The Ability of Pineapple Callus Regeneration (*Ananas comosus* L.) from Sipahutar North Sumatra Indonesia with in Vitro Culture

Fauziyah Harahap¹, Nikmatul K Harahap¹, Ely Djulia¹, Dirga Purnama¹, Herbert Sipahutar², Rosmayati², Suci Rahayu², Periseuein F Zega¹, Rifa Fadhilah Munifah Hasibuan³

¹Department of Biology, Faculty of Mathematics and Natural Sciences, Medan State University, Jl. William Iskandar Pasar V Medan Estate, Medan, 20221, Indonesia
²Department of Biology, Faculty of Mathematics and Natural Sciences, University of North Sumatera, Jalan Biotechnology No.1, USU Campus Medan, 20155, Indonesia
³Department of Agronomy and Horticulture, Faculty of Agriculture, Agriculture University, Bogor, Indonesia.

Corresponding author : fauziyahharahap@gmail.com

Abstract. Pineapple (*Ananas comosus* L.) from Sipahutar is an herbaceous plant from Sipahutar District. Pineapple is canning industry sourced from Sipahutar pineapple has the opportunity to be developed in North Sumatra. Propagation through in vitro culture is an alternative method to solve this problem. The purpose of this research was to determine the regeneration ability of pineapple callus derived from callus induction with the addition of 2,4-D 1 ppm and BAP 1 ppm. The callus were regenerated using the addition of Kinetin (0; 0.5; 1; 1.5 ppm). Each treatment was carried out 4 times. The results showed: 1) the fastest time of emergence of shoots (5 days) came from 0.5 ppm, 2) formed the most shoots (8 shoots) resulting from the addition 0 ppm and 0.5 ppm, 3) the most number of leaves (7.75 leaves) resulted from light treatment with the addition kinetin 0.5 ppm and 1 ppm, 4) the longest leaf length (4.44 cm) from dark treatment with kinetin 0.5 ppm, 5) the highest plant height (5.32 cm) from dark treatment with 0.5 ppm kinetin. This shows that the concentration of 0.5 ppm kinetin with the dark treatment is the best concentration to stimulate the formation of pineapple shoots.

1. Introduction
Pineapple or "Pineapple" (*Ananas comosus* L.) is a fruit plant that has a distinctive taste and aroma. Indonesia is known as the third largest exporter of pineapple and canned juice after the Philippines and Thailand. Pineapple canning industry has a great opportunity to be developed in Indonesia [1]. For industrial scale, conventional pineapple plant propagation is less effective because the number of seedlings produced is very limited and requires a quite long time. Pineapple is a plant that is almost always propagated vegetatively by conventional means by planting crowns or by using saplings [2]. To achieve large-scale development, traditional propagation is not effective, because the number of
seeds produced is limited and requires a long time. Propagation through tissue culture is an alternative technique that can be done, especially using callus culture [3].

Plant can be regenerated from unorganized tissue called callus tissue that induced by a variety of growth regulating hormones [4]. Callus induction is the initial stage in indirect embryogenesis. Callus is a collection of amorphous cells that occur from tissue cells that divide continuously [5, 6]. Callus culture is carried out on plant explants to rejuvenate cells in explants that are isolated and grown in a controlled environment [5]. Callus culture is important to be done to see the ability of explant in forming callus also the ability to grow on continuous regeneration media that can be used in somaclonal utilization, genetic transformation, and secondary metabolite production.

Regeneration of plants from cells and tissues that are not yet mature enough (callus) is the best plant regeneration way, especially plants that have recasiltrant species of monocotyledonous plants characteristic in this case, pineapple plant [7]. Callus can be regenerated and sub cultured by taking part of the callus and transferring it to new culture media. Regeneration of buds from callus explants is a complex process, many factors affect, including plant genotypes, balanced of cytokines and auxin growth regulators both inside and outside cells and callus physiology.

