 1000 ASL (Above Sea Level) or more. The purpose of this research is to analyze the phylogenetic patterns of genus *Megophrys* in Java and Sumatra based on the analysis of 16s rRNA gene. We also analyzed the zoogeography to understand the history of relationship and the establishment of these islands. We took samples on 12 localities: 10 areas of Sumatra and 2 areas of Java. We used Qiamp Blood and Tissue Kit to extract the DNA from tissues. Qualitative analysis of DNA was done by running electrophoresis on 1% agarose gel. Qualitative analysis of DNA was done by running electrophoresis on 1% agarose gel. Results show that Clade A has 2 subclasses: I and II. Subclade I consists of Sumatran specimens, whereas subclade II consists of Javanese specimens. Subclaude I and II have paraphyletic connections. Clade B classified as politomy clade consists of *Megophrys* sp. from West Pasaman, *Megophrys* sp. from West Sumatra and *Megophrys* sp. from Pasawaran, Lampung. The result shows that *Megophrys* sp. of subclade I (clade A) and clade B, belongs to the same group, both of them were originated from Sumatra. *Megophrys* sp. subclade I (clade A) originated from Tanggamus (Lampung) and West Lampung (Lampung) separated with 2 species of *Megophrys* sp. of clade B originated from Pasawaran (Lampung).

Keywords: 16s rRNA, Java, *Megohprys*, Sumatra, zoogeography

INTRODUCTION

Java and Sumatra islands emerged at the end of Miocene era. It has impact to the diversity of fauna living there [1]. Genetic factor contributes greatly on determining the relationships among fauna, as well as being the key for studying it's distribution time [2]. Amphibia is one of a genetic information series that can be used as good relationship comparison. Due to its small roaming, high dependence on microhabitat, and population limitations, it makes amphibia an attractive option on understanding a historical biogeography [2,3,4].

Genus *Megophrys* (Anura: Megophryidae) is a member of amphibians that live isolated at an altitude of 1000 ASL (Above Sea Level) or more [5]. This frog is easily recognized by the 'hornlike' eyelids [6]. Javan horned frog, *Megophrys montana,* is an endemic species from Java [5]. Other species were dispersed in Sumatra, Malayan horned frog *M. nasuta*, was also found spread from Yala, Thailand, Malay Peninsular, Sumatra, Kalimantan, to the Natuna Islands [7]. *Megophrys* were not an endangered genus in the world, but their presence are limited to certain of localities [8]. The purpose of this research is to analyze the phylogenetic patterns of genus *Megophrys* in Java and Sumatra based on the analysis of 16s rRNA gene. We also analyzed the zoogeography to understand the history of relationship and the establishment of these islands.

MATERIALS AND METHODS

Data Collection

We took samples on 12 localities: 10 areas of Sumatra and 2 areas of Java. Sampling was done by catching directly method on hills and mountains above 1000 mdpl (Table 1). Samples preserved by injection of 70% alcohol in the heart of the animals. DNA obtained from animals by dissecting abdomen right to the chest, to get DNA sample of liver or tissue on the femur. We added SDS and EDTA to the tissues then inserted into the sample tube. The animal samples were

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injected with formaldehyde solution thoroughly then soaked in alcohol (wet preserved).

DNA Extraction, Isolation, and Amplification

We used Qiamp Blood and Tissue Kit to extract the DNA from tissues. Procedure was done by took 25 µL of the tissue, added by 180 µL ATL buffer. The solution homogenized by vortex. Homogenate added with 20 µL of proteinase K, then homogenized using vortex. Homogenate solution incubated in waterbath at 56°C to complete a lysis solution.

The solution homogenized for 15 seconds, added by 200 µL of AL buffer, homogenized, then added by 200 µL of absolute ethanol (96-100%), re homogenized. Homogenate solution took using micropipette into 2 mL collection tube (equipped with Qiamp mini spin column). Solution centrifuged at 6000 x g (8000 rpm) for 10 minutes. Qiamp Mini spin column was placed on new 2 mL collection tube, added with 500 µL of the AW1 buffer, centrifuged at 6000 x g for 1 minute. Qiamp Mini spin columns were placed into 1.5 mL microtube, added 200 µL of AE buffer, then incubated for 1 minute at room temperature. After that, the solution was centrifuged at 6000 × g for 3 minutes.

Qualitative analysis of DNA were done by running electrophoresis on 1% agarose gel. Electrophoresis started by a gel-making process. Mixture consist of 0.2 grams of agarose powder, 20 mL TBE buffer, and 1 µL EtBr solution. Agarose powder poured into a beaker glass, added 20 mL TBE solution, then boiled. Boiled gel cooled down for a while then added by 1 µL EtBr. The gel poured into the a gel tray with the well comb in place. Agarose gel were loaded into gel electrophoresis box and added 1 µL Loading dye and 1 µl extract DNA on each gel's lane. Marker (100bp Benstop) put at the first lane of the gel. Negative control made by put 1 µL loading dye into the gel's lane. Filled gel's run at 90 V for ± 45 minutes. Electrophoresis results were documented using Gel Doc- UV Transiluminator.

PCR mix solution's composition in each PCR tube are 3.6 µL ddH20, 5 µL PCR mix (Go Taq Green), 0.2 µL primer of 16 SBR (forward) (5'-CCG GTC TGA AC TCA GAT CAC GT-3 '); 0.2 µL primer of 16 SAR (reverse) (5'-CGC CTG TTT ATC AAA AAC AT-3 '); 0.5 µL extract of DNA. Forward and reverse primer stocks were eluted by mixed 1 µL primer with 9 µL Free Nuclease Water or TE buffer (Tris - EDTA). PCR's cycle repeated for 34 times by predenaturation at 95°C for 3 minutes, denaturation at 95°C for 30 seconds, annealing at 52°C for 60 seconds, extension at 72°C for 60 seconds, and post-extension at 72°C for 7 minutes for each cycle. The PCR product was purified with PCR sequence added by PCR kit. The amplified gene was examined on 2% agarose gel to perform electrophoresis.

Table 1. *Megophrys* sp. samples used on this research

Sample's Code	Species	Locality	Island	Accession Number
Megophrys sp_10	*Megophrys* sp.	Pasawaran District, Lampung	Sumatra	MG012872
Megophrys sp_11	*Megophrys* sp.	Pasawaran District, Lampung	Sumatra	MG012881
Megophrys sp_7	*Megophrys* sp.	West Lampung, Lampung	Sumatra	MG012873
Megophrys sp_5	*Megophrys* sp.	Ogan Komering Ulu District, South Sumatra	Sumatra	MG012874
Megophrys sp_6	*Megophrys* sp.	Tanggamus District, Lampung	Sumatra	MG012875
Megophrys sp_9	*Megophrys* sp.	West of Pasaman District, West Sumatra	Sumatra	MG012876
Megophrys sp_8	*Megophrys* sp.	Pandeglang, Banten	Java	MG012877
Megophrys montana	*Megophrys montana*	Bandung, West Java	Java	MG012878
Megophrys sp_1	*Megophrys* sp.	Toba Samosir, North Sumatra	Sumatra	MG012882
Megophrys sp_2	*Megophrys* sp.	Humbang Hasundutan, North Sumatra	Sumatra	MG012879
Megophrys sp_3	*Megophrys* sp.	Karo, North Sumatra	Sumatra	MG012883
Megophrys sp_4	*Megophrys* sp.	Mandailing Natal, North Sumatra	Sumatra	MG012880

Data Analysis

The isolated DNA that has been amplified were sequenced at Uniseck Laboratory GATC, Germany. The quality of sequences were checked and combined for forward and reverse sequences using AB Sequence Scanner. The results confirmed using BLAST to confirm the species. Sequence data has been submitted to GenBank (https://www.ncbi.nlm.nih.gov) and can be accessed online after January 2017. Sequences data from the genus Megophrys used to compare and outgroup species built by taken molecular data from GenBank (http://www.ncbi.nlm.nih.gov) (Table 2).

The sequence result aligned using MEGA 6.0. Reconstruction of phylogenetic trees was performed by using Maximum Parsimony (MP), Maximum Likelihood (ML), and Bayesian Inference (BI) the analyzed using PAUP * 4.0b10 [9]. General Time Reversible (GTR) of DNA

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evolution was analyzed using the gamma parameter [10]. The ML modeling on PAUP were analized using Jmodeltest program. BI and Bayesian Posterior Probabilities (BPP) were estimated using Mr.Bayes 30b4 [11]. Analysis of BI by using Monte Carlo Markov Chains (MCMC) for 6000000 generations and burn in = 3,000,000. MP analyzed for non parametric bootstrapping for 1000 pseudoreplication on PAUP and 100 replications on ML analysis [12]. Genetic distance analyzed using MEGA 6.0.

Table 2. Sequences Data of the Genus *Megophrys* and Outgroup Species From GenBank

Species	Accesion Number	Locality
M. acuta	KJ579124	Guangdong, China
M. nankiangensis	AY526200	Sichuan, China
M. obesa	KJ831312	Guangdong, China
M. omeimontis	AY561307	Sichuan,China
M. spinatus	AY526205	Sichuan,China
Leptobrachium mangyanorum	GQ995556	Philippines
L. tagbanorum	GQ995552	Philippines

RESULT AND DISCUSSION

Phylogenetic Tree of Genus *Megophrys*

Clade A has 2 subclasses: I and II (Fig. 1). Subclade I consists of Sumatran specimens, whereas subclade II consists of Javanese specimens. Subclaude I and II have paraphyletic connections. These results are supported by high MLBS values (MPBS = 82%; MLBS = 57%; BPP = 100%) [13]. The Iaa and Iab group have a monophyletic relationship, but the data is only supported by MPBS (MPBS = 97%; MLBS = 65%; BPP = 71%). Boostrap values in phylogenetic tree branches were considered significant (70%) [13]. The Iaa group consists of *Megophrys* sp. (1) originated from Toba, *Megophrys* sp. (2) from Humbang Hasundutan, *Megophrys* sp. (3) from Karo, and *Megophrys* sp. (4) from Mandailing Natal (North Sumatra).

Megophrys sp. originated from Toba Samosir and Mandailing Natal were closely related to p-distance = 0 and supported with high bootstrap values (MPBS = 99%; MLBS = 100%; BPP = 100%). *Megophrys* sp. originated from Humbang Hasundutan and Karo also shows a close relationship (p-distance = 0). *Megophrys* sp. of Toba Samosir and Karo are closely related and considered as the same species (p-distance = 0.01). *Megophrys* sp. (4) from Mandailing Natal is the sister lineage of the three species with p-distance = 0.01 which is still related in the same species (MPBS / MLBS / BPP = 100%).

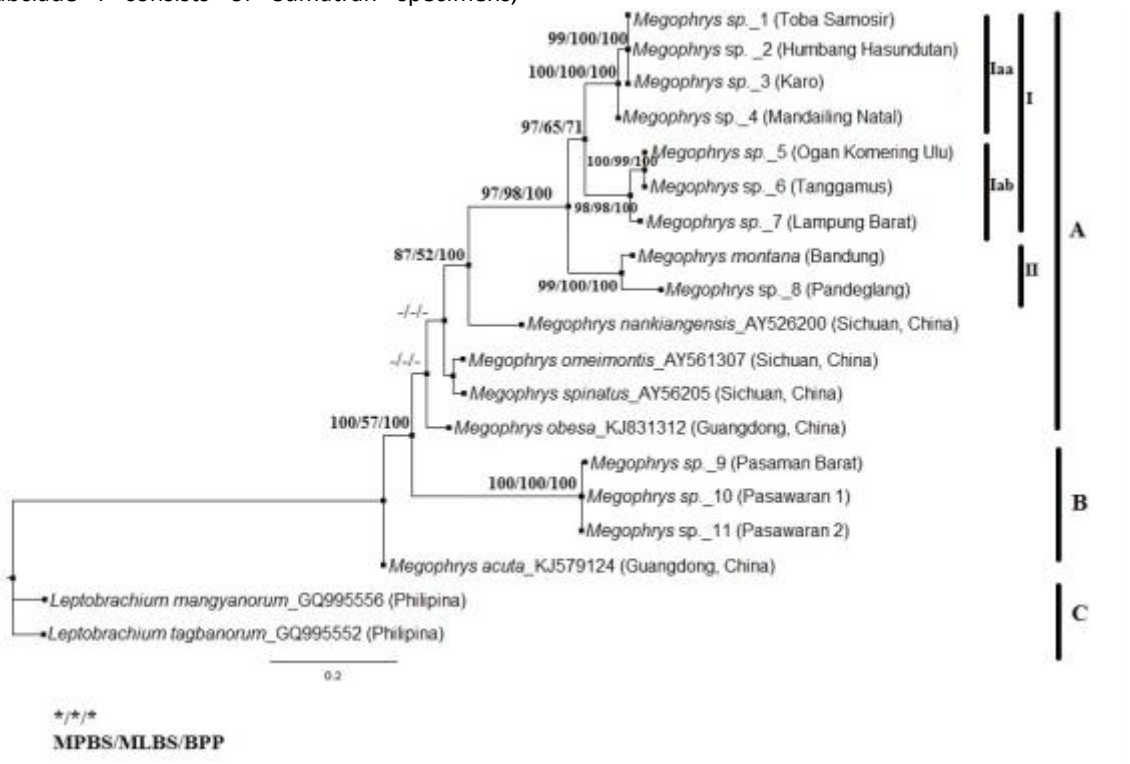

Figure 1. The Phylogenetic Tree of Genus *Megophrys* from Java and Sumatra based on 16S rRNA Analysis

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All species in the Iab group were closely related and probably still in a species (p-distance <0.05). The Iab group consists of *Megophrys* sp. (5) from Ogan Komering Ulu, *Megophrys* sp. (6) from Tanggamus, and *Megophrys* sp. (7) from West Lampung (Lampung). Based on p-distance analysis, *Megophrys* sp. from Ogan Komering Ulu and Tanggamus are in a close relationship (p-distance = 0.00) (MPBS = 100%; MLBS = 99%; BPP = 100%). *Megophrys* sp. from West Lampung is the sister lineage of *Megophrys* sp. from Ogan Komering Ulu and Tanggamus (MPBS = 98%; MLBS = 98%; BPP = 100%). *Megophrys* sp. from Ogan Komering Ulu and West Lampung are still closely related in a species (p-distance = 0.03). *Megophrys* sp. from West Lampung and Tanggamus showed intraspecies relationship (p-distance = 0.03). Based on the p-distance value, the three species of *Megophrys* sp. of the Iab group are still closely related in the same species.

Subclade II consists of *M. montana* species originated from Bandung, West Java and *Megophrys* sp. (8) from Pandeglang, Banten. Based on p-distance analysis, *M. montana* from Bandung and *Megophrys* sp. from Pandeglang isnot included as the same species, shown by p-distance = 0.06 (MPBS = 99%; MLBS = 100%; BPP = 100%). *M. nankiangensis* (Sichuan, China) is a sister lineage of the Ia and Ib groups, and was the only species supported by high bootstrap MPBS and BPP values (MPBS = 82%; MLBS = 57%; and BPP = 100%).

Clade B classified as politomy clade consist of *Megophrys* sp. (9) from West Pasaman, *Megophrys* sp. from West Sumatra (10) and *Megophrys* sp. (11) from Pasawaran, Lampung. The three species of *Megophrys* sp. closely related to on p <0.05 (MPBS / MLBS / BPP = 100%) value. The three species of *Megophrys* sp. have 98% resemblance to *M. nasuta*. Uniquely, *M. acuta* (Guangdong, China) considered as the sister lineage of the three species of *Megophrys* sp. from Pasaman Barat, Pasawaran 1, and Pasawaran 2.

Zoogeography

The result shows that *Megophrys* sp. of subclade I (clade A) and clade B, belongs to the same group, both of them were originated from Sumatra. *Megophrys* sp. subclade I (clade A) originated from Tanggamus (Lampung) and West Lampung (Lampung) separated with 2 species of *Megophrys* sp. of clade B originated from Pasawaran (Lampung). Hypothetically, the location of the species is close to each other and should be able to construct the same clade. The p-distance values of these species indicate a considerable genetic distance to each other (p – distance> 0.020). The topography of the Bukit Barisan Mountains, most likely, affects this phenomenon. Other areas that have splited between subclade I (clade A) and clade B are Ogan Komering Ulu, Tanggamus, West Lampung, Pasaman and Pasawaran, surrounded by highlands, hills, up to mountains that its height more than 2,000 meters. Those barriers can be lead to the separation and genetic isolation process.

The construction of phylogenetic tree shows the relation of zoogeography, where the dispersal areas of *Megophrys* sp. took place on clade A (subclass I and subclass II) consists of northern, western, southern area of Sumatra, and Java (Fig. 2). Meanwhile, *Megophrys* sp. on clade B separated into subclass I which is also originated from Sumatra. Subclade II that was consist of *M. montana* from Bandung, West Java and *Megophrys* sp. from Pandeglang Banten have a proximity to subclass I originated from West Lampung, Ogan Komering Ulu, and Tanggamus. The dispersal of species *Megophrys* sp. from the northern part of Sumatra to the southern part of Sumatra was indicated by the species on subclade II and clade B. It can be explained by the geographic history of the Pleistocene epoch (1.8-0.01 MYA), when rivers from northern Sumatra and the western part of Malay Peninsula, formed a stream known as the Straits of Malacca nowadays. The ancient river system provided spreading pathways for amphibia [2].

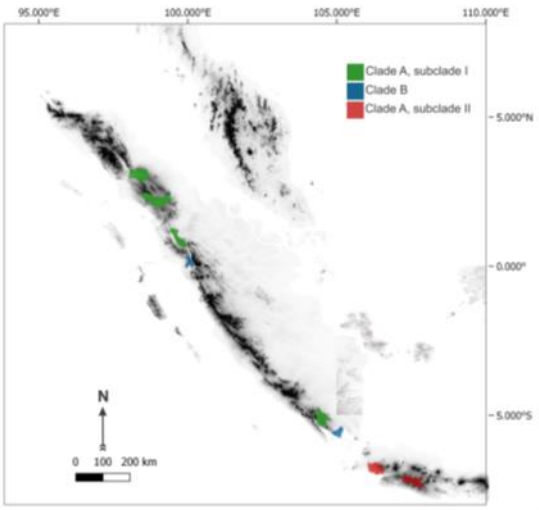

Figure 2. Distribution Map of *Megophrys* species from Java and Sumatra based on Phylogenetic Analysis

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Twenty five percents of species from Java Island come from Borneo and 43% of species may come from Sumatra. A total of 20 non-endemic species of Java are also known in Sumatra. These species was predicted that had reached Java in the last few millenium. Several species from Sumatra which commonly found in Java, can be found in lowland areas [5]. The exchange of fauna between Java and Sumatra is likely occured several times during the Pleistocene period due to the merge of the two islands and the loss of the Sunda strait [2].

Geologically, Sumatra has been much more stable than Kalimantan or the Malay Peninsula. During the epoch of Miocene, when most of Kalimantan and the Malay Peninsula areas might still be a mainland, the Sumatra's areas were mostly flooded and separated, then fragmented into a small volcanic island [14]. The exchange of amphibia may also occured in the epoch of Miocene (23.5-5 MYA) and Pliocene (5-1.8 MYA) affects to the spread of *M. montana* in Java [5].

The exchange of fauna occured after the formation of a broad topographic areas at the beginning of the epoch Pleistocene (Fig. 3). This exchange allowed fauna from the Malay Peninsula to have several similarity to Kalimantan and Sumatra fauna. However, it actually has greater similarity to Sumatra fauna. The regression of the sea level between Sumatra and the Malay Peninsula at that time, reached up to 30 meters, allowing a sub-aerial dispersal route between the Malay Peninsula and Sumatra.

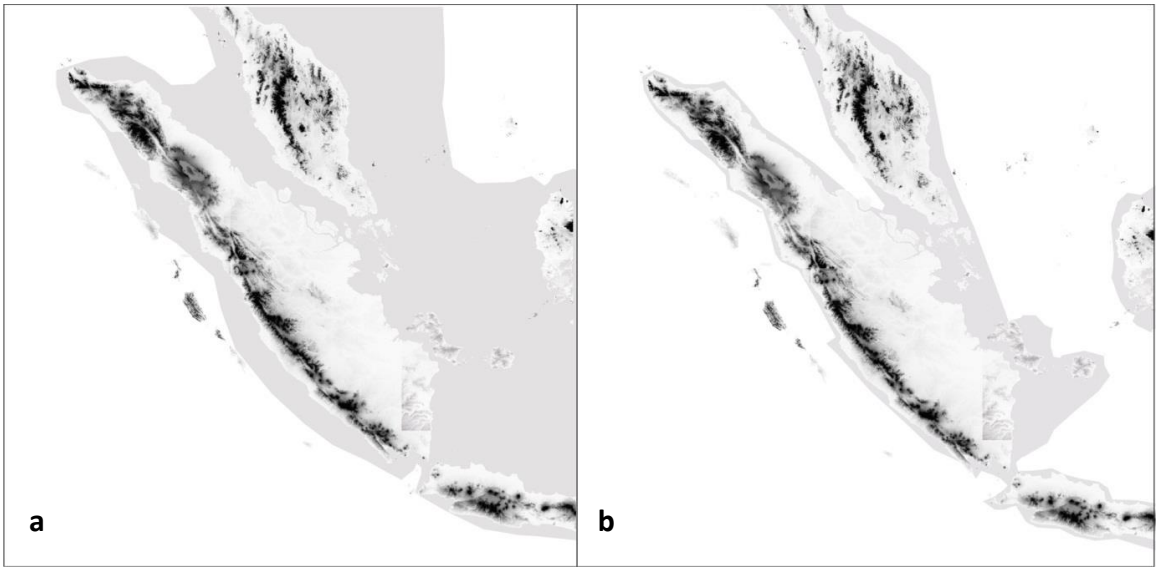

Figure 3. Siltation of the Sunda Strait which Resulted in the Merge of Java, Sumatra and the Malay Peninsula During the Pleistocene Epoch (redrawn from Inger and Voris [2])
Description: a) At the maximum lowering of sea level (120 meters from current surface-below present level);
b) At 30 meters from the current surface

This spread of the species were carried out by the non-endemic fauna of Sumatra, the most diversity species originated from the Malay peninsula. *Megophrys nasuta*, as one of the non-endemic species, can breed in ancient rivers that was still connected the two lands. It is different with the exchange of the species on Malay peninsula and Sumatra, where between Sumatra and Kalimantan was more difficult to be carried out into, because of the connected periods for both islands are shorter. This phenomenon considered as the reason why the pattern among the fauna has a less similarity [2].

Species's distribution of the genus *Megophrys*, one them is *M. nasuta*, started from the Malay Peninsula and Sumatra. *M. nasuta* experienced a sympatric speciation in Kalimantan and a long period of isolation in the Tertiary era. *M. nasuta* probably reached its distribution areas as well on modern period when the Pleistocene epoch, when the seawater regression was maximized. The ability to reproduce and tolerance to dry climates indicate that these species are able to survive at the relatively dry Pleistocene epochs on Sundaland [2].

Sympatric speciation could be defined as the result of reproductive isolation without any geographical isolation [15]. Geographical isolation occurs when the distance between population exceeds the ability of individuals to

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spread or when the environment is not appropriate thus hindering the spread of population. Geographical barriers are an extrinsic factor of the environment which is affects all individual, independent and genotype.

Unclear and random situations could arise because of the extrinsic barrier of the environment that affects the biological nature of each individual. Sympatric speciation also defined between populations that initially used two different habitats but then experienced a free distribution [16]. Coyne and Orr [17] predicted that the species arised from sympatric speciation, by the most of them have overlapping geographic ranges. The spatial scale to determine the overlapping areas must be balanced with the organism's dispersal ability. In principle, species originated through sympatric speciation can be allopathric at over time [18].

Clade that predicted as a construction through sympatric speciation must be a sister species or monophyletic. All speciation processes occured in several geographical contexts and depend on the spatial distribution of biodiversity and considered as mechanisms of speciation. The geographical structure of habitats always plays a role on the process of sympatric, parapatric, and allopathic speciation, and also on the population divergence then lead to the change from allopathric to sympatric [19] or vice versa [18].

Allopatric speciation can occurs through the separation of one population into an ecologically divided area, but physically, it is not an appropriate habitat [20], or it may be said that the allopathic speciation is a divergence niche [21]. Population which comes from an allopathic speciation, experienced by individuals from different areas, impacts to the limits of species' range and its distribution [22]. Barrier supports a new population to find an appropriate and suitable habitat. The existing barrier types are influenced by the presence of substantial physographical transitions or other geological features, such as rivers and mountains as a range of inhibiting species's dispersal [23]. Thus, the effect of the barrier appears as an intrinsic response of species with wide environmental variations. Barrier types may vary geographically, so they may change over a short period of time [24].

Based on the nature of the cycle and the rate process of the ecological changes, this process then called as soft vicariance and become important in a speciation process [25]. In the case of physical inhibition, where the distribution was geographically limited, the effect of climate on a broad population distribution may has no effect. The isolation occured under these conditions is not due to the reduction of distribution capabilities, but rather to the intrinsic ecological conditions that have been applied as barriers [26]. These barrier's condition is depicted on the Sumatra Island. From the northeast to the southwest, the geology of the Sumatra Island is characterized by the sedimentary basins, and several of mountain ranges which has formed volcanic arc and fractals [27].

In addition, Sumatra Island, especially on the South area, has so many valleys. These valleys occured as the result of volcanic activity in the subduction zone. Sundaland or Sundanese plate, including Sumatra, since the early of Tertiary era, was generally experienced a slope towards the South [28]. Then, the subduction system in Sumatra as we can see on the west coast of Sumatra and southern Java has begun since the epoch of Oligocene. However, the formation of Barisan Mountains which arised from this subduction process, was began at the end of the Miocene epoch, around the epoch of Plio-Pleistocene (1.8-0.01 MYA) [29]. This volcanic activity then continually conceals some of the surface of the South Sumatra's basin [30], thus separates the species between subclade I (clade A) and clade B, even though it is located in an adjacent area.

CONCLUSIONS

The reconstruction of phylogenetic trees shows 3 main clades: clade A (parts of Sumatra, Java, and China/GenBank), clade B (Sumatra), and clade C (outgroup). The dispersal of genus Megophrys in Sumatra is predicted through the ancient river flows from the Malay peninsula in the north to Sumatra and Java during the Pleistocene era. The species dispersals were likely possible because of the silting of the Sunda Strait. The Barisan Mountains, which ranges from north to south and also the basins and valleys became a greater barrier for subclass I (clade A) and clade B. Genus Megophrys were estimated to experience a sympatric speciation and distributed during the Pliocene era when the regression of sea water was maximized.

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The Effect of Explant Types and Kinetin Concentration on In Vitro Callus Induction in *Vetiveria zizanioides* (L.) Nash

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Abstract

The aims of this research were to evaluate the effect of explant types and several kinetin concentrations on in vitro induction and growth of callus vetiver (*Vetiveria zizanioides* (L.) Nash). Crown and tiller of vetiver were cultured on Murashige and Skoog's (MS) media supplemented with combination of 2,4-D 0.75 ppm and several kinetin concentrations (0, 0.3, 0.5, 0.75, and 1) ppm. The induction and growth of callus were influenced by type of explant and concentration of kinetin. Formation and growth of callus on tiller explant were faster than crown explant. Callus on tiller explant were formed one week after culture, while callus from crown explant were formed at four weeks after culture. Callus growth on tiller explant also was better than crown explant. Eight weeks after culture, callus fresh weight from tiller explant was 0.35 ± 0.09 g, while callus fresh weight from crown explant was only 0.16 ± 0.08 g. The addition of kinetin in the medium combined with 2,4-D was able to increase callus growth and the optimum concentration of kinetin used was 0.5 ppm. The addition of kinetin more than 0.5 ppm in the medium decreased the callus fresh weight.

Keywords: callus, crown, in vitro culture, tiller, *Vetiveria zizanioides*.

INTRODUCTION

Vetiver grass (*Vetiveria zizanioides* (L.) Nash) is a perennial grass from the family Graminae (Poaceae). It has been utilized as various fields such as industries, economies, and environments [1]. Utilization of vetiver in the various fields is caused by the essential oil contents. The need for essential oils increases along with the development of the medicines, perfumes, cosmetics, and aromatherapy industries. The needs of essential oils in the world, especially vetiver oil is 300 tons per year. However, Indonesia is only able to supply about 28% with the price of essential oil production of approximately 25-30 tons per year [2]. One of the attempts which can be used to increase the essential oil is cell culture. Cell culture is a technique which can be used to increase the production of bioactive compounds in plants. Through tissue culture, bioactive compounds can be produced continuously, controlled and manipulated environment to obtain optimum results [3].

Increasing bioactive compound through cell culture has been carried out by some methods, one of them is callus culture. Callus culture has increased quinone compound in *Peritassa campestris* about 2.5 folds [4] and isopenoid in *Artemisia annua* L. 2.5 folds higher than compounds produced *in vivo* [5]. Thus, the attempt to obtain a callus culture method in vetiver is necessary.

Plant growth regulator is one of the factors which can influence the success of callus cell culture. One of the growth regulator has been used to promote callus formation is kinetin. The use of kinetin in callus induction has been done in *Andropogon gerardii* [6]. In addition, the success of cell culture or callus culture growth is also affected by explant type used. Callus induction has been performed from shoot base explant in *Vetiveria zizanioides* [7] and tiller explant in *Pennisetum purpureum* Schum [8]. However, the used of tiller explant in vetiver plants for callus induction has never been done. Therefore, in this research, the callus induction will be performed by tiller explant in vetiver on media supplemented with several kinetin concentrations. The objectives of the current study were to determine the effect of explant type and kinetin concentration on formation and growth of callus in vetiver.

MATERIALS AND METHODS

Plant material

Plants of *Vetiveria zizanioides* (L.) Nash. were collected from Sengklek, Pamalayan Village, Bayongbong District, Garut, West Java. These plants were washed by using running tap water, then trimmed about 4 cm from the base of shoot and separated from the root. The trimmed plants were disinfected using 96% alcohol for a minute,

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sterilized using 80% commercial whitening agent (containing 5.25% NaClO) for 25 minutes, and finally rinsed by using sterile aquadest twice each for five minutes. Sterile trimmed of plant was cleft, then the crown (part of plant intercourse between tiller and root system) and tiller were used as explant.

Callus Induction

The crown and tiller explant measured ± 0.2 cm from the base were cultured on MS basal medium containing 2,4-D 0.75 ppm and kinetin (0, 0.3, 0.5, 0.75, and 1) ppm. The cultures were incubated at room temperature 25-26^{0}C with 600 lux light intensity for 8 weeks. Each treatment was repeated three times (three bottles). Each bottle consists of one explant for crown and three explants for tiller. The observation parameters were formation and growth of callus including the time of first callus formation, morphology, and callus fresh weight.

RESULT AND DISCUSSION

Callus were induced from crown and tiller explant cultured on MS basal media supplemented with 2,4-D alone or combination of 2,4-D and kinetin. Callus started to form from the edge of explant and then followed by the entire of surface explant. These calli tend to be yellow to whitish yellow and transparent in color. Induction and growth of callus in vetiver is affected by explant types and concentration of kinetin in the medium. However, there are not interaction between explant type and kinetin concentration on formation and growth of callus.

The type of explant had significant effect on formation and growth of callus in vetiver. Callus formation from tiller explant were faster than callus formation from crown explant. Callus from tiller explant started to form a week after culture, while callus from crown explant were formed four weeks after culture. In addition of the faster callus formation, the callus growth from tiller explant was also better than crown explant (Fig. 1).

At 8 weeks after culture, the callus fresh weight from tiller explant was 0.35 ± 0.09 g, while the fresh weight callus from crown explant was only 0.16 ± 0.08 g (Fig. 2). It was shown by the distinct difference in callus growth from both of two type of explants, biomass of callus from tiller explant was higher twice than biomass of callus from crown explant.

Addition of 0.3-1 ppm kinetin in combination with 0.75 ppm 2,4-D did not significantly influence the formation and growth of callus. However, the addition of kinetin in the medium in combination with 0.75 ppm 2,4-D tended to increase callus growth. On the other hand, when the concentration of kinetin was higher than 0.5 ppm (0.75-1 ppm) callus growth began to decrease. Callus fresh weight on medium containing 2,4-D alone was only 0.17 ± 0.07 g, while on medium containing 0.75 ppm 2,4-D in combination with 0.3-1 ppm kinetin was 0.23 ± 0.13 – 0.28 ± 0.15 g. The addition of 0.5 ppm kinetin in the medium produced the highest callus fresh weight than the other treatments, it was 0.32 ± 0.12 g (Fig. 3).

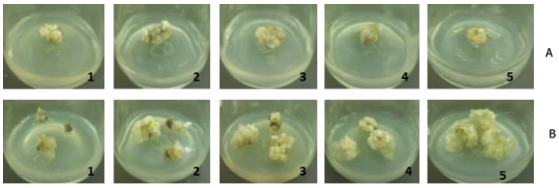

Figure 1. Formation and growth of callus from crown explant (above, A) and tiller explant (bottom, B) on MS basal medium supplemented with combination of 2,4-D 0.75 ppm and several kinetin concentrations.
Description: A1 and B1 without kinetin (control), A2 and B2 kinetin 0.3 ppm, A3 and B3 kinetin 0.5 ppm, A4 and B4 kinetin 0.75 ppm, A5 and B5 kinetin 1 ppm.

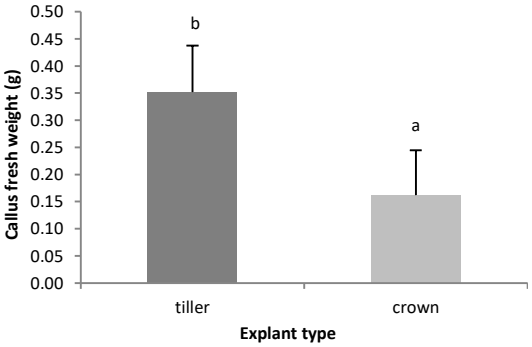

Figure 2. Effect of explant types on vetiver callus fresh weight at 8 weeks after culture.
Note: the same letter of each bar showed no significant on BNT test (α=0.05)

Increased fresh weight in medium supplemented with kinetin was quite large, however there was no significant difference in statistical analysis on biomass of callus on medium only containing 2,4-D alone. This is probably due to the very high variation of callus fresh weight on each treatment.

Some factors such as genotype of plant, explant source, basal medium, and growth regulator in the medium affect the formation and growth of callus [9]. Explant has important role on the success of callus initiation. Explant of

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young tissue generally more responsive than the oldest tissue [10]. Explant from meristematic tissue forms the callus faster than dorman tissue [11].

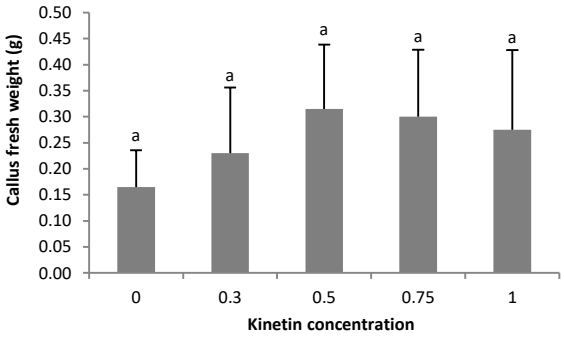

Figure 3. Effect of kinetin concentration in combination with 2,4-D 0.75 ppm on callus fresh weight at 8 weeks after culture.
Note: the same letter of each bar showed no significant on BNT test (α=0.05)

In *Trigonella foenum* Graecum L., the percentage of callus formation from hypocotyl explant was higher than cotyledon explant [12]. In the study [13], the highest percentage of callus formation in *Sphenostylis stenocarpa* Hochst. was formed from leaf and stem compared with root explant. Nodal explant showed better response on callus induction in *Citrus jambhiri* that better than leaf and root explant [14]. While the results of research [15], showed that callus growth in *Ricinus communis* from cotyledon explant was better than root explant.

The difference response of callus formation on each explant were caused by the different physiology condition of each explant. The success of callus formation is increasing with using young tissue as explant source. The tissue that growth actively on earlier of growth period is an excellent explant source, because the ability of the organ division process on the older tissue decreased [16]. According to previous study, the young tissue is generally proliferate easier than old tissue [17].

The success of the callus induction depends on the type and concentration of growth regulator. Callus formation was affected by auxin or combination between auxin and cytokinin. Kinetin is one of growth regulator which often combined with auxin to promote the callus formation [18]. Kinetin promotes the development of cell and affects the development of cell physiology [19]. The addition of kinetin could induce the callus from leaf explant in *Talinum paniculatum* [20] and shoot tip explant in *Shorgum bicolor* [21].