Growth regulators are one of the most important factors for successful culture. In tissue culture, cytokines plays an important role as pioneers in division and act in the formation of meristematic tissue leading to organ formation, especially shoot formation [8]. Indole-1-acetic (IAA), 6-BenzylAminoPurine (BAP) and kinetin are hormones or growth regulators that are often used in callus induction besides 2,4 D [9]. Kinetin is known as a type of cytokine PGR which is widely used for buds multiplication because it has the ability to stimulate the formation of buds with high concentrations (more than 1ppm) that are not easily damaged when the media is sterilized [10, 11]. Kinetin is known to produce an increased ability to regenerate callus wheat cultivars [9]. Based on his research on the Matthiolaincana plant, the buds length and number of buds increased along with increasing concentrations of kinetin given [12]. There is an effect of callus growth with a kinetin concentration of 1.0 mg / l [13]. Rashid also has the same idea about the using of kinetin [9].

The purpose of this research is to determine the regeneration ability of Sipahutar pineapple plant which is given PGR kinetin addition derived from pineapple plant callus induced by the addition of 2,4D PGR and BAP for light and dark treatment.

2. Material and Methods

2.1. Research Place and Time
This research was conducted at YAHDI Tissue Culture Laboratory, located at Perum Pelabuhan Jl. Lambung No.16 Tanah 600 Medan Marelan. The research was conducted from August 2017 to April 2018.

2.2. Population and Sample
The population in this research was the whole pineapple plantlet (Ananas comosus L.) Sipahutar pineapple varieties from in vitro subcultures in the Johor Tissue Culture Laboratory, Ministry of Agriculture Laboratory. The sample in this research was callus derived from callus induction with the addition of PGR 2,4D and BAP for light and dark treatments.

2.3. Tools and Materials
The tools that used in this research were autoclaves, stirring rods, beaker glass, culture bottles, petri dishes, measuring cups, scissors, hand sprayers, millimeter paper, label paper, Laminar Air Flow Cabinet (LAFC), bunsen burner, refrigerators, pans, pH indicator, tray / basket, volume pipette, tweezers, spatula, culture rack, scapel, analytical scale, dripping pipette, tissue, rubber band, paper, plastic, napkin and stationery.
While the ingredients used in this study were pineapple callus induced by the addition of PGR 2,4-D and BAP, MS (Murashige&Skooog) media, kinetin growth regulators (0; 0.5; 1; 1.5 ppm), 70% alcohol, 96% alcohol, 0.1 N HCl, 0.1 N NaOH, sterile aquadest, detergent, chlorox, formalin.

2.4. Research Design
This research used an experimental method, where the first time to do was to induce callus from explants of pineapple plants induced by the addition of PGR 2,4D and BAP for light and dark treatment. Next, the best callus derived from the results of the induction is transferred to be regenerated by subculture. Callus regeneration was carried out using a Completely Randomized Design (CRD) factorial with 4 treatments. As for the factors of this research are the additions of kinetin by treating four levels of kinetin concentration (0; 0.5; 1; 1.5 ppm). The variables observed were callus formation time, number of buds, number of leaves, leaf length, and plant height.

3. Results
3.1. Callus Regeneration
3.1.1. Time of Formation of Buds. Observation time of formation of buds on pineapple explants is done from the first day to the 14th day. From the observations was found that the most rapid treatment of buds appeared was the K₀.5 treatment (kinetin 0.5 ppm) which was 5.75 days after subculture in the treatment origin of the light treatment callus and the 5th day for the treatment of the origin of the dark treatment callus (Table 1). Followed by treatment K₀ (kinetin 0 ppm) which was day 7.5 (light) and K₀ ppm day 6 (dark). K₁ treatment (kinetin 1 ppm), which is 6.75 days after subculture (light) and 7th day (dark). Followed by the treatment of K₁.5 (kinetin 1.5 ppm) that appears at the 8.75 days (light) and the 8.25 days (dark).

| PGR Treatment | Day- (Explants origin) | Light | Dark |
|---------------|-----------------------|-------|------|
| K₀₀ (₀ Kinetin 0 ppm) | 7.5 | 6 |
| K₀.5 (₀ Kinetin 0.5 ppm) | 5.75 | 5 |
| K₁ (₀ Kinetin 1 ppm) | 6.75 | 7 |
| K₁.5 (₀ Kinetin 1.5 ppm) | 8.75 | 8.25 |

From table 1 shows the most rapid buds formation resulted from 0.5 ppm kinetin treatment and significantly different from 3 other treatments. This means that the earliest time the formation of buds, the acceleration of bud formation required optimum Kinetin dose 0.5 ppm [14].