Some research results also showed that the callus formation was affected by explant type used and growth regulator in the medium. In *Vetiveria zizanioides*, the addition of 1 ppm kinetin combination in 2,4-D dan IAA produced callus from shoot base explant 47% [22], whereas 1 ppm kinetin promoted optimum callus growth 85% from axillar bud explant with combination in NAA [23]. The result of other research showed that the addition of 0.5 ppm kinetin in the medium in combination with 2,4-D produced callus 32.78% from leaf disc and 20% hypocotyl explant in tomato [24]. In addition to the type of explant, growth regulator also as important factor that affected the success of callus induction. On leaf explant in *Sauropus androgynous*, the addition of 1 ppm kinetin in combination with NAA in the medium resulted callus formation 20%, while the addition of 0.5 ppm kinetin promotes callus formation only 13% [25]. The addition of kinetin in combination with NAA promotes the callus formation from root explant in *Sphenostylis stenocarpa* Hochst. On root explant in *Sphenostylis stenocarpa* Hochst., the addition of 3 ppm kinetin in the medium produce the percentage of callus higher than 1.5 ppm kinetin, respectively 50% dan 16% [13].

CONCLUSION

Formation and growth of callus in vetiver were affected by explant type and kinetin concentration supplemented in the medium. The formation and growth of callus from tiller explant were faster and better than crown explant. In addition, the formation and growth of callus were also affected by kinetin in the medium. The addition of 0.3-1 ppm kinetin in combination with 0.75 ppm 2,4-D in the medium increased callus growth. However, higher concentration of kinetin, which were 0.75-1 ppm, the callus growth started to decrease.

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Assessing the Genotoxic Potentials of Methomyl-based Pesticide in Tilapia (*Oreochromis niloticus*) Using Micronucleus Assay

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Abstract

Pesticides are recognized as serious pollutants in the aquatic environment with the potential to cause genotoxic on the aquatic organism, especially fish. The micronucleus (MN) assay has been used to evaluate genotoxicity of many compounds in polluted ecosystems such pesticides. im of this study to determine genotoxic effect of methomyl-based pesticide on tilapia (*Oreochromis niloticus*). Fish were exposed to six different concentrations base on range finding test (0 ppm, 3.2 ppm, 4.2 ppm, 6.5 ppm, 8.7 ppm and 10 ppm) of methomyl-based pesticide. The micronucleus were collected from peripheral blood erythrocyte of fish after 96 h exposure. Peripheral blood samples smears were stained with Giemsa, MN frequencies were counted and statistically analyzed using one-way ANOVA. The result of this study showed after 96 hours exposed to methomyl-based pesticide, at concentration 0 ppm causes 0% mortality, at concentration to 3.2 ppm causes 30% mortality, at concentration 4.2 ppm causes 60% mortality, at concentration 6.5 ppm causes 70% mortality, at concentration 8.7 ppm causes 80% mortality, at concentration 10 ppm causes 100% mortality of fish test. Lethal Concentration 50 (LC50 - 96 hours) of methomy-base pesticide towards tilapia (*O. niloticus*) is 4.015 ppm. Through micronuclei assay during 96 hour exposure of methomyl-based pesticide, the result shows that frequencies of micronuclei in erythrocyte of fish test at concentration at 0ppm is 12‰, 18‰ and 16‰; at concentration at 3.2ppm is 33‰, 26‰ and 29‰; at concentration at 4.2ppm is 41‰, 38‰ and 46‰; at concentration at 6.5ppm is 68‰, 81‰ and 82‰; at concentration 8.7ppm is 133‰, 130‰ and 137‰; at concentration 10ppm is 163‰, 166‰ and 156‰. It revealed that methomyl-based pesticide exposure induced after 96 h significantly ($P<0.05$) increased genotoxic potentials simultaneous with increased concentration.

Keywords: Genotoxic, Methomyl, Micronucleus Assay, Pesticide, Tilapia.

INTRODUCTION

Pesticides are widely used throughout the world in agriculture to protect crops and in public health to control diseases[1]. Although a type of pesticide aimed to turn off a group or species specific targets, but in substance is poisonous against all of an organism either organism target and non target as in fishes [2].

Methomyl (C5H10N2O2S), S-methyl-1-N-[(methylcarba-moyl)-oxy]-thioacetimidate, is an carbamate pesticides which widely used in many agricultural countries to protect crops or plant against insects because of its broad biological activity, relatively rapid disappearance and high efficiency [3,4]. The entry of the pesticides remains as methomyl into the agricultural irrigation will pollute the environment [5]. Several study about consequences of the methomyl pesticide exposure to fish has done. Where certain concentration exposure of methomyl pesticide can cause acute poisoning to death [6].

Exposure of organisms to xenobiotics such as pesticides, insecticides, herbicides and other synthetic materials is a serious matter in environmental and toxicological chemistry. Cypermethrin, as one of insecticides, is highly toxic to fish and aquatic invertebrates [7].

Fish is common aquatic animal that provide a good model for monitoring the toxicity of pesticide such methomyl in aquatic systems because they are extremely sensitive to pollutants, have the ability to metabolize xenobiotics and exhibit a very high bioaccumulation rate of dissolved chemicals relative to their concentration. Moreover, Fish also used for the potential of mutagenic and carcinogenic study of pesticide contaminants present in aquatic [8-11].

In present study, Micronucleus test in fish erythrocyte used as a sensitive indicator for evaluation and assessment of the genotoxic potential environment [12]. Micronuclei is a secondary nucleus that smallest than primary nucleus.

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Appearance micronucleus can used as indicator of genotoxic activity in fish body [13,14]. Moreover, occurance of micronucleus in blood erythrocyte can used as of cellular deviation and other genetic demage because of pesticides exposure [15].

In this study, we choose Tilapia as animal test because of their potential for future aquaculture [16]. They are hardy, prolific and fastgrowing tropical fishes, low on the food chain, and adaptable to all kind environtment [17]. Major producers of Tilapia are developing countries, including China, Indonesia, Philippines, Thailand, Honduras, Ecuador and Costa Rica. The main objective of this study was to determine effect of methomyl-based pesticide at dependent doses against micronucleus frequency on erythrocyte of Tilapia (*O. nilotius*).

MATERIALS AND METHODS

Treatment Preparation

Materials

Methomyl-based pesticide was purchased from agriculture market in Batu, East Java, Indonesia as Lannate 25 WP. In this work, Tilapia fish (±9-12cm) were purchased from Technical Application Unit of Freshwater Fish (UPT Perikanan Air Tawar), Sumberpasir, Malang, East Java.

Acclimatization

The fishes were holded in tank and fed with commercial feed once per day. After 14 days holding periode, fishes were classified into 6 group of 10 fishes, then trasfered and acclimatized into aquarium with aeration system (size 60x30x25cm) for 2 days. If less that 3% of fish population are dead during 48 hours, its mean the Tilapia population treatment that will be considered worthy for testing. But if over than 3% of fish population are dead, the fish should replaced with the new fish from holding tank then reacclimatized for 2 days.

Critical Range Test

This test was conducted to determine the upper range (N) and the bottom range (n) of methomyl-based pesticide on fish test. This section was conduted 96 hours by observed the fish test mortalitas level. The used concentration of methomyl-based based on Guthrie and Perry method [18].

Definitive Test

Devinitive test carried out to determine methomyl-based pesticide concentration that cause 50% mortality of fish population (LC50). Based on critical range test, the concentration of methomyle-base pesticide was 0 ppm, 3.2 ppm, 4.2 ppm, 6.5 ppm, 8.7 ppm dan 10 ppm. The concentration was modified from progressive concentration table of Bowman dan Rand [19] with 96 hours exposure.

Data Collection

Micronuclei Assay

After 96 hours exposure of methomyl-based pesticide, erythrocute blood from each fish group was sampled and smeared on clean microscope slides. After fixation in absolute methanol for about 20 min, the slides were air-dried and stained with 10% of giemsa for about 25 minutes. Six slides of 1.000 erythrocyte that sampled from each Tilapia *(O. niloticus)* were scored [20], observed and coding by using microscope (Olympus CX21) with 400X magnification to determine the frequency of micronucleus cell and other different pattern of morphologically altered erythrocyte and then counted as cell per 1000 (‰) [21]. The micronucleus frequency then counted base on Betancur formulation [22].

$$\text{Mikronuclei Frequency} = \frac{\textbf{Mikronuclei x (1000)}}{\textbf{Total Cell Counted}}$$

Data analysis

Data analysis in this study using probit analysis to determine the relative toxicity (LC50) of chemicals on living organisms. LC50 was base on fish level of Tilapia *(O. niloticus)* mortality at definitive test after 96 hours methomyl-based exposure. Moreover, the data of micronucleus was analysis using One Way Anova to determine effect of methomyl-based pesticide against current parameter response.

RESULT AND DISCUSSION

Critical Range test

Preliminary test carried out to obtain an upper range and lower range concentration of methomyl-based pesticide against Tilapia. Figure 1 show that after 96 hours exposure causes 100% mortality at concentration ≥10 ppm and 20% mortality at concentration 1 ppm. However, no mortality happened at concentration 0.1 ppm. Base on the data, concentration 0.1 ppm can be used as lower range of methomyl-based concentration and concentration 10 ppm used as upper range.

The preliminary test carried is essential to determine the limits of the range of critical concentration of methomyl-based pesticide (critical range test) against Tilapia *(O. niloticus)*, which became the basis of the determination on the concentrations used in definitive test or toxicity tests. That concentration can cause most of the

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deaths was close to 50% and lowest mortality approach 50% [23].

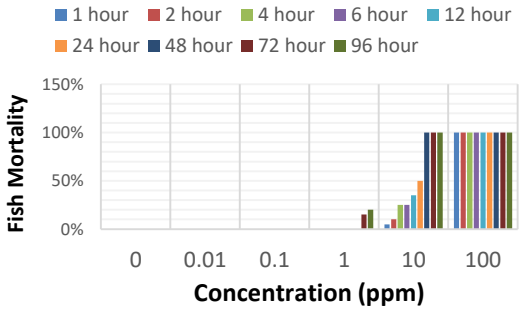

Figure 1. Fish Mortality during critical range test

Definitive test

Base on lower and upper range of methomyl-based pesticide on Tilapia, the variative concentration for definitive test determined base on logarithmic scale of Bowman dan Rand [19] (0 ppm, 3.2 ppm, 4.2 ppm, 6.5 ppm, 8.7 ppm and 10 ppm).

The result of definitive test (Figure 2) showed methomyl-based pesticide exposure was no fish mortality (0% population of fish test) after 96 hours exposure at concentration 0 ppm. During 96 hours methomyl-based pesticide exposure, in concentration 3.2 ppm exposure showed that mortality start happening after 6 hours exposed as much as 10% population of fish and total of fish mortality during 96 hours exposed as much as 30% of population. In concentration of methomyl-based pesticide at 4.2 ppm, fish mortality start happening after 4 hours exposed as much as 10% population of fish and total of fish mortality during 96 hours exposed as much as 60% population of fish. In concentration of methomyl-based pesticide at 6.5 ppm showed fish mortality start happening after 4 hours exposed as much as 10% population of fish test and total of fish mortality during 96 hours exposed as much as 70% population of fish. Concentration of methomyl-based pesticide at 8.7 ppm showed fish mortality start happening after 2 hours exposed as much as 10% population of fish test and total of fish mortality during 96 hours exposed as much as 80% population of fish. Concentration of methomyl-based pesticide at 10 ppm, fish-mortality start happening after 2 hours exposed as much as 10% population and total of fish mortality during 96 hours exposed as much as 100%.

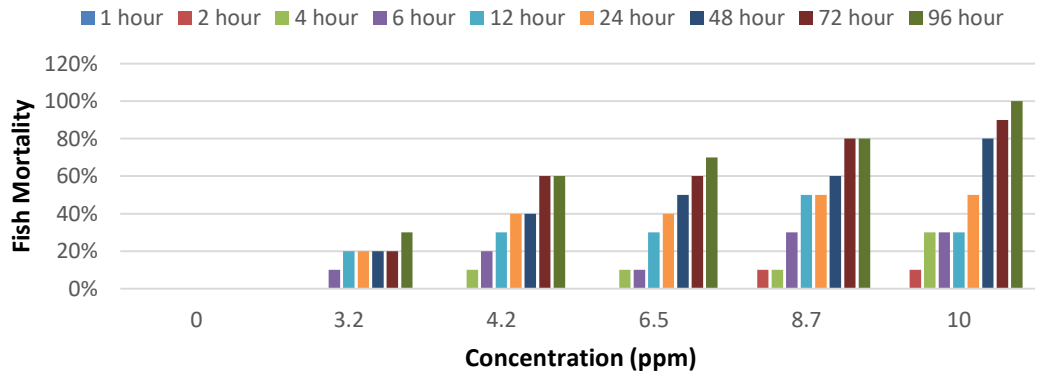

Figure 2. Percentage Mortality of Tilapia (*O. niloticus*) During the Definitive Test

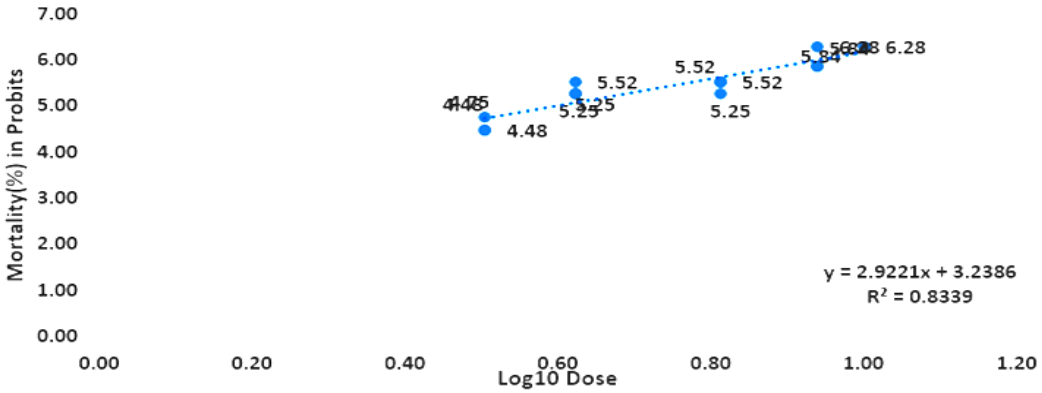

Figure 3. Probit Analysis of LC50-96 hour of methomyl-based pesticide

During 96 hours exposed, lower mortality of Tilapia *(O. niloticus)* occurred at concentration 3.2

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ppm (30% population) and the higher occurred at concentration 10 ppm (10% population). Moreover, in each doses (3.2, 4.2, 6.5, 8.7, and 10 ppm), lower mortality occurred in beginning of methomyl-based exposure (1 hour exposed) and higher mortality occurred in the end of exposure (96 hours exposed). The data showed that toxicity of methomyl-based exposure against Tilapia increased simultaneously with increased of doses and time exposure. A chemical toxicity increased against organism simultaneously with increasing of dose and time of exposure [24].

Base on the result of probit analysis that show in Figure 3, can be find out of LC50 96 hours methomyl-based against Tilapia (*Oreochromis niloticus*). Line equation Y = $\mathbf{2.9221x + 3.2386}$ and the value of LC50 96 hours is 4.015 ppm that causes 50% death of the population of fish test.

Micronuclei assay

In the present study, the micronuclei test in fish usually based on erythrocytes and it observed that there was a basal level of measurable spontaneous micronuclei formation in *O. mossambicus* which was also observed in most of the fish species [11]. Micronuclei assay provide information as a simple bioindicator for chromosomal aberrations not available from other methods: (i) the consolidated effect of a variety of environmental stresses on the health of an organism, population, community, and ecosystem (ii) warning of harmful effects to human health based on the responses of wildlife to pollution, and (iii) the effectiveness of remediation efforts in decontaminating waterways [25]. Counting of micronuclei is faster and less demanding of technical than scoring of chromosomal aberrations, the micronuclei assay has been widely used for chemicals screening that cause these types of damage and also it demonstrate that micronuclei test in fish can be used for the genotoxicity assessment in environment [26]. In the present study micronuclei frequencies in the fish peripheral blood erythrocytes after 96 hours exposure in different concentration of metomyl pesticide (Fig. 4) show significant increase in micronuclei frequencies ($p<0.05$).

The result of micronuclei assay on fish against exposure of methomyl-base show in Figure 4. At concentration 0 ppm of methomyl-based pesticide, frequencies of micronuclei was 12‰, 18‰ and 16‰. At concentration 3.2 ppm, after 96 hours exposure showed that frequencies of micronuclei was increase up to 33‰, 26‰ and 29‰. At concentration 4.2 ppm, after 96 hours exposure showed that frequencies of micronuclei was increase up to 41‰, 38‰ and 46‰. At concentration 6.5 ppm, after 96 hours exposure showed that frequencies of micronuclei was increase up to 68‰, 81‰ and 82‰. At concentration 8.7 ppm, after 96 hours exposure showed that frequencies of micronuclei was increase up to 133‰, 130‰ and 137‰. At concentration 10 ppm, after 96 hours exposure showed that frequency of micronuclei was increase up to 163‰, 166‰ and 156‰. The micronuclei frequencies in tilapia *(O. niloticus)* erythrocyte were significantly increased ($p<0.05$) simultaneously increased concentration and ime of exposure of methomyl-based pesticide.