![Figure 1. The average time of budsformation](image-url)
3.1.2 Number of Buds. Observation of the buds number formed on pineapple explants is done from the first day until the 12th week. From the observations was found that the treatment with the most number of buds was the K0.5 treatment (kinetin 0.5 ppm) which is as many as 7.75 buds (light) and 8 buds (dark). Then the treatment K1.5 (kinetin 1.5 ppm), which is 7.5 buds (light) and 6 buds (dark). Followed by the treatment of K0 (kinetin 0 ppm) i.e. 6.75 buds (light) and 8 buds (dark). And the last is the treatment of K1 (kinetin 1 ppm) which is as many as 6 buds (light) and 7 buds (dark).

| PGR Treatment       | Average of buds number |
|---------------------|------------------------|
|                     | Light  | Dark  |
| K0 (Kinetin 0 ppm)  | 6.75   | 8     |
| K0.5 (Kinetin 0.5 ppm) | 7.75   | 8     |
| K1 (Kinetin 1 ppm)  | 6      | 7     |
| K1.5 (Kinetin 1.5 ppm) | 7.5    | 6     |

From table 2, can seen that the highest number of buds resulted from 0.5 ppm kinetin treatment and significantly different from the other 3 treatments. Increasing the kinetin dose is not produced by increasing the number of buds produced.

3.1.3 Number of Leaves. Observation of the leaves number in pineapple explants is done from the first week to 12th week after subculture. From the observations was found that the treatment that had the most leaves was the K0.5 treatment (kinetin 0.5 ppm) with 7.75 leaves (light) and 6 leaves (dark). Followed by the treatment of K1 (kinetin 1 ppm) with 7.75 leaves (light) and 5 leaves (dark). The PGR K0 (kinetin 0 ppm) treatment was followed by 5.75 leaves (light) and 7.25 leaves (dark). And the last is the treatment of K1.5 (kinetin 1.5 ppm) which is 4.75 leaves (light) and 6.5 leaves (dark).

| PGR Treatment       | Average number of leaves |
|---------------------|--------------------------|
|                     | Light  | Dark  |
| K0 (Kinetin 0 ppm)  | 5.75   | 7.25  |
| K0.5 (Kinetin 0.5 ppm) | 7.75   | 6.25  |
| K1 (Kinetin 1 ppm)  | 7.75   | 5     |
| K1.5 (Kinetin 1.5 ppm) | 4.75   | 6.5   |
The highest number of leaves was produced from light treatment with the addition of 0.5 ppm and 1 ppm kinetin and significantly different from other treatments (Table 3). For the dark treatment, the highest number of leaves was produced from the Kinetin 0 ppm treatment. This means that without the addition of kinetin produced the highest number of leaves.

![Figure 3. Average of leaves number](image)

Based on the figure 3 above, can be concluded that the most number of leaves appeared in 0.5 ppm kinetin PGR treatment both at the origin of the light and dark treatment callus. While the smallest number of leaves that appeared was in the treatment of 1.5 ppm kinetin PGR both at the origin of the light and dark callus.