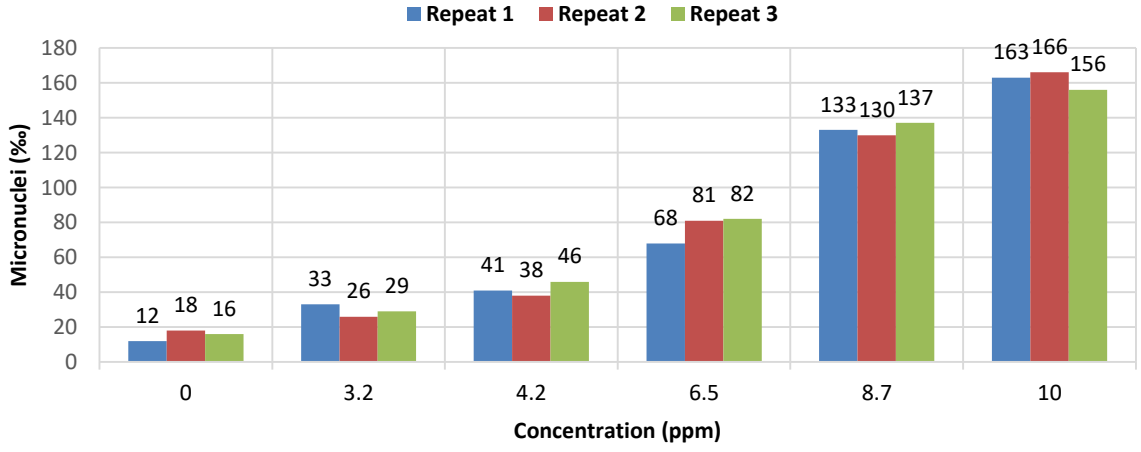

Figure 4. Micronucleus Frequency in Tilapia (*O.niloticus*) Erythrocyte

The lowest micronuclei frequencies were recorded after 96 hours exposure at concentration 0 ppm. The highest micronuclei frequencies were recorded after 96 hours at concentration 10

ppm. The micronucleus assay test in tilapia erythrocyte has been used for accessing genotoxic potential [27]. It also has been used for the detection of breaked strand in aquatic species [28]. Figure 4 showed that genotoxic potential of methomyl-based pesticide against erythrocyte of Tilapia *(O. niloticus)* increased simultaneously with increased concentration. The concentrations and the exposure period of pesticide may be the reason for relatively high micronuclei frequencies recorded in pesticide treated fish. The present study also reports that dose and time dependent of some pesticide exposure (Chlorpyrifos, malathion, cypermethrin, lambda-cyhalothrin and Buctril) can increase micronuclei induction in the peripheral blood erythrocytes of fish (*O. mossambicus*) [27].

CONCLUSION

Through definitive test, fish test after 96 hours exposed by methomyl-based pesticide, at concentration 0 ppm causes 0% mortality, Increasing concentration to 3.2 ppm causes 30% mortality, Increasing concentration to 4.2 ppm causes 60% mortality, Increasing concentration to 6.5 ppm causes 70% mortality, Increasing concentration to 8.7 ppm causes 80% mortality, Increasing concentration to 10 ppm causes 100% mortality of fish test. Lethal Concentration 50 (LC50 - 96 hours) of methomy-base pesticide towards Tilapia *(O. niloticus)* is 4.015 ppm.

Through micronuclei assay during 96 hours exposure of methomyl-based pesticide, the result show that frequencies of micronuclei in erythrocyte of fish test at concentration at 0ppm is 12‰, 18‰ and 16‰; at concentration at 3.2ppm is 33‰, 26‰ and 29‰; at concentration at 4.2ppm is 41‰, 38‰ and 46‰; at concentration at 6.5ppm is 68‰, 81‰ and 82‰; at concentration 8.7ppm is 133‰, 130‰ and 137‰; at concentration 10ppm is 163‰, 166‰ and 156‰. The frequencies simultanously increase with increased of concentration and time periode of exposure. The increased frequencies of micronuclei in Tilapia erythrocyte meant that genotoxic potential simultaneously increased.

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Phylogenetics and Biogeography of Cobra (Squamata: *Naja*) in Java, Sumatra, and Other Asian Region

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Abstract

The separation of Sumatra and Java occurred at the end of the Miocene (10 mya) to the end of Pliocene (1.8 mya). The existence of ecological variations and geographic barriers inhibits gene flow through the isolation of adaptation, geography, reproduction, inbreeding, and leading to population segregation. Cobra (genus *Naja*) distribution became greatly influenced by the geologic condition and sea level. This study was conducted by phylogenetic analysis towards the 16S rRNA gene. Survey was done with Visual Encounter Survey (VES) method on 6 locality. There were 3 location in Sumatra Island and the others in Java Island. Sample from other Asian region was obtained from Genbank, which are 11 individuals from China, Thailand, and Nepal. DNA extraction was done according to the QIAmp® DNA Mini Kit standard protocol. The forward and reverse 16S sequences are combined with the Sequencher™ version 4.1.4 program, then in BLAST (Blast Local Alignment Search Tool) at www.ncbi.nlm.nih.gov. Phylogenetic analyzes of clade A (MP = 60, ML = 54, BI = 88) indicate the presence of division into two monophyletic subclade (AI and AII). Subclade AI consists of groups of Cobra from Sunda (Thailand, Sumatra and Java). Subclade AII is a group of species *N. kaouthia* originating from Chumpon Province, Southern Thailand with (MP = 96, ML = 95, BI = 100). Clade B is divided into two subclasses (BI and BII). The result is supported by bootstrap value MP = 93, ML = 99, BI = 100. *N. atra* of Fujian Province is a sister lineage of the same species from Jiangxi Province (MP = 86, ML = 86, BI = 100).

Keywords: Asian, 16s rRNA, Java, Cobra, Sumatra.

INTRODUCTION

The evolution of South Asia's diversity was occurred in Paleocene epoch (60 mya-million years ago), when the Malay Peninsula, Java, Sumatra, and Borneo still merged as a big continent, called as sundaland. Then, the formation of the volcanic rings during Eocene (40 mya) and sea level fluctuations at the beginning of Miocene (23.03 mya) initiated the separation between the Malay Peninsula and Borneo. On the late Miocene, sea level fluctuations occured again and half of Java Island were flooded by water. The separation of Sumatra and Java as well as Borneo rotational changes occurred at the end of the Miocene (10 mya) to the end of Pliocene (1.8 mya) [1]. The movement of the earth's plate during the land evolution was separate several regions of Asia. In addition, Sumatra Island is estimated to occured in last 25 million years ago (late Miocene) and divided into west and east regions by Bukit Barisan mountains. The existence of ecological variations and geographic barriers inhibits gene flow through the isolation of adaptation, geography, reproduction, inbreeding, and leading to population segregation [2]. Cobra (genus *Naja*) distribution became greatly influenced by the geologic condition and sea level [3]. This study was conducted by phylogenetic analysis towards the 16S rRNA gene. The purpose of this study is to estimate the relationship of *Naja* genus in the island of Java, Sumatra, and other Asian region based on the 16S rRNA mithocondrial gene. Zoogeography analysis is also examined for more advance understanding between the correlation and evolution of Sundaland.

MATERIALS AND METHODS

Sampling and Preservation

Survey was done with Visual Encounter Survey (VES) method on 6 locality. There were 3 location in Sumatra Island and the others in Java Island (Table 1). Sample from other Asian region was obtained from Genbank, which are 11 individuals from China, Thailand, and Nepal (Table 2). VES method identification was done based on morphological characteristics, then preserved by alcohol 70%. The preserved sample tissue was further extracted and cured in EDTA 4%.

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Table 1. Cobra (Genus*: Naja*) Samples Used on This Research

Species	Locality	Island	Accession Number
Naja sputatrix	Malang, East Java	Java	MG012885
Naja sputatrix	Bondowoso, East Java	Java	MG012888
Naja sputatrix	Cilacap, Central Java	Java	MG012889
Naja sumatrana	Mandailing Natal, North Sumatra	Sumatra	MG012884
Naja sumatrana	Medan, North Sumatra	Sumatra	MG012886
Naja sumatrana	Bengkulu, Sumatra	Sumatra	MG012887

Table 2. Sequences Data of the Genus Megophrys and Outgroup Species from GenBank

Species	Accesion Number	Locality
Naja sumatrana	JN687929.1	Thailand
Naja kaouthia	GQ359757.1	Thailand
Naja kaouthia	EU624269.1	Thailand
Naja atra	EU729432.1	Fujian, China
Naja atra	HM439991.1	Jiangxi, China
Naja atra	EF413645.2	Guangdong, China
Naja atra	EF413642.2	Guangdong, China
Naja atra	EU729431.1	Yunnan, China
Naja naja	GQ359756.1	Nepal, India
Naja naja	EU624270.1	Nepal, India
Ophiophagus hannah	JN687931.1	Thailand

DNA Extraction

DNA extraction was done according to the QIAmp® DNA Mini Kit standard protocol. A total of 25 mg of tissue was cut in tiny pieces and placed in 1.5 ml microtube. The tissue was given 180 µL of ATL buffer and 20 µL of K-proteinase. Samples were incubated in waterbath-shaker, and was done in 56°C for 1-3 hours, then added 200 µL of AL buffer. Sample incubation at a temperature of 56°C was done for 10 minutes, then added 200 µL of 90-100% ethanol. Each procedure is terminated with vortex. Samples were pipetted and inserted into 2 mL collection tube and placed in Dneasy Mini Spin. Centrifugation was done at 8000 rpm (one minute), supernatant is then discarded. Spin Column was placed in new collection tube and was given 500 µL of AW1 buffer. Sample then centrifuged on 8000 rpm (one minute), supernatant is then discarded. Spin column then placed in new collection tube (2 mL) and was added 500 µL of AW2 buffer. Centrifugation was done at 14.000 rpm (3 minute), supernatant is then discarded. Spin column then placed in 1.5 or 2 mL microtube. DNA was thawed with 200 µL AE buffer in the middle of spin column. Incubation was done at one minute (15-25°C), then centrifugated at 8000 rpm (1 minute).

Qualitative Analysis

Qualitative analysis was done by agarose gel (1%) electrophoresis [4]. Gel was made using 0.3 g agarose and 30 mL Tris-Boric-EDTA (TBE) with pH 8. Gel was made inside Erlenmeyer flask until boiled a little. Warm agarose was given 1 µL EtBr and then homogenized. Homogenate was poured slowly into gel cast, and allowed to harden. The hardened gel was inserted into electrophoresis chamber and soaked in TBE (pH 8). DNA sample is mixed wit loading dye, then inserted into wells as well as the DNA marker ladder 1 kb (1:1). Electrophoresis then conducted at half voltage for 45-60 minute. The results were documented with Gel Doc.

DNA Amplification

The components were placed in microtube: 3.6 µL of dH_2O; 5 µL of PCR mix (Go Taq Green); 0.2 µl of 16 SAR primer (F) 5'CGCCTGTTTATCAAAAACAT-3' and 16 SBR (R) 5'-CCGGTCTGAACTCAGATCACGT3' [5]; 0.5 µL of DNA sample. PCR was set at: Hot start at 95°C (3 minute), denaturation at 95°C (30 second), annealing at 52°C (60 second), extension at 72°C (60 second), and post-extension at 72°C (7 minute). PCR cycle was repeated 34 times.

Phylogenetic Analysis

The forward and reverse 16S sequences are combined with the Sequencher™ version 4.1.4 program, then in BLAST (Blast Local Alignment Search Tool) at www.ncbi.nlm.nih.gov. As outgroup, *Ophiophagus hannah* (Family Elapidae) is used with DNA sequence data that is accessed from the site www.ncbi.nlm.nih.gov. Both sample sequences and outgroup species were aligned with ClustalW analysis on the MEGA6.0 program.

The reconstruction of phylogenetic tree was done by Maximum Parsimony (MP) analysis, with bootstrap 1000 in PAUP 4.0b10a program. The Jmodeltest 3.06 program is used for the best model of likelihood analysis. Analysis of MP, Maximum Likelihood, and Bayesian Inference were performed on Mr.Bayes program ver 3.1.2 MCMC 10 million generation. Haplotype Network Analysis is done with DNAsp program and Bandelt Network.

RESULT AND DISCUSSION

Qualitative Analysis

The results of electrophoresis of all *N. sputatrix* DNA samples and *N. sumatrana* showed the presence of DNA with an ampicton size of

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500-600 bp (Fig. 1). An amplycone length range of 550-590 bp [6] is the result obtained from the amplification of 16S rRNA gene with 16 SBR primary and 16 SAR as reverse and forward respectively.Visualization of DNA bands describes the purity of DNA resulting from isolation process, hence the visualized DNA can be used as a further molecular analysis material [6].

Genetic Distance of *Naja*

The determination of new species on *N. haje* is indicated by the percentage of p-Distance value of the 16S rRNA gene ≥5% [7]. The genetic characteristics of a species are influenced by differences in geographical conditions, while geographical barriers can alter the genetic distance of a population [8]. The p-Distance results showed a low value (1-2%) between the species of *N. sputatrix* (Java) and *N. sumatrana* (Sumatra) . The obtained value is supported with a similarity rate of 96-97% in the BLAST analysis. The p-Distance value between *N. sumatrana* from Sumatra and Thailand is low (p-Distance 1%), which is supported 97-98% similarity level on BLAST result. The p-Distance value between *N. atra* (China), *N. sputatrix* (Java), and *N. sumatrana* (Sumatra) is high (p-Distance 4-16%). The p-Distance value between *N. kaothia* and *N. sputatrix* (Java) and *N. sumatrana* (Sumatra) is low (p-Distance 2-3%), while p-Distance value between *N. kaouthia* with *N.atra* and *N. naja* classified as high (p-Distance 9-12%). The p-Distance analysis concluded that *N. sputatrix*, *N. sumatrana*, and *N. kaouthia* were spread in the Sundaland and had close phylogenetic relationships. Based on the p-Distance value of ≥5% for the determination of new species, the three *Naja* species may be possible still within the same species (Table 3).

Phylogenetic Analysis

The phylogenetic analysis of *Naja* genus based on 16S rRNA (MP=98, ML=80, BI=100) shows the formation of two clades of *Naja* (A and B), and one *outgroup,* which is *Ophiophagus hannah* (C) (Fig. 2). The results of this study led to the idea to review the results of research by Philip [9] which states that *Ophiophagus hannah* is a sister lineage of the genus *Naja*.

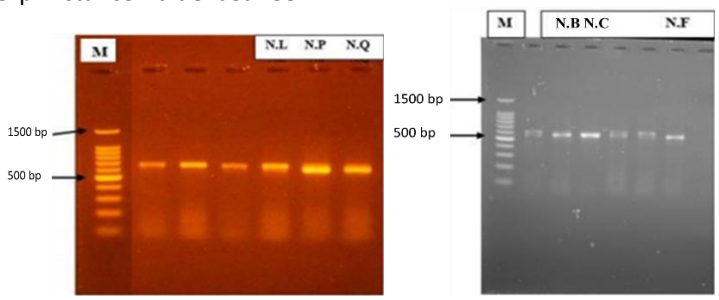

Figure 1. Electrophoresis result of Naja DNA sample by 16SAR and 16SBR primers. **Description:** M=marker; N.L.=*N. sumatrana* (Bengkulu); N.P.=*N. sputatrix* (Bondowoso); N.O.=*N. sputatrix* (Cilacap); N.B.=*N. sputatrix* (Malang); N.C.=*N. sumatrana* (Mandailing Natal); N.F.=*N. sumatrana* (Medan).

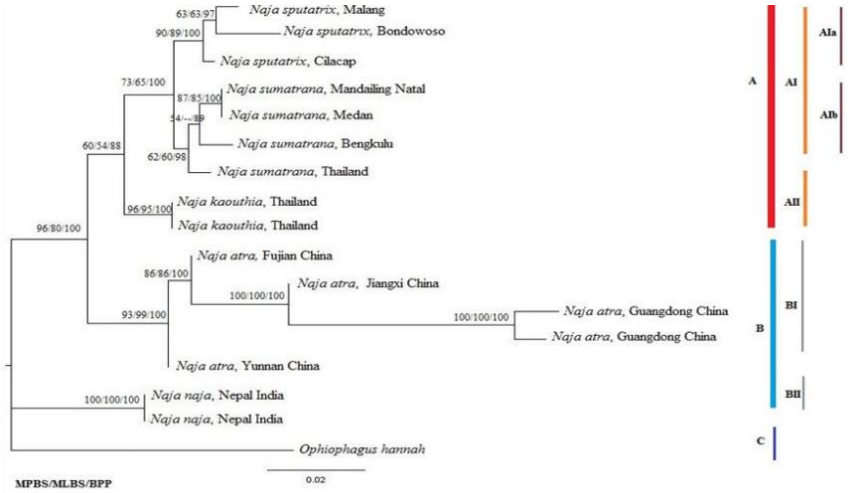

Figure 2. The Reconstructed Phylogenetic Tree of the *Naja* Genus Based on the 16S rRNA Gene from Java, Sumatra and Some Areas of Asia. **Description:** MPBS = Maximum Parsimony, MLBS = Maximum Likelihood, BPP = Bayesian Inference, sign-indicates no bootstrap values on phylogenetic tree branches.

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Table 3. Value of Uncorrected p-Distance of Genus *Naja* (Laurenti, 1768) Based on mtDNA 16S rRNA

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
N. sputatrix [1]																
N. sputatrix [2]	0.01															
N. sputatrix [3]	0.00	0.01														
N. sumatrana [4]	0.02	0.02	0.02													
N. sumatrana [5]	0.02	0.02	0.02	0.00												
N.sumatrana [6]	0.02	0.03	0.02	0.01	0.01											
N. sumatrana [7]	0.02	0.02	0.02	0.01	0.01	0.02										
N. atra Fujian [8]	0.03	0.04	0.04	0.04	0.04	0.04	0.03									
N. atra Jiangxi [8]	0.06	0.06	0.06	0.06	0.06	0.06	0.05	0.02								
N. atra Guangdong [8]	0.14	0.14	0.14	0.14	0.14	0.14	0.13	0.10	0.07							
N. atra Yunnan [8]	0.03	0.04	0.03	0.03	0.03	0.04	0.03	0.00	0.02	0.10						
N. atra Guangdong [8]	0.12	0.13	0.13	0.12	0.12	0.13	0.12	0.08	0.06	0.02	0.09					
N. kaouthia [7]	0.02	0.03	0.03	0.03	0.03	0.03	0.03	0.02	0.04	0.12	0.02	0.10				
N. kaouthia [7]	0.02	0.03	0.03	0.03	0.03	0.03	0.03	0.02	0.04	0.12	0.02	0.10	0.00			
N. naja [9]	0.06	0.07	0.06	0.06	0.06	0.06	0.05	0.06	0.08	0.15	0.05	0.14	0.06	0.06		
N. naja [9]	0.06	0.07	0.06	0.06	0.06	0.06	0.05	0.06	0.08	0.15	0.05	0.14	0.06	0.06	0.00	
Ophiophagus hannah	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.08	0.10	0.18	0.08	0.17	0.08	0.08	0.09	0.09

Notes: 1=Malang, 2= Cilacap, 3= Bondowoso, 4= Mandailing Natal, 5= Medan, 6= Bengkulu, 7= Thailand, 8= China, 9= Nepal.

Clade A

Phylogenetic analyzes of clade A (MP = 60, ML = 54, BI = 88) indicate the presence of division into two monophyletic subclade (AI and AII). Subclade AI consists of groups of Naja from Sunda (Thailand, Sumatra and Java). Phylogenetic analysis of subclade AI (MP = 73, ML = 65, BI = 100) divided the subclade into two monophyletic groups (AIa and AIb). The grouping of AIa group (MP = 90, ML = 89, BI = 100) indicates the division of *N. sputatrix* originating from East Java (Malang, Bondowoso) and Central Java (Cilacap). The construction of the phylogenetic tree shows that *N. sputatrix* from Cilacap is a sister lineage of *N. sputatrix* from Malang and Bondowoso. *Naja sputatrix* from Malang and Bondowoso were identified as sister species or close relatives (MP = 63, ML = 63, BI = 97).

According to BLAST results, the AIa group (*N. sputatrix* from Java) is similar to *Naja sumatrana* and *Naja kouthia* with 96-97% similarity level. Wuster and Thrope [10] show that *N. kouthia* and *N. sumatrana* in Asia are one group with *N. sputatrix* and represent a lineage with appropriately similar morphology or distribution and ecological characteristics. The results showed that *N. sputatrix* from Malang and Bondowoso (Fig. 3) had closer relationship than *N. sputatrix* from Cilacap, in accordance with similar morphological characters (brown scales on *N. sputatrix* from Malang and Bondowoso, while black on *N. sputatrix* from Cilacap).

Das [3] revealed that N. sputatrix has 1189 mm (SVL) and155 mm (TL). Meristic characters are two nasal scales, no loreal scales, one supraocular scales, one preocular scoop, three postocular scales, supralabial scales, nine infralabial scales, 19 dorsal central scales, smooth surfaces, 180 ventral scales, and anal scales are divided. Generally, there is no pattern behind the hood, but if there is, it usually resembles the shape "V". The presence of small scale accessories [11] on the head scales is not directly related to the process of adaptation, but in contrast, color is possible to be one of the adaptation factor. Populations in Central Java to West Java have dominant blackish color and occupy a thick humid rainforest, while the population from East Java has a silver to brownish scales, occupying habitat with dry soil conditions.

The AIb group (*N. sumatrana*) originating from Sumatra has a similar likelihood of 97-98% with the same species from Thailand, while it has a similarity of 95-96% with *N. kaouthia* (AII) of Thailand, in accordance with BLAST results. The result of BLAST is in accordance with the result of phylogenetic tree reconstruction using bootstrap value: MP = 62, ML = 60, BI = 98, which indicate that *N. sumatrana* species from Indonesia is the sister lineage of *N. sumatrana* from Thailand. The groupings performed on AIb (MP = 54, ML = -, BI = 89) indicate that *N. sumatrana* from Bengkulu is identified as sister lineage with the same species from Medan and Mandailing Natal. Meanwhile, *N. sumatrana* from Medan and Mandailing Natal is a sister species (MP = 87, ML = 85, BI = 100).

The phylogenetic reconstruction of the AIb group is in accordance with the research of Wuster and Thrope [10], i.e. the comparison of Sumatran *N. sumatrana* with similar species from South Thailand and the Philippines based on morphological characters. Some of the morphological characters observed in the juvenile phase of *N. sumatrana* are bright and striking colors on the back, dark base that surrounds the body, and a dot mark on the back of the head. The adult of *N. sumatrana* has a whole black body color, and only brightly colored in it's head scales (Fig. 4). Climatic conditions

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affect the distribution process of this species in Southern Thailand resulting in competition between *N. sumatrana* and *N. kaouthia*. *N. sumatrana* has a body length of 1.5 m with a strong body, large head differs from the neck, round muzzle, and round hood (adult) or oval (juvenile). This species has a single preocular scales, postocular scales of two to three, seven supralabial, four infralabial, 179-206 ventral scales, 40-57 subcaudal scales. The back color is related to geographical origin and size [3].

Subclade AII is a group of species *N. kaouthia* originating from Chumpon Province, Southern Thailand with (MP = 96, ML = 95, BI = 100). The results of the phylogenetic reconstruction trees in accordance with the results of research Wuster [12] which showed that three evolutionary lines separated from the lineage of the genus *Naja*. The main sub-lineage representing the *Naja* genus of Asia represented by *N. naja* where the reconstructed phylogenetic tree by Bayesian analysis shows that *N. kaouthia* is a sister lineage of *N. atra* supported by a valid matching value (bootstrap 95%).

Clade B

Clade B is divided into two subclasses (BI and BII). Sequences with valid matching values from the BI subclass *N. atra* are from China (Fujian, Guangdong, Jiangxi, Yunnan province) are taken from GenBank. The result is supported by bootstrap value MP = 93, ML = 99, BI = 100. *N. atra* of Fujian Province is a sister lineage of the same species from Jiangxi Province (MP = 86, ML = 86, BI = 100). *N. atra* of Guangdong Province is a sister lineage of the same species of Jiangxi Province with a strong matching value (MP = 100, ML = 100, BI = 100).

Subclade BII is *N. naja* from Nepal (India), and has a valid topology (MP = 100, ML = 100, BI = 100). *N. naja* according to the results of the reconstruction of phylogenetic trees is the species with the closest kinship with outgroup species *Ophiophagus hannah*. The results of the reconstruction are in accordance with research from Wuster [12] and Wallach [13], where *N. naja* are separated from clade of *N. kaouthia*, *N. sputatrix*, and *N. Sumatrana.*

Figure 3. *N. sputatrix* Based On the Color Difference of Scales Among Localities. **Description**: A1. Bondowoso with whole body, A2. Ventral scales, A3. Head Scale; B1. Malang, with whole body, B2. Ventral scale, B3. Head scales.

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Figure 4. *N. sumatrana* Muller, 1890. (a) Bengkulu; (b) Medan

Naja Biogeography

The divergence time of Elapidae occurs at the beginning of the Neogenic Period (approximately 16 mya) to the end of the Cretaceous subperiod [14]. The distribution of this family is related to the evolution of Sundaland. There are two main factors affecting the distribution of cobra in the Malay Peninsula and Indonesia, i.e. ecological conditions and sea surface changes [3].

The separation of Elapidae occurred in about 16 mya [16]. *N. sputatrix* is recorded only in Indonesia (Java, Bali, Lombok, Sumbawa, Flores, and possibly the surrounding islands). The abundance of *N. sputatrix* in Java is varies depend on locality [17]. Ecological conditions is found to be correlated with patterns of diversity among *N. sputatrix*, and occur in various climatic zones in Java and the Sunda Islands. The phylogenetic trees reconstruction and the value of p-Distance show that *N. sputatrix* in Malang (East Java) with Bondowoso (East Java) is closely related.

Separation of Java begins at late Miocene, which accompanied by sea water fluctuations that cause half of Java to be submerged by wate (Fig. 5). Separation of Java and Sumatra occurs during the end of Miocene (10 mya) to the end of Pliocene (1.8 mya) [1]. The process of land separation causes a geographical isolation on *N. sputatrix*. Geographical isolation ultimately limits the spread of this species due to its inability to adapt to the Sumatra, Kalimantan, and other Asian environments. This insights explain why N. sputatrix is only found in Java. Furthermore, at the beginning of the Pleistocene (250,000 years ago) there was a decrease of sea water surface, allowing the distribution of *N. sumatrana* in southern Thailand. This species is able to adapt to climatic and ecological conditions in Thailand and is able to spread from Sumatra and Kalimantan [18].

N. kaouthia that used in this study was came from Southern Thailand. *N. kaouthia* experienced separation with the divergence time of 1-0.8 mya [16]. The species is distributed almost in various regions: Myanmar, Thai Peninsula, South Laos, Cambodia, South Vietnam, northern Peninsular Malaysia, India, Nepal, Bangladesh, South China, Sichuan Province, and Yunnan [3]. *N. kaouthia* throughout the mentioned region is the result of the evolution of the Sundaland. The Distribution was began in the early Eocene to the late Oligocene (25 million years ago), when Burma, Malay Peninsula, Java and Sumatra were still in the mainland [2].

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N. naja is separated from Elapidae family with divergence time of 1-0.8 mya [16]. The relation of this species with another *Naja* in Sundalandis quite distant. This evidence can be explained by the separation proccess of Indian and African land that occurred at the end of the Mesozoic era (120 mya) to the beginning of the Cenozoic era (65 mya). The expansion of the ocean floor that occurred during the Tertiary period led to the movement of the Indian mainland towards the Asian Continent (Fig. 5). The movement of mainland India resulted in the occurrence of collisions and continues on the formation of the Himalayas and the Tibet plateau [19]. The *N. naja* species subsequently experienced an allopathic specia-tion due to the separation of population by the ecological barrier (Himalayan Mountains).

N. atra has a divergence time of about 3 mya. Nanling and Luoxiao mountains do not become a barrier in the distribution of *N. atra* in mainland China, where in this study is represented by the provinces used as sampling data (Fujian, Yunan, Jiangxi and Guangdong). The existence of *N. atra* in Taiwan, an island separated from mainland China was seen as any divergence split progress. However, *N. atra* in Taiwan differs from the species of mainland China. *N. atra* in Taiwan has a distinct genetic character and shows the separation of the phylogenetic tree lineage between populations in mainland China and Taiwan. Formation of Taiwan Island occurred during the Miocene, thenit was separated from mainland from the southeast coast of mainland China by Taiwan Strait. Climate change during the Pleistocene caused most of the Taiwan Strait to become a landmass, thus providing an opportunity for *N. atra* to migrate from mainland China [20].

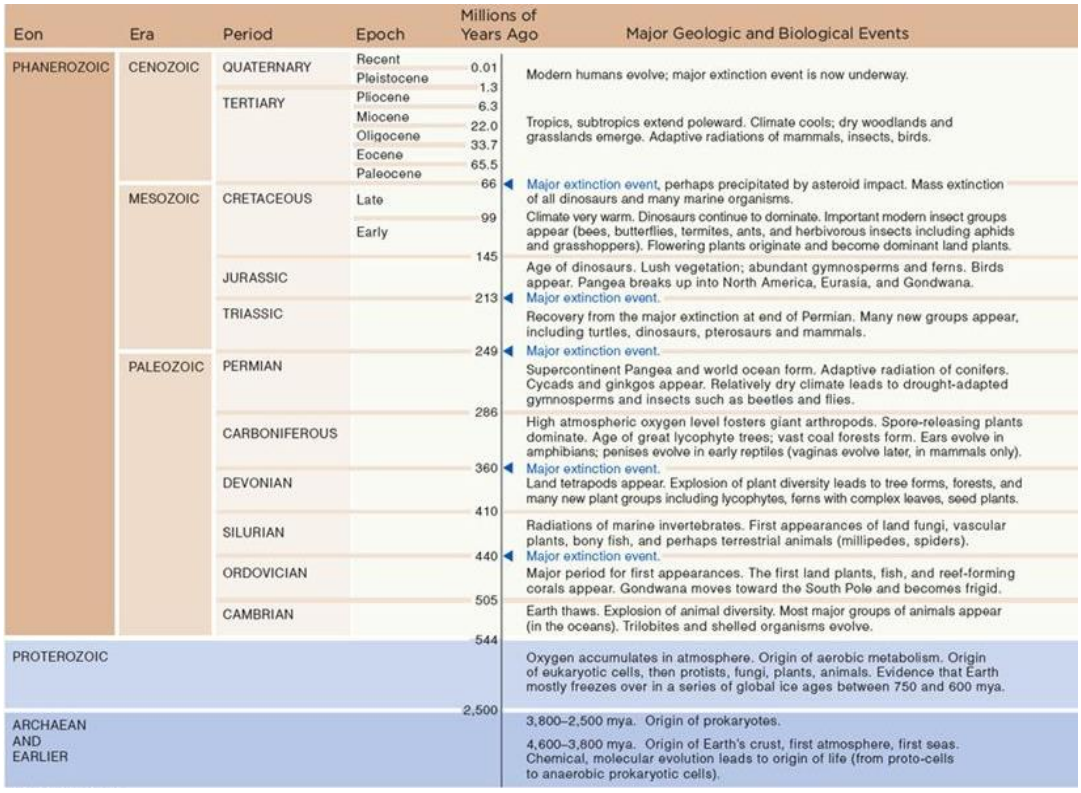

Figure 5. Geological Time Scale and Some Important Events [15]

CONCLUSION

N. sputatrix from Java is similar to *Naja sumatrana* and *Naja kouthia* with 96-97% similarity level. The results showed that *N. sputatrix* from Malang and Bondowoso had closer relationship than *N. sputatrix* from Cilacap, in accordance with similar morphological characters. *N. sumatrana* from Sumatra has a similar likelihood of 97-98% with the same species from Thailand. *N. atra* of Fujian Province is a sister lineage of the same species from Jiangxi Province (MP = 86, ML = 86, BI = 100). *N. atra* of Guangdong Province is a sister lineage of the same species of Jiangxi Province with a strong matching value. *N. naja* is the species with the closest relationship with outgroup species *Ophiophagus hannah*.

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In Vitro Morphogenesis Responses of Various Explant in *Physalis angulata* L.

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Abstract

The objective of this research was to identify morphogenesis responses of in vivo and in vitro-derived explant of *Physalis angulata* L. Explants were cultured on MS medium containing 3% sucrose solidified with 1.3% agar supplemented with combination of 6-benzylaminopurine (BAP), indole acetic acids (IAA), naphthalene acetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D). All explant types have morphogenesis ability. Capability for shoot regeneration was observed in high number of shoot tip (96.1%) and nodes explants (75.2%). Callus formation was dominantly produced by in vitro leaf explants. In vivo leaf had low responses for shoot/root regeneration and callus formation. These results provided an alternative choice of the explant suitable for either plant regeneration or callus formation.

Keywords: callus, cotyledonary nodes, explant types, shoot regeneration.

INTRODUCTION

Herbal medicine has been used for centuries to treat various problems of health conditions. A lot of medicinal plants have been used to treat illness and promote health. *Physalis angulata* L. is a member of Solanaceae family which has been widely used for traditional medicine [1]. A wide variety of species have different and specific herbal characters. As herbal medicine this plant is used to cure various disorders like asthma, kidney, bladder, jaundice, gout, inflammations, cancer, digestive problems and diabetes [2]. *Physalis angulata* has also been reported has antimicrobial [3] and antiparasitic activity [4].

Increasing interest to *P. angulata* is not accompanied by their availability in high number. Generally *P. angulata* is grow wild in the waste land, near road, in the forest and cultivated field. In addition this plant has not been widely cultivated. High exploitation of this plant will lead to extinction. This plant is generatively propagated through seeds which take a time to yield a mature plant. Therefore the alternative technique to produce a lot of regenerated plants in a relative short time is necessary.

Plant tissue culture is micropropagation technique which can be initiated by various tissues as explant. The most commonly used explants are shoot tips, organ, and axillary bud or meristem. Shoot regeneration of *P. minima* were initiated from leaf, shoot tips and root explants [5]. While the efficient in vitro regeneration of *P. peruviana* was achieved from node, internode and leaf explants [6]. High number in vitro shoots of *P. angulata* has been produced by axillary meristem [7] and leaf disc explants [8]. This study was to identify the potential of in vivo and in vitro explant types of *P. angulata* in shoot/root regeneration through in vitro system. This knowledge is expected to support the efforts to conserve biodiversity of medicinal plants especially *P. angulata*.

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MATERIALS AND METHODS

Plant materials and explant source

Explants were derived from in vivo plants and in vitro tissue culture. In vivo explant was young leaf derived from wild grown *P. angulata* plant in the maize field. In vitro nodes and leaves were obtained from three weeks old shoot culture while shoot tips were derived from two weeks old in vitro seedling.

Surface sterilization of in vivo explants

Young leaf was excised from plant source and washed thoroughly in running tap water for 30 min. Further, it was surface sterilized with 70% (v/v) ethyl alcohol (30 sec) followed by 10 % (v/v) Clorox (10 min) in Laminar Air Flow under aseptic conditions. Finally, leaf explants were rinsed thoroughly (3 times) with sterilized aquadest for 5 min each to remove traces of Clorox.

Medium and culture conditions

All explant types were cultured on MS salt basal medium [9] with a source of carbon sugars 30 $g.L^{-1}$ and solidified with agar 13 $g.L^{-1}$. Combina-

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tions of cytokinin (BAP, Kinetin) and auxin (IAA, 2,4-D and NAA) were used to observe morphogenesis responses and the potency of explant to produce shoot. The media was adjusted to pH 5.8 by adding NaOH/HCl 0.1N and sterilized by autoclave at 121°C, 1 atm for 15 min. All cultures were maintained in continuous light intensity of 600 lux at a temperature of 22 ± 2°C.

Culture initiation stage

All explant types were cultured in Benzyl adenine (BA) (1, 3, 4 mg.L^{-1}) + IAA 0.1 mg.L^{-1}; BAP 3.5 mg.L^{-1} + Kinetin 0.4 mg.L^{-1} and BAP 2 mg.L^{-1} + 2,4-D 0.1 mg.L^{-1} to observe the response of cultured explants. The sterilized in vivo leaf was cut into ± 0.5 cm^2 pieces. Each piece was laid upon the medium with its abaxial side face the medium surface. While in vitro leaf was cultured as a whole with the same orientation. Nodes and shoot tip were cultured in media in an upright position. Each experiment was repeated thrice with three replicates. High shoot produced explants were selected and cultured into shoot multiplication medium containing combination of BAP+NAA, BAP+2,4-D, BAP+IAA and BAP+Kin.

Shoot multiplication

The explant type showed high percentage of morphogenesis or organogenesis response in culture initiation stage was selected to culture in shoot multiplication medium containing BAP combined with other cytokinin (kinetin) and auxin, namely BAP 0.1 mg.L^{-1} + NAA 0.5 mg.L^{-1}; BAP 2 mg.L^{-1} + 2,4-D 0.1 mg.L^{-1} ; BAP (3.5 and 4 mg.L^{-1}) + Kin 0.4 mg.L^{-1} and BAP (0.5 ; 1.0 ; 3.0 ; 4.0 mg.L^{-1} + IAA 0.1 mg.L^{-1}. Shoot number was recorded after 4 weeks of culture.