3.1.4. Leaf Length. Observation of leaf length in pineapple explants was conducted from the first week to 12th week after subculture. From the observations, it was found that the treatment that had the longest leaf length was the K0.5 treatment (kinetin 0.5 ppm) with 3.75 cm (light) and 4.44 cm (dark). Followed by the treatment of K0 (0 ppm kinetin) with 3.63 cm (light) and 4.33 cm (dark). Followed by the treatment of K1 (kinetin 1 ppm) which is 3.6 cm (light) and 3.74 cm (dark). And the last treatment was K1.5 (kinetin 1.5 ppm) which was 3.37 cm (light) and 3.59 cm (dark).

| PGR Treatment | Day- (Explants origin) |
|---------------|------------------------|
|               | Light | Dark |
| \(^a\)K0 (\(^b\)Kinetin 0 ppm) | 3.63 | 4.33 |
| \(^b\)K0.5 (\(^c\)Kinetin 0.5 ppm) | 3.75 | 4.44 |
| K1 (\(^\text{d}\) Kinetin 1 ppm) | 3.6 | 3.74 |
| \(^\text{d}\)K1.5 (\(^\text{d}\) Kinetin 1.5 ppm) | 3.37 | 3.59 |

From table 4, can seen that the highest leaf length resulted from 0.5 ppm kinetin treatment and was significantly different from the other 3 treatments. Increasing the kinetin dose is not followed by an increase in the resulting leaf length, even an increase in the kinetin dose causes a decrease in leaf length.

The longest leaves were in PGR kinetin 0.5 ppm treatment both at the origin of the light and dark treatment callus. Whereas the shortest is on the treatment of 1.5 ppm PGR kinetin both at the origin of the light and dark callus. The difference of light and dark treatment results can be seen in figure 4.
3.1.5. Plant Height. Observation of plant height in pineapple explants was carried out from the first week to 12th week after subculture. From the observations it was found that the highest explant is K₀,₅ treatment (kinetin 0.5 ppm) treatment which was 4.02 cm (light) and 5.32 cm (dark). Followed by the treatment of K₀ (kinetin 0 ppm) with 3.84 cm (light) and 4.86 cm (dark). Followed by the treatment of K₁ (kinetin 1 ppm) with 3.75 cm (light) and 4.36 cm (dark). And the last treatment was K₁,₅ (kinetin 1.5 ppm) which was 3.72 cm (light) and 4.08 cm (dark).

The highest plantlet is in 0.5 ppm kinetin PGR treatment both light and dark callus origin. Whereas the lowest height is in 1.5 ppm PGR kinetin treatment both light and dark origin callus. More details about plant height result can be seen in figure 5.
4. Discussion

Tissue culture is a technique for multiplying plants from cells or plant tissue in glass tubes containing media that contains complete nutrition with sterile conditions [5, 15]. Plant parts which are used as starting material for in vitro culture are called explants [16]. Explants will develop into a complete plant if cultured on the appropriate media. The pattern of its development can occur directly (not through callus formation) or indirectly (through callus formation), can be sub cultured by taking a portion of the callus and transferring it to a new culture medium. Through the right induction system, callus can develop into complete plants so that the supply of plant seedlings through callus propagation is very beneficial [17].

Plant growth regulators have an important role in plant regeneration in in vitro culture. Growth regulators which are often used in in vitro culture are auxins and cytokines [11, 14]. Auxin and cytokinin play an important role in plant regeneration because the ability to induce callus formation [18]. In general, auxin is able to induce callus proliferation and growth, whereas cytokines play a role in stimulating regeneration and buds growth. Plant regeneration through callus explants is influenced by auxins and cytokines in the callus that determine the direction of differentiation. In some studies, the combination of auxin and cytokine can affect the appearance of callus. Combination of plant growth regulator 2ip (auxin) and kinetin (cytokine) resulted in increased regeneration ability of wheat plant culture [9].

From table 1, can be seen that the 0.5 ppm kinetin treatment resulted the earliest buds formation and different significantly from the other 3 treatments. This means that to accelerate the formation of buds, the optimum dose of kinetin is needed, which is 0.5 ppm. From this treatment, it was seen that an increase the kinetin dose was not followed by the speed of buds formed. The optimum dose of kinetin is needed for the maximum buds formation [14, 5].