RESULT AND DISCUSSION

Initiation stages

Shoot and root regeneration were observed in all explant types except for shoot tips. Almost all (96%) shoot tips were capable of shoot regeneration but there was no shoot tips that produced root (Fig. 1). Nodes not only showed high frequency of shoot (75.2%) and root (57.1%) regeneration but also callus formation (38.9%). High number of in vitro leaf explants produced callus in almost medium tested. Callus formation was observed in 51.8% in vitro leaf explants which more dominant than shoot (35.7%) and root (7.1%) regeneration. Callus formation in basal leaf blade apparantly is due to active cellular growth of wounding respons (Fig. 3C). In vivo leaf explants showed low frequency of shoot (5.6%) and root (10.1%) organogenesis as well as of callus formation (11.1%). In the combination of cytokinin:auxin the in vitro shoot tips and nodes had high response in shoot induction and regeneration than in vitro and in vivo leaf explants.

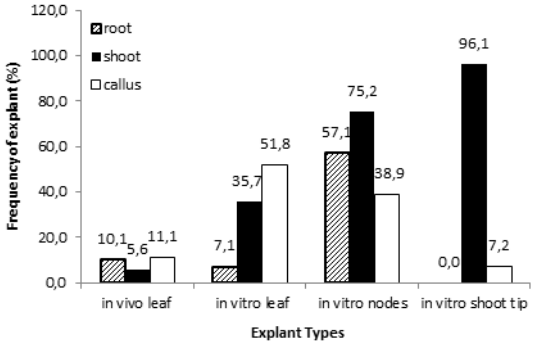

Figure 1. Frequency of morphogenesis response (shoot – root organogenesis and callus formation) derived from different types of explant

Organogenesis response of all explant types was influenced by plant growth regulator (PGR) supplemented in culture medium. High percentages of shoot tips and nodes explant regenerated shoots in all combination of PGR (Fig. 2). Almost 100% of shoot tips were capable of producing shoot (Fig. 3A) in all types of culture media. Adventif shoots were also produced by > 90% nodes on media containing BAP (3 and 4 mg.L^{-1}) + IAA (0.1 mg.L^{-1}) (Fig. 3B) and BAP (2 mg.L^{-1}) + 2,4-D (1 mg.L^{-1}). However, in the media supplemented by BAP (1 mg/L) + IAA (0.1 mg.L^{-1}) and BAP (3.5 mg.L^{-1}) + Kin (0.4 mg.L^{-1}) shoots was only produced by <50% of nodes explant. Shoots were only produced by 5.6% of in vivo leaf (Fig. 3C) and 35.7% of in vitro leaf explants when they were cultured in media containing BAP (4 mg.L^{-1}) + IAA (0.1 mg.L^{-1}). These results indicated that combination of BAP+IAA, BAP+Kin and BAP + 2,4-D were suitable for promoting shoot regeneration in nodes and shoot tips explants.

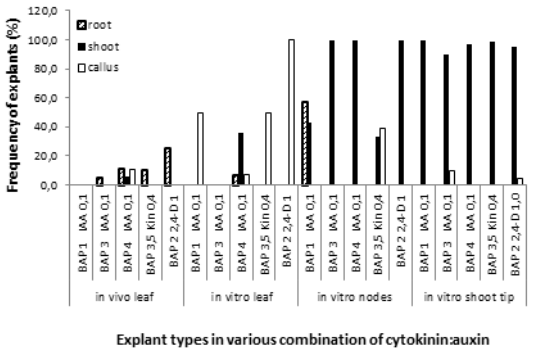

Figure 2. Frequency of morphogenesis response of four explant types in various combination of cytokinin:auxin

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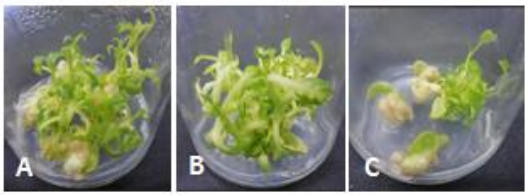

Figure 3. Shoot multiplication of *P. angulata* derived from different explant types in shoot induction medium after 4 weeks of culture. A. shoot tips explant, B. nodes explant, C. in vitro leaf explant.

Type of explant is an important factor for the success of tissue culture methods [10,11]. Parts of plants commonly used as explants are stem, leaf, petiole, cotyledon and hypocotyls. Factors that affect the response of explants were: genotype, physiological condition of donor plants, source or origin of explants, and explant position on the donor plant. The different morphogenesis responses of each explant types on the same medium can be due to the different levels of endogenous growth hormone in the different types of explant [12]. Morphogenesis process is the formation of new cellular structures which is highly related to not only chemical modulators such as plant growth regulators, but also the competence levels of cell, physiological stage and performance of gene control [13]. It is related to competence of cell in answering signs of extrinsic and intrinsic factors which promote cell determination.

This results shows that shoot tip of in vitro seedling of *P. angulata* was superior to other types of explant for shoot induction. Low morphogenesis response (< 30%) of in vivo leaf explants is accordance with the statement of Yildiz [10] that explants derived from field have the low growth response.

Shoot multiplication derived from shoot tips explants

Since almost all shoot tips explants showed good responses to shoot regeneration (Fig. 2) therefore the effect of other PGR combinations was observed on its competence to shoot multiplication. Eight weeks after culture the number of shoot/explants ranges from 1.3 to 6.8 shoots/explant in all culture medium tested (Fig. 4). Combination of BAP + NAA produced shoots with the lowest number (1.3 shoots/explant) rather than any other PGR combinations. Explants cultured in medium BAP + NAA only produced single shoot and followed by root induction (Fig. 5A). The number of shoots produced in this medium was not significantly different from that produced by medium BAP 0.5 mg.L^{-1} + IAA 0.1 mg.L^{-1} (1.7 shoots/explant). However medium BAP + NAA promoted single shoot with wide leaf (Fig. 5D). Meanwhile, the other concentration of BAP+IAA could induce direct shoot regeneration with the relative similar number (4.9-6.8 shoots/explant) (Fig. 5B-C, E-F). BA alone or combined with auxin also produced high shoot number in *P. minima* [14-15] and *P. peruviana* [16]. High shoot number (152.8 $\pm$ 0.40) was produced by leaf disc explants of *P. angulata* in MS+BAP+IAA+GA$_3$ after the third subculture [8]. This results showed that shoot tips derived from in vitro seedling is potentially used as explants to support the development of micropropagation of *P. angulata* because the ability to regenerate shoot in various PGR combinations. Routine subculture should be done to obtained higher shoot number.

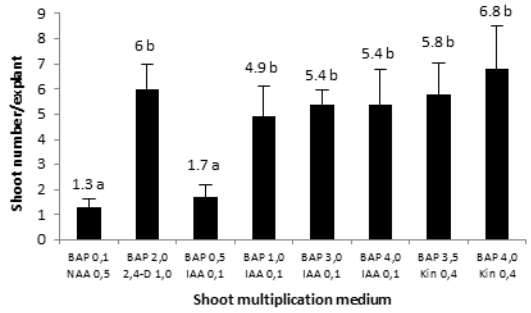

Figure 4. Shoot number derived from in in vitro shoot tips explants in various combinations of cytokinin: auxin eight weeks after culture. The number followed by the same letter are not significantly different at the 5% level (mean$\pm$SD, n = 3).

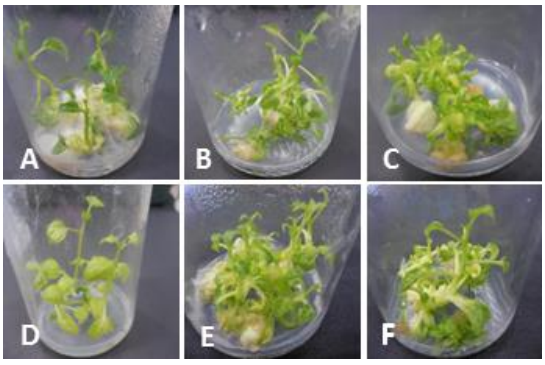

Figure 5. Shoot multiplication derived from in vitro shoot tip explants in various combination of hormone cytokinin:auxin eight weeks after culture.
A. BAP 0.1 mg.L^{-1} + NAA 0.5 mg.L^{-1}
B. BAP 2 mg.L^{-1} + 2,4-D 1 mg.L^{-1}
C. BAP 4 mg.L^{-1} + Kin 0.4 mg.L^{-1}
D. BAP 0.5 mg.L^{-1} + IAA 0.1 mg/L mg.L^{-1}
E. BAP 1 mg.L^{-1} + IAA 0.1 mg.L^{-1}
F. BAP 4 mg.L^{-1} + IAA 0.1 mg.L^{-1}

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CONCLUSION

Shoot tip of *P. angulata* derived from in vitro seedling was the most responsive explant to shoot regeneration and mutiplication in various combination medium BA+auxin/cytokinin. On the contrary, in vivo leaf explant showed low respons in morphogenesis.

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Effects of Fish Oil and Tomato Powder Supplementation in Mojosari Ducks Diet on Egg Quality

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Abstract

This research aimed to examine the effects of fish oil and tomato powder supplementation on egg quality of Mojosari ducks. A total of one hundred and forty Mojosari ducks (consisted of 20 male and 120 female ducks), aged at 40 weeks old, were randomly allotted into 20 flocks (each flock consisted of 1 male and 6 female ducks). Method used was experiment in a Completely Randomized Design with 5 treatments and 4 replications. Dietary treatments used were T_0: basal diet (control), T_1: basal diet + 1% FO, T_2: basal diet + 2% FO, T_3: basal diet + 1% FO + 1% TP; T_4: basal diet + 2% FO + 1% TP. Data were analyzed using one-way analysis of variance (ANOVA). Result showed that supplementation of fish oil and tomato powder did not significantly affect (P>0.05) external egg quality (egg shape index, shell surface area, shell thickness and specific gravity). Dietary treatments also had no significant effect (P>0.05) on internal egg quality (albumen volume, yolk volume, albumen index, yolk index and Haugh unit). It is concluded that there was no detrimental effect of fish oil and tomato powder supplementation in Mojosari ducks diet on external and internal egg quality.

Keywords: antioxidant, Haugh unit, laying duck, omega-3 fatty acids, poultry.

INTRODUCTION

In the last decade, there is a growing interest in the development of functional food. This food is aimed not only to satisfy hunger but also to provide health benefit for consumers. In the livestock industries, one kind of functional food is omega-3 egg. This egg is developed by adding dietary omega-3 sources into laying-type poultry diet. The omega-3 content in the diet will be absorbed in the body and then will be deposited into the egg yolk.

Fish oil is the most common dietary source of omega-3. Fish oil contains omega-3 in the amount of 26.03% [1]. In another study, fish oil had higher enrichment of long chain omega-3 in the yolk compared to other source of dietary omega-3 such as flaxseed and marine algae [2]. However, fish oil is highly susceptible to oxidation, which may cause detrimental effect on egg quality. Consequently, antioxidant addition is required in laying-type poultry diet containing fish oil.

Tomato is one of potential natural antioxidant. Major phytochemical compound in tomato is carotenoid, particularly lycopene [3]. Beside that, tomato also contain other antioxidant compounds such as ascorbic acid and phenolic compounds [4]. Previously, it was reported that lycopene supplementation could increase total antioxidant capacity in liver of breeding hens [5]. Phenolic compounds were also noted could delay oxidation in fish oil [6]. In addition, ascorbic acid supplementation could decrease serum malondialdehyde (MDA) concentration in broiler [7]. These mentioned study showed that tomato powder had a potency to prevent fish oil oxidation, which then could minimize detrimental effect of fish oil addition in the diet. Therefore, this research aimed to examine the effects of fish oil and tomato powder supplementation on external and internal egg quality of Mojosari ducks.

MATERIALS AND METHODS

Object Study

A total of 140 Mojosari ducks which was consisted of 20 male and 120 female ducks, aged at 40 weeks old were used in this study. The ducks were randomly distributed into 20 flocks (each flock consisted of 1 male and 6 female ducks). The ducks were fed twice daily in the amount of 160 g.bird^{-1}day^{-1}, while drinking water was supplied *ad libitum*. Basal diet used in this study consisted of yellow corn, soybean meal, rice bran, meat bone meal, grit, soybean oil, tapioca flour, mineral premix, vitamin premix, DL-methionine and salt. Proportion of feedstuff

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and calculate nutrient content of basal diet shown in Table 1.

Table 1. Proportion of feedstuff and calculated nutrient content of basal diet

Feedstuff	Proportion (%)
Yellow corn	48.00
Soybean meal	20.00
Rice bran	13.20
Meat bone meal	8.00
Grit	5.00
Soybean oil[1]	2.00
Tapioca flour[2]	1.00
Mineral premix	2.00
Vitamin premix	0.50
DL-methionine	0.20
Salt	0.10
Calculated nutrient content	
Metabolizable energy, Kcal.kg^{-1}	2,863
Crude protein, %	19.34
Crude fat, %	4.88
Crude fiber, %	4.09
Calcium, %	3.25
Phosphor, %	0.50
Lysine, %	1.06
Methionine, %	0.54
Methionine + Cysteine, %	0.85

Notes: [1]Proportion of soybean oil was replaced by fish oil according to treatment

[2]Proportion of tapioca flour was replaced by tomato powder according to treatment

Data Collection and Analysis

Method used in this study was experiment in a Completely Randomized Design (CRD) with five treatments and 4 replication. Treatments used in this study were T_0: basal diet (BD as control), T_1: BD + 1% fish oil, T_2: BD + 2% fish oil, T_3: BD + 1% fish oil + 1% tomato powder and T_4: BD + 2% fish oil + 1% tomato powder. Dietary treatment were lasted for 4 weeks.

At the end of experimental period, 60 eggs (3 eggs from each replication) were randomly collected for external and internal egg quality analysis. External egg quality analysis includings: egg shape index, shell surface area, shell thickness and Haugh unit. Internal egg quality analysis includings: albumen volume, yolk volume, albumen index, yolk index and Haugh unit. Data of external and internal egg quality was tabulated and then analyzed using one-way ANOVA.

RESULT AND DISCUSSION

External Egg Quality

Effects of fish oil and tomato powder supplementation in Mojosari ducks diet on external egg quality shown in Table 2. Result showed that supplementation of fish oil in combination with tomato powder in T_3 and T_4 tend to give higher egg shape index and shell surface area as compared to control (T_0) and fish oil supplementation (T_1 and T_2). However, statistical analysis showed that there were no significant effect (P>0.05) of dietary treatments on egg shape index and shell surface area of Mojosari ducks. Previously, it was also reported that fish oil and tomato powder supplementation in laying chicken diet did not significantly affect egg shape index and shell surface area [8].

Table 2 showed that dietary fish oil and tomato powder supplementation had no significant effect (P>0.05) on shell thickness and specific gravity of Mojosari ducks egg. Shell thickness of Mojosari ducks egg were in the range of 0.26-0.27 mm, while specific gravity were remain similar in all treatments (1.09). In line with this finding, dietary fish oil supplementation in laying chicken also had no significant effect on egg shell thickness [9]. The use of fish oil also had similar effect on egg specific gravity compared to sunflower oil, cotton oil, corn oil, flaxseed oil, soybean oil, olive oil, tallow oil and rendering oil [10].

Table 2. Effects of Fish Oil and Tomato Powder Supplementation in Mojosari Ducks Diet on External Egg Quality

Variables	T_0	T_1	T_2	T_3	T_4
Egg shape index (%)	82.10±1.23	82.51±0.85	82.22±1.56	83.10±0.72	84.20±2.54
Shell surface area (cm^2)	68.06±0.89	69.24±1.62	69.41±1.14	70.64±0.76	71.08±5.00
Shell thickness (mm)	0.26±0.02	0.27±0.01	0.27±0.01	0.27±0.03	0.27±0.03
Specific gravity	1.09±0.00	1.09±0.01	1.09±0.00	1.09±0.01	1.09±0.00

Internal Egg Quality

Effects of fish oil and tomato powder supplementation in Mojosari ducks diet on internal egg quality shown in Table 3. Result showed that there were no effect of dietary treatments on albumen and yolk volume of Mojosari ducks. Albumen volume of Mojosari ducks were in the range of 30.75-31.34 mL, while yolk volume were in the range of 15.08 – 16.58 mL. In previous finding, dietary fish oil supplementation in laying chicken diet also had no effect on albumen and yolk proportion percentage [11].

Result showed that dietary fish oil and tomato powder supplementation had no effect on Haugh unit of Mojosari ducks egg. Haugh unit were in

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the range of 80.60-83.89. Similarly, it was reported that laying chicken fed diet supplemented with fish oil, either with or without antioxidant sources (herbal mixture or synthetic antioxidant) had no effect on Haugh unit [12]. In another study, addition of tomato powder in laying chicken also did not affect Haugh unit [13].

Table 3. Effects of Fish Oil and Tomato Powder Supplementation in Mojosari Ducks Diet on Internal Egg Quality

Variables	T_0	T_1	T_2	T_3	T_4
Albumen volume (mL)	31.00±1.05	30.75±0.83	30.83±1.45	31.34±1.22	30.92±0.99
Yolk volume (mL)	16.00±1.25	15.08±0.17	15.33±0.00	16.58±2.24	16.17±0.84
Albumen index	0.08±0.01	0.08±0.01	0.10±0.04	0.08±0.02	0.10±0.01
Yolk index	0.42±0.04	0.44±0.02	0.46±0.02	0.42±0.03	0.46±0.03
Haugh unit	80.60±7.01	81.45±2.23	82.84±5.63	81.43±3.71	83.89±5.37

In this study, there were no effect of dietary treatments on all parameter of egg quality of Mojosari duck. This result may be due to the low level of fish oil supplementation. In another study, addition of fish oil at 5.5% could reduce egg yolk and albumen of broiler breeder [14]. It could be stated that supplementation of fish oil until 2% had no detrimental effect on egg quality. Moreover, in this study, the duration of treatment was only 4 weeks. This short duration of experimental exposure maybe not enough to give significant response on egg quality. It was previously reported that lycopene supplementation in quails diet could increase Haugh unit after 90 days of experimental treatment [15].

CONCLUSION

The conclusion of this research is that there were no detrimental effect of fish oil and tomato powder supplementation in Mojosari ducks diet on external and internal egg quality.

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Comparison of DNA Extraction Methods Between Conventional, Kit, Alkali and Buffer-Only for PCR Amplification on Raw and Boiled Bovine and Porcine Meat

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Abstract

Detection of porcine contamination in food material by employing PCR techniques is integral in halal food confirmation. However, PCR is both costly and laborious, particularly in DNA isolation method. This study explores several different methods in DNA extraction for PCR amplification in bovine and porcine raw and boiled meat samples. Four methods for DNA extraction (conventional PCI method, DNA isolation kit, alkaline-based method, and a DNA lysis buffer-only from the same kit) was employed followed by PCR using primers from previous studies and compared for DNA quality and quantity (in six replicates) and PCR amplification on the best three DNA samples. This study shows that in all samples, the conventional method had the best DNA yield based on nanodrop measurement, followed by an alkali-based method, buffer-only method, and DNA isolation kit. Each method except lysis-buffer only had at least one sample with good DNA quality. Conventional and isolation kit showed reliable positive PCR detection for all porcine and bovine samples (92% positive). Using the alkaline-lysis method, DNA was amplified reliably on boiled meat samples (83% positive). Lysis-buffer-only method did not show consistent PCR amplification on the samples used (50% positive). The conclusion was that conventional PCI method and DNA isolation kit showed high reliability in PCR amplification of bovine and porcine meats, both raw and boiled. While high DNA yield was obtained using the alkaline-lysis method, PCR amplification was only successful on boiled samples. Lysis-buffer only method yielded in poor DNA quality and was not able to result in reliable DNA amplification.

Keywords: DNA extraction methods, halal, Porcine food contaminants.

INTRODUCTION

The consumption of meat products as a food staple was regulated in several major religions (e.g. Islam, Christianity and Judaism) mainly in the form of prohibitive consumption of porcine meat [1]. In many countries, a commitment to this principle is by the certification of meat products being sold to consumers by religious or government bodies, in which food free from porcine products is deemed *halal* in Islamic regulation, or *kosher* in Judaism. Unfortunately, contamination of porcine meat in halal-certified has been shown to occur in several countries, including Indonesia [2,3].

Detection of porcine meat product can be accomplished using several different methods. Commonly used, due to its high sensitivity and specificity, are DNA amplification methods, such as PCR [4,5,6]. However, this method, while reliable, has some drawbacks. Firstly, PCR method requires special equipment and relatively costly reagents, although cheaper than other methods [7]. Secondly, conventional DNA isolation methods (such as phenol-chloroform isoamyl-alcohol or PCI) are also costly, labor-intensive, time-consumptive, and requires toxic chemicals [8]. Thus, while having a high reliability, these methods are prohibitive to be used on a day to day basis.

Several DNA isolation methods which are less time-consuming or costly has been attempted and published. While conventionally a kit is used to isolate DNA, one researcher reported the use of its accompanying lysis buffer with successful DNA amplification, cutting the DNA isolation time to 20 minutes after overnight incubation for preparation [9]. Other researchers have attempted to isolate DNA using an alkaline-lysis method, which uses a simple heat and alkaline treatment method to lyse the cells and isolate the DNA [8,10]. While these two methods have shown success in DNA isolation and PCR amplification for detection in raw meat, its reliability compared side by side to the established kit and conventional PCI has not been explored.

Furthermore, detection of porcine contamination in meat products is a more important issue. In this case, heat treated meat, for instance, could potentially damage DNA and complicate the detection for porcine contamination [7]; although DNA was shown to be resilient to heat damage [11]. However, the effectiveness of current DNA isolation protocols,

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as well as novel and rapid isolation methods, on heat-treated meat had not been compared. Therefore, in this study, we attempt to compare DNA isolation methods in the quantity and quality of extracted DNA and its success in PCR amplification for the detection of porcine material in raw and boiled meat.

MATERIALS AND METHODS

Samples Preparation

Bovine and Porcine meat samples was purchased from a local market in Malang, Indonesia. Meat samples were weighed to approximately 50 g before further handling and stored at -20°C until use. These samples were separated into two groups: a raw meat and a boiled meat group. Bovine and Porcine meats were mixed with tapioca flour with a 10:1 ratio (meat g. flour g^{-1}), and left for 15 minutes in boiling water, to stimulate the creation of meatballs. Both raw and boiled meat was weighed at 80 g and 150 g and distributed in 1.5 mL microtube for DNA extraction.

DNA Extraction Protocols

Samples were subjected to four DNA extraction and purification methods: a conventional PCI method [12], isolation kit method, alkaline-lysis method [9], and a lysis-buffer only method [10], in 6 replicates. Each method was validated using quantitative method (Nanodrop Spectrophotometer ND-1000). DNA extraction and PCR amplification were conducted in Central Laboratory for Life Science (LSIH), University of Brawijaya, Malang, Indonesia.

Conventional PCI Method

Modified from Erwanto *et al* [3], DNA was extracted by addition of 150 mg of sample with 500 µL TNE buffer, 50 µL SDS 10% and 10 µL Proteinase K and incubated at 42°C for 18 hours. Following this, 40 µL phenol (pH 8.0), 5 µL NaCl 5M, and 400 µL PCI (pH 8.0) was added and incubated at 37°C for 1 hour. The solution was centrifuged (Sigma-Santorius 318K) at 3000 rpm for 5 minutes in room temperature, and the supernatant was transferred and added with 50 µL of NaCl 5 M and 800 µL of ethanol and incubated in -20°C for 1.5 hours. DNA was retrieved by centrifugation at 8000 rpm for five minutes in 4°C. The resulting pellet was added with 600 µL absolute ethanol, 300 µL ammonium acetate 5 M, and 500 µL TB buffer and incubated at -20°C for ten minutes, before a final centrifugation at 13.000 rpm for five minutes in 4°C. Dried pellets were eluted using 50 µL of TE buffer.

Isolation Kit Method

The isolation kit used in this method was the Jena Bioscience DNA Isolation Kit (Jena Bioscience, Jena, Germany). Samples were first aliquoted at 80 mg in microtubes, and DNA extraction method was following manufacturer's instructions.

Alkaline Lysis Method

Modified from Buntjer *et al* [10], each sample was added with 2 mL NaOH (0.5 M per gram), vortexed and incubated at 100°C for 10 minutes. The resulting solution was centrifuged at 13.000 rpm for 2 minutes at 37°C twice, with supernatant transferred into a new microtube each time. The supernatant was then added with ammonium acetate 5 M at 0.5 times volume, added with absolute ethanol at 2-3 times volume, and incubated at -20°C for 15 minutes. The solution was centrifuged for at 13.000 rpm for five minutes to obtain pellets which were then washed using 500 µL of 70% ethanol and recentrifuged at the same settings. Elution was done in 100 µL TE buffer.

Lysis-Buffer Only (Modified ASL Method)

Modified from Alaraidh [9], each sample (80 mg) was extracted using the lysis buffer provided in the Jena Bioscience DNA isolation kit. Each sample was added with 300 µL lysis buffer and 2 µL RNase A, and vortexed prior to 8 µL proteinase K, and incubated at 60°C for 5 minutes. The mix was then added with 300 µL binding buffer, and placed on ice for 5 minutes before centrifugation at 10000 g for 5 minutes. The supernatant was transferred into a spin column for purification, following instruction from the manufacturers. DNA was eluted using a 50 µL elution buffer and stored at -20°C.

DNA Amplification Method

The oligonucleotide primers [13], were synthesized by Integrated DNA Technology, Singapore. According to this study [13], oligonucleotides used were forward primer SIM 5'- GAC CTC CCA GCT CCA TCA AAC ATC TCA TCT TGA TGA AA -3', used for both porcine and bovine samples, and a bovine reverse primer 5'- CTA GAA AAG TGT AAG ACC CGT AAT ATA AG -3' and porcine reverse primer 5'- GCT GAT AGT AGA TTT GTG ATG ACC GTA-3'. The amplicon length for porcine samples and bovine samples would be 398 bp and 274 bp, respectively.

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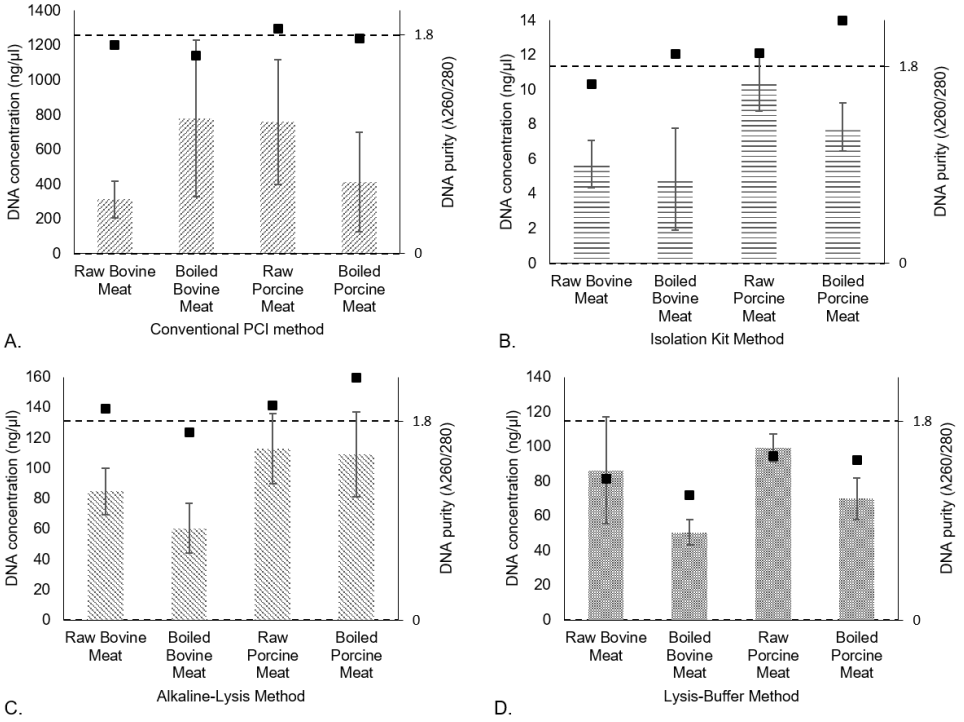

Figure 1. DNA yield and quality (denoted by symbol ■) from A. conventional PCI method, B. isolation kit, C. alkaline-lysis, and D. lysis buffer method. The conventional method showed very high yield and good DNA quality in all samples.

The annealing temperature of these primers was optimised for both porcine and bovine samples using DNA samples obtained from PCI method. For porcine samples, five temperatures were tested, whilst seven temperatures were tested for bovine samples. The optimum annealing temperature was determined to be 64°C for bovine samples and 58°C for porcine samples (data not shown).

DNA amplification was carried out using five µL Go-Taq PCR mix, 0.5 µL for each forward and reverse primers, three µL of nuclease-free water, and one µL of the DNA samples itself. PCR was carried out with a 5 minute 95°C pre-heating prior to 35 cycles of amplification for bovine samples (30 seconds at 94°C, 30 seconds at 64°C, and 45 seconds at 72°C) and porcine samples (30 seconds at 94°C, 30 seconds at 58°C, and 45 seconds at 72°C).

Detection of Amplified Products

PCR products were analysed using electrophoresis using a 1.5% Agarose gel. DNA band imaging was performed using a Gel Doc.

RESULT AND DISCUSSION

Conventional PCI Method Showed Reliable DNA Isolation Compared to Other Methods

A comparison of DNA yield and DNA quality (as measured by Nanodrop) obtained using the methodology previously described is shown in Figure 1, with good DNA quality defined as having a λ260/280 of 1.8 – 2.0, as described in a previous literature [14].

Conventional PCI method was found to have the highest DNA yield with an average concentration of 566.16± 370.05 ng.µL^{-1} (up to 60 times of other methods), while also having a good quality with an average quality for all samples of 1.74±0.12. On the other hand, both alkaline lysis and lysis-buffer method showed good DNA yield (91.78±29.32 ng.µL^{-1} and 76.45±24.73 ng.µL^{-1}, respectively), with good quality for alkaline lysis method (1.94±0.31) and a very low quality for lysis-buffer method (1.34±0.17). Lastly, isolation kit yielded the lowest DNA concentration (averaging at 7.21±2.85 ng.µL^{-1}), although having a good DNA quality (1.92±0.39).

Overall, based on DNA yield and quality of the control methods (PCI and isolation kits), these findings suggest that conventional PCI method was the most reliable method for DNA extraction, with high concentration obtained from raw and boiled porcine or bovine meat. These results support previous findings, as PCI method has been used in many DNA isolation studies in this field [3,7,12,15]. Compared to PCI method, isolation kit method showed relatively low DNA yield, albeit having good DNA quality. These results

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were also concurrent with many researchers in this field, whereas DNA yield in various kit assays was often shown to be significantly lower compared PCI method [16]. PCR amplification, however, seems to be dependent than other factors aside from high DNA yield, whereas PCR amplification was shown to be successful on DNA yield as low as five $ng.\mu L^{-1}$ [17].

As for the unconventional methods tested in this study, alkaline-lysis method showed good reliability in DNA extraction for these samples, while lysis buffer method showed good DNA isolation, albeit having poor quality. Results from alkaline-lysis method used in this study showed comparable DNA yield from other studies using similar methods [8], and higher than the original method [10]. Lysis-buffer only method differed from the previous article, in which a DNAzol® Direct from Qiagen was used; however, the DNA isolation yield quality was not reported [9].

PCR Amplification Showed Varied Results Depending on DNA Isolation Method

PCR amplification results summarized in table 1. Amplification was conducted on a cytochrome b gene with a common forward primer and specific reverse primers, as first described previous literature [18]. The size calculated from these samples were different than previously reported [13], whereas bovine samples, a band size of 320 bp was calculated, and from porcine samples a band size of 360 bp was calculated.

Table 1. Summary of PCR amplification from raw and boiled bovine and porcine meat samples

DNA isolation method	Samples	Positive results/ replicate
Conventional (PCI)	Raw bovine meat	2/3
	Boiled bovine meat	3*/3
	Raw porcine meat	3/3
	Boiled porcine meat	3/3
Isolation Kit	Raw bovine meat	3/3
	Boiled bovine meat	2/3
	Raw porcine meat	3/3
	Boiled porcine meat	3/3
Alkaline-lysis	Raw bovine meat	3*/3
	Boiled bovine meat	3/3
	Raw porcine meat	1*/3
	Boiled porcine meat	3/3
Lysis-Buffer Only	Raw bovine meat	0/3
	Boiled bovine meat	1/3
	Raw porcine meat	2*/3
	Boiled porcine meat	3*/3

Notes: (*) denotes hazy, unclear, or weak bands

Based on this study, conventional and isolation kit method showed a 92% PCR amplification from all samples. Alkaline-lysis success in amplification was 83%, and lysis-buffer method had only a 50% chance. Control methods, therefore, showed more reliability in the detection of porcine and bovine samples, and alkaline-lysis had a higher chance of detection compared to the lysis-buffer method. It should be noted that while some bands were unclear/weak, all bands showed the same band size corresponding to bovine and porcine samples.

Previous research has noted the importance of DNA isolation methods in successfully amplifying a DNA product [16,17], in which this research also concurs. Amplification failure may occur in samples having high PCR inhibitors, including various proteins found in meat samples, as described elsewhere [19]. While both conventional and isolation kit methods provide efforts to minimalize these inhibitors, unconventional methods used in this study do not. Therefore, the presence of these inhibitors may explain the low reliability in using lysis-buffer method for PCR amplification, in which false negatives occurred. Similarly, this may also explain the increased amplification of gene products using the alkaline-lysis method on boiled meats, whereas previous studies indicate that boiling as an inexpensive method to remove PCR inhibitors [20].

CONCLUSION

Conventional methods for DNA isolation in this study, which was PCI method and isolation kit method, showed high reliability in detection of bovine and porcine meat samples, both raw and boiled for a short time; despite the low DNA yield for isolation kit method. In the unconventional method tested in this study, DNA isolation using alkaline-lysis method resulted in a higher reliability in PCR amplification on boiled samples compared to raw samples, while also having a high DNA yield and good DNA quality. On the other hand, lysis-buffer method only showed poor results in DNA isolation as well as PCR amplification.

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Study of Glucosamine Production from Shrimp Shells by Fermentation Using *Trichoderma harzianum*

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Abstract

Shrimp shells are one of chitin sources in Indonesia which is potential to be converted into multifunctional glucosamine. This research was aimed to study the glucosamine production by fermentation using *Trichoderma harzianum*. Method used was experimental fermentation with pH treatment of 3-5 and fermentation duration of 10-20 days, and designed using Response Surface Methodology (RSM). Results showed that fermentation duration of 10-20 days has reached the optimum point of glucosamine production from shrimp shells using *T. harzianum*. The highest production of D-glucosamine in fermentation using *T. harzianum* occurred on initial pH of 5.41 and fermentation duration of 15 days (18,294.95 ppm), while the highest N-Acethyl-D-Glucosamine production occurred on initial pH of 3.00 and fermentation duration of 20 days (127,000.00 ppm).

Keywords: fermentation, glucosamine, shrimp shells, *T. harzianum*.

INTRODUCTION*

Shrimp is one of the important export commodities for fishing industry in Indonesia. Majority of shrimp export from Indonesia is done in form of frozen unshelled shrimp, therefore shrimp shell is considered as waste or unused by-products. The shrimp waste produced is around 30-35% of total weight of a shrimp [1]. Shrimp waste, such as shells can cause environmental problem if they are left unutilized.

Meanwhile, shrimp shells contain 25-40% of protein, 45-50% of calcium carbonate and 15-20% of chitin, depend on the types of shrimp [2]. Thus, shrimp shells can be used as a source of protein, calcium and chitin. Among those three components, the most potential component, in terms of economic value, is chitin. This is because chitin and its derivatives are very useful to be used in many industrial fields [3].

Chitin and its derivatives are commonly used in several fields, such as medical and health, i.e. they can be used as immunoadjuvant (non-specific stimulator of immune response) and basic material to make surgical suture [4]. N-acetyl glucosamine compound, the product of chitin hydrolysis, is used in treatment of osteoarthritis and used as supplement [5,6]. In aesthetic field, chitin derivatives can be used as basic ingredients for cosmetics, toothpaste, body cream and hair treatment products. In textile, they are widely used as coating material for cellulose fiber, nylon, cotton and wool [4].

Glucosamine is one of chitin derivatives that are widely used. Glucosamine production can be done physicochemically [7-11], enzymatically [12-15] and microbiologically [16]. To produce glucosamine chemically is relatively fast, but the chemical compounds used are dangerous for the environment, meanwhile to produce glucosamine enzymatically, the limitations are on the stability of enzyme and enzyme extraction cost that are quite expensive [17,18]. To produce monomer (likes glukosa, glucosamine) using microorganism is more advantageous because it is easier, more practical and the waste does not pollute the environment [19].

Glucosamine production using microorganisms is usually done by fermentation. There are many kinds of microorganisms, including bacteria and molds that can produce chitinolytic enzyme that can break down chitin into glucosamine. One of the microorganisms that can produce chitinase quite actively is *Trichoderma*. One of the strains, *T. viride*, produces chitinase enzyme with enzyme activity value of about 210.14 $U.mg^{-1}$ [20].

Trichoderma have several strains and in this research, the characteristics of *Trichoderma harzianum* in fermenting chitin into glucosamine with different pH condition and fermentation duration using RSM method, was studied.

MATERIALS AND METHODS

Method used in chitin fermentation was experimental method with design and analysis using Response Surface Methodology. Treatment

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or random variables applied in this fermentation experiment were pH (X_1 = 3, 4, 5) and fermentation duration (X_2 = 10, 15, 20 days). The determination of fermentation duration was based on report [21,22] that optimum fermentation duration of *Trichoderma* was 15 days, whereas the pH of fermentation was based on optimum pH for *T. harzianum* growth [23], i.e. pH of 5.6. The experimental matrix was derived by Software Design Expert, as can be observed on Table 1. The fixed factor was nutrition composition inside the fermentation media (KH_2PO_4 and $MgSO_4$) and fermentation temperature, i.e. about 30°C (room temperature). Therefore, the treatment applied in this research was combination from initial pH column and duration column, i.e. 21 treatments (run column).

Table 1. Experimental Design

Std	Run	Block	Initial pH	Duration (days)
11	1	Block 1	5.41	15.00
21	2	Block 1	4.00	15.00
12	3	Block 1	5.41	15.00
3	4	Block 1	5.00	10.00
16	5	Block 1	4.00	22.07
2	6	Block 1	3.00	10.00
15	7	Block 1	4.00	22.07
4	8	Block 1	5.00	10.00
5	9	Block 1	3.00	20.00
20	10	Block 1	4.00	15.00
17	11	Block 1	4.00	15.00
14	12	Block 1	4.00	7.93
19	13	Block 1	4.00	15.00
9	14	Block 1	2.59	15.00
13	15	Block 1	4.00	7.93
6	16	Block 1	3.00	20.00
10	17	Block 1	2.59	15.00
8	18	Block 1	5.00	20.00
18	19	Block 1	4.00	15.00
7	20	Block 1	5.00	20.00
1	21	Block 1	3.00	10.00

Note: Std = Standard

Preparation of Chitin from Shrimp Shells

Chitin isolation from *Vannamei* shrimp shells was performed by demineralization step using HCl and deproteination using NaOH [24]. First, *Vannamei* shrimp shells were washed and dried under sunlight until dry and then milled using Buhrmill and sieved using 60 mesh sieve to obtain shrimp shells powder.

Shrimp shells powder was then added with 1.5 M HCl with ratio of 1:15 (w/v) between sample and solvent. The mixture was then heated at temperature of 70-80°C for 4 hours under agitation of 50 rpm, and was filtered afterwards. The solid obtained was washed with aquadest to remove the remaining HCl. Then, the solid was dried in the oven with temperature of 70°C for 24 hours and cooled inside the desiccator, to obtain low mineral shrimp shells powder.

Demineralized shrimp shells powder was put into beaker glass and added with 3.5% NaOH solution with ratio of 1:10 (w/v). The mixture was then heated at temperature of 65-70°C for 4 hours under agitation of 50 rpm. The mixture was filtered to obtain chitin solids and washed with aquadest until neutral pH is obtained.

Fermentation of Chitin from Shrimp Shells using *T. harzianum*

Fermentation of chitin using *T. harzianum* was performed using semi solid fermentation system. The substrate consists of 5 gram of chitin, 0.05 gram of KH_2PO_4, 0.25 gram of $MgSO_4$, 0.05 gram of urea, 1 gram of glucose, and 25 ml of aquadest [25].

The substrate was then sterilized using autoclave with temperature of 121°C for 15 minutes. When the substrate has cooled down, pH was set according to the treatment using 0.1 M NaOH and/or 0.1 M HCl. Each substrate was added with 1 mL of *T. harzianum* spore and fermented at room temperature using shaker fermenter.

Chitinolytic Activity Test by Clear Zone Measurement

Chitinolytic activity test was done by observing the clear zone formed on agar media enriched with chitin, surrounding the growth of molds. Agar-chitin media was made from PDA added with 0.5% colloidal chitin (w/v media) [26, 27] and acidified using 50% lactic acid for about 0.14% from total volume of media [28]. This media was then sterilized using autoclave with temperature of 121°C, pressure of 1 atm for 15 minutes. The media was then poured into Petri dish and let solidify. After the media has solidified, a well with 6 mm diameter was made, aseptically. Then, 20 µL of *T. harzianum* spores with density of $20.48\times10^7.mL^{-1}$, $38.48\times10^7.mL^{-1}$, $63.52\times10^7.mL^{-1}$, $158.72\times10^7.mL^{-1}$, were respectively added into the well (6.0 mm diameter), and incubated at temperature of 35°C for 48 hours. Chitinolytic activity was determined by measuring the clear zone formed, i.e. by subtracting diameter of clear zone formed from diameter of well.

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Glucosamine Analysis using Spectrophotometry Method [29]

About 0.01 gram of fermented sample was dissolved uisng 1 mL of 0.25 M CH_3COONa to obtain concentration of 10,000 $mg.L^{-1}$. This solution was then diluted gradually until concentration of 12 ppm and 100 ppm were obtained. Each sample (concentration of 12 ppm and 100 ppm) was reacted with same concentration of PITC. The absorbance of sample solution of 12 ppm concentration that has been added with PITC was determined using UV-Vis spectrophotometer at wavelength of 273 nm to obtain absorbance of D-Glucosamine compound. Meanwhile, the absorbance of sample solution of 100 ppm concentration that has been added with PITC was determined using UV-Vis spectrophotometer at wavelength of 584 nm to obtain absorbance of N-Acethyl-D-Glucosamine compound. These absorbance results were then plotted on standard curve of D-Glucosamine and N-Acethyl-D-Glucosamine.

Standard curve of D-Glucosamine and N-Acethyl-D-Glucosamine were prepared by dissolving 0.01 gram of standard D-Glucosamine or N-Acethyl-D-Glucosamine in 10 mL of 0.25 M CH_3COONa and allowed to stand for 24 hours (to stabilize the solution). After that, standard D-Glucosamine or N-Acethyl-D-Glucosamine solution with concentration of 1,000 ppm as a stock solution was obtained. The stock solution was then diluted to obtain concentration of 3, 6, 9 and 12 ppm. About 10 mL of each solution was added with 10 mL of PITC solution, homogenized for 5 minutes and derivatives compound, i.e. phenylthiourea (PTH), was obtained. The absorbance of this compound can be determined according to the wavelength used. In this case, PITC solution was prepared by dissolving PITC using methanol that has the same concentration with glucosamine standard solution.

RESULT AND DISCUSSION

Characteristics of Chitin from Shrimp Shells

Table 2 shows that chitin used has not fulfilled the commercial chitin standard in terms of protein content, although its moisture content, mineral content and deacetylation degree have fulfilled the commercial chitin standard. High protein content in chitin indicates that deproteination process was not enough. This might be caused by insufficient NaOH concentration and/or insufficient deproteination process. Effectivity of deproteination process depends on the concentration of basic solution and temperature used [30]. Besides, protein content of chitin is related to its moisture content [30]. Lots of protein matrix that have been degraded cause hydrophilic properties of protein to bind water molecule becomes weaker[33]. As a result, the moisture content will become less [31]. It is shown by the moisture content of shrimp shells which is about 60-65% [32].

Table 2. Characteristics of Chitin and Dried Shrimp Shells

Parameter (%)	Dried Shrimp Shells	Chitin	Commercial Chitin
Moisture	10.07± 0.26	6.69±0.21	<10*
Protein	42.41±4.21	13.2 ±1.42	<1*
Mineral	19.75±0.39	1.42±0.09	<2.5*
Deacetylation degree	-	35.16	15-70**

Note : *Commercial food grade chitin[33];
**Chitin standard Protan Laboratories[34]

Chitinolytic Activity of *T. harzianum* Molds

Chitinolytic activity of molds is defined as the ability of molds to degrade chitin into other simpler compounds, e.g. glucosamine by chitinase enzyme. This activity can be measured by clear zone formation around the wells, paper disc or colony [20,35,36]. In this research, chitinolytic activity obtained based on clear zone formation was used to determine the amount of spores added during chitin fermentation. The results of clear zone based on density of *T. harzianum* spores can be observed on Table 3.

Table 3. Clear zone formation based on density of *Trichoderma harzianum* spores

Density (spore.mL^{-1})	Clear zone (mm)*
20.48×10^7	6.52 ± 0.73
38.48×10^7	5.93 ± 0.91
63.52×10^7	4.67 ± 1.28
158.72×10^7	2.95 ± 0.17

***Note**: clear zone = diameter of clear zone – diameter of well

The size of clear zone formed shows the amount of N-acetylglucosamine formed from chitin breakdown by chitinase enzyme. The larger the clear zone, the higher amount of chitinase formed, which means the higher the chitinolytic activity [36]. Based on Table 3, it can be observed that the largest clear zone diameter for *T. harzianum* molds was obtained from spores with density of 20.48 x 10^7 spores.mL^{-1}, with clear zone of 6.52 ± 0.73 mm, and then decreased as the spore density was increased. The smallest clear zone occurred at density of 63.52×10^7 spores.mL^{-1} with clear zone of 2.95 ± 0.17 mm. This might happen because the spore density of 20.48 x 10^7 spores.mL^{-1} was the most effective;

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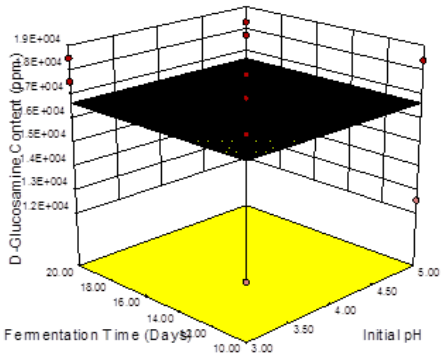
therefore there was no growth competition among the molds. Thus, *T. harzianum* can work optimally to degrade chitin during fermentation. Chitin degradation is done by chitinase enzyme that has different activity based on the types and stage of growth of microorganisms, and factors which influence the growth of microorganisms, such as pH, temperature and fermentation period [12,37,38]. Chitinase activity from *Trichoderma* sp. and *T. viridae* was 33.19 IU.mL^{-1} and 18.77 IU.mL^{-1}, respectively [20].

Glucosamine Production

Glucosamine that is obtained from chitin fermentation can be in form of D-glucosamine and N-Acethyl-D-Glucosamine. The correlation between pH and fermentation duration of shrimp shells chitin for glucosamine production according to RSM program can be observed on Figure 1.

A) D-Glucosamine



B) N-Acethyl-D-Glucosamine

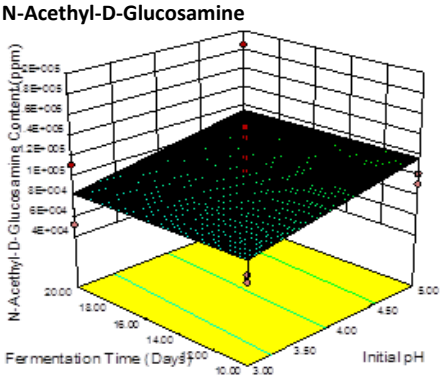


Figure 1. Three-dimensional RSM graph of correlation between pH and fermentation duration of shrimp shells chitin for glucosamine production using *T. harzianum*

Three-dimensional RSM graph on Figure 1 shows the flat shape of correlation between pH and fermentation duration of shrimp shells chitin for glucosamine production, which means there was no optimum point. This phenomenon shows there was no significant effect of fermentation duration and pH on D-Glucosamine and N-acetyl-D-Glucosamine obtained. This might happen because the fermentation duration was too long; therefore the production of D-Glucosamine is already maximum since the first observation (day 10) and there was only slight difference in glucosamine content on the following observations. It is based on a previous research that stated the production of chitinase by fermentation using *T. harzianum* reached its highest amount after 72 hours (day 3) when *T. harzianum* mycelia starter was used and after 96 hours (day 4) when *T. harzianum* spore starter was used [39].

This statement is also supported by another research that stated chitinolytic activity of *T. harzianum* reached its optimum amount in between day 2 and day 3 [40]. No significant change of glucosamine production after it has reached its optimum point is also in accordance with a research [41] which stated after 172 hours, glucosamine fermentation using *T. harzianum* mold starter did not undergo significant increase. Because there was no optimum point for glucosamine production, the glucosamine production can be observed based on interaction between pH and fermentation duration, as can be seen on Figure 2.

Figure 2 shows that the highest D-glucosamine amount obtained from pH treatment of 5.41 and fermentation duration of 15 days, which produces D-glucosamine content of 18,294.95 ppm. This amount of glucosamine is still higher compared to other research reports. Fermentation using chitinase from *Pseudomonas* sp. was optimum after 35 hours, which produces 0.164 mg.mL^{-1} 164 ppm of glucosamine [14], using *Actinomycetes* ANL-4 starter produces highest glucosamine about 9,784.25 ppm on day 5 [29], using *Aspergillus* sp. BCRC 31742 produces highest glucosamine of 7,480 ppm [42].

The highest N-acetyl-D-Glucosamine production in fermentation of shrimp shells chitin using *T. harzianum* obtained from pH treatment of 5 and 10 days of fermentation, which produces N-acetyl-D-Glucosamine of 127,000.00 ppm. This amount of glucosamine is still higher compared to other research reports. Fermenta-tion using *Aspergillus rogulosus* 501 on day 10 obtained N-acetyl-D-Glucosamine of 2,228 ppm [43], fermentation using enzyme combination from *T. harzianum* and *T. reesei* was highest after 72 and 96 hours of incubation, which was about 4,040 ppm [39], fermentation using *T. harzianum* was

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highest after 212 hours, which was about 190 ppm [40].

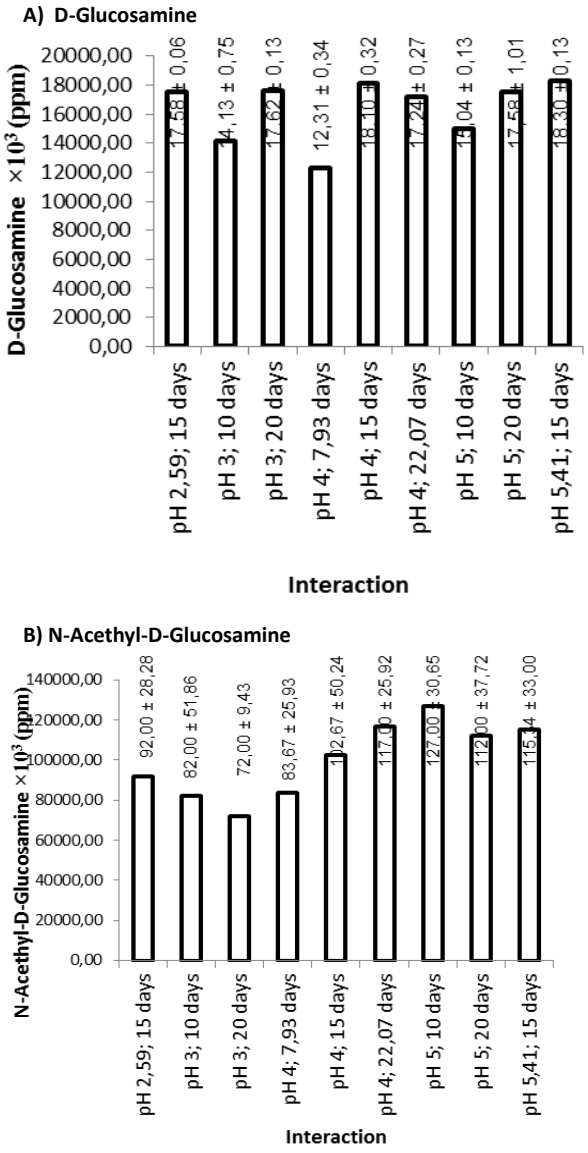

Figure 2. Graph of interaction between fermentation factors and their influence on glucosamine content

CONCLUSION

The fermentation duration applied on optimization of fermentation of shrimp shells chitin for glucosamine production using *T. harzianum* was too long. The highest production of D-Glucosamine from fermentation using *T. harzianum* occurred on initial pH of 5.41 and fermentation duration of 15 days, which reached about 18,294.95 ppm, whereas the highest production of N-Acethyl-D-Glucosamine occurred on initial pH of 3.00 and fermentation duration of 20 days, which reached about 127,000.00 ppm.

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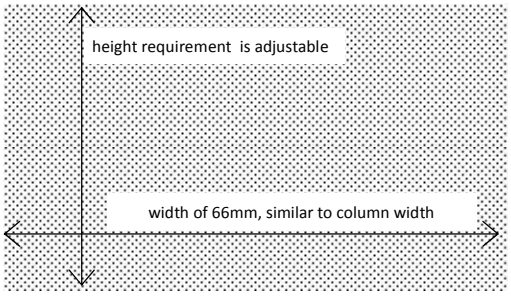

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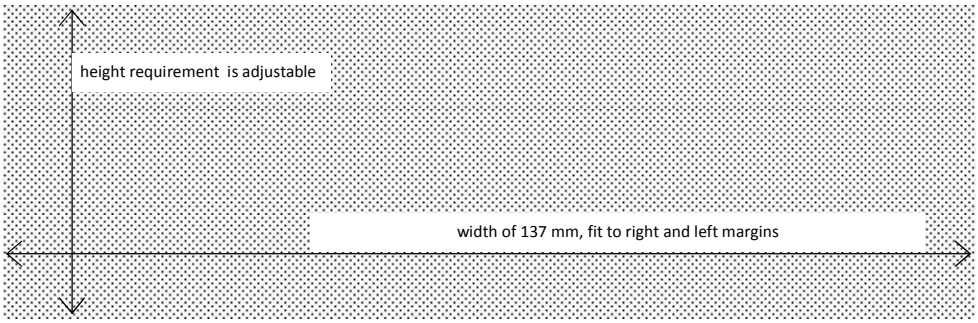

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