In this research, growth regulators used to stimulate buds growth are kinetin with concentrations of 0, 0.5, 1, 1.5 ppm. From the results obtained show that cytokines are very influential on the time of formation of buds, number of buds, number of leaves, plant height and leaf length. Where in all observations parameters for the formation of buds, the number of shoots, number of leaves, leaf length, and plant height the best treatment is the 0.5 ppm kinetin PGR treatment both at the origin of light and dark treatment calluses. While the lowest was in the 1.5 ppm PGR kinetin treatment both of the light and dark origin callus.

From this research, it was seen that the highest number of leaves was produced from light treatment with the addition of 0.5 ppm and 1 ppm kinetin. While for the dark treatment, the highest number of leaves was produced from the 0 ppm kinetin treatment. This means that without the addition of kinetin produced the highest number of leaves in the dark treatment, while in light treatment requires the addition of 0.5 to 1 ppm kinetin to produce the highest number of leaves. From the results of this research known that light causes endogenous growth regulators do not work optimally to produce the
highest number of leaves. So it takes the addition of kinetin from outside to produce the highest number of leaves. As for dark treatment, endogenous growth regulators can work optimally to produce the most number of leaves. Need further research whether the light treatment will causes kinetin cannot work optimally.

Based on data, can be conclude that the addition of kinetin growth regulators included in the cytokines has a significant effect on the regeneration of pineapple explants callus (Ananascomosus). Cytokines are compounds that can increase cell division in plant tissue and regulate plant growth and development, as well as kinetin (6-furfurylaminopurine) [19]. Kinetin is one of the cytokines commonly used in plant tissue culture because it has an important function in the development of callus and regeneration. Kinetin does not play a role in callus initiation but in the case of callus regeneration plays a huge role to increase the proliferation and formation of buds in tissue culture [20].

The addition of higher cytokine concentrations compared to auxin concentrations will initiate multiplication of buds [6]. The multiplication process of plant buds requires the presence of external growth hormones especially cytokine groups such as kinetin because in order to get high levels of multiplication of buds, internal cytokines are still lacking [21].

The buds is a part of the plant that obtained from vegetative propagation, which grows in order to produce offspring on the plant. The formation of buds shows the success of the regeneration of inoculated explants on tissue culture media. Callus resulting from explant callus induction can differentiate to form buds. The faster the buds appear, the faster the material produced for propagation of plants.

Buds is categorized grow if you see the green protuberances (± 2 mm) in the callus that has formed. The bulges are adventitious shoots that will grow into new buds. The formation of adventitious shoots is affected by the interaction between auxin and cytokine [6]. The buds that are formed come from the elongation of buds at the buds of plant stems and buds that come from the differentiation of callus tissue in explants.

![Figure 8](image8.png) **Figure 8.** Result of plant height measurement

![Figure 9](image9.png) **Figure 9.** Callus regeneration after 12 weeks of observations

Callus regeneration from callus explants is a complex process, because it is influenced by lots of factors, including genotype factors [22], explants type and balance of growth regulators in the medium [23] and callus physiology conditions [24]. Factors that influenced the success of callus regeneration include plant species, origin of explants, type and concentration of growth regulators [25].

This research showthat the increase of kinetin dose causes the decrease of leaf length. It is understood that there is an optimum dose needed to produce the maximum leaf length. Increasing the kinetin dose causes inhibition of cells in the leaves to develop. Need further research, structurally whether cell repetition occurs repeatedly but does not provide an opportunity for cells to differentiate properly, as a result of repeated leaves cell division, which causes the leaves do not grow normally.

Some plants takes a long time to regenerate, because the use of endogenous cytokines is insufficient for the formation of buds, in addition to auxin, the growth regulator cytokine also needs to
be added to the media [26, 27]. Cytokines play a role in stimulating the growth and development of plants, especially adventitious buds. Without the addition of cytokines into the planting medium, the callus is unable to organogenesis to form buds because there is no interaction between auxin and endogenous cytokines with auxin and cytokines. The replacement of exogenous auxin and cytokines will change the level of endogenous cell growth regulators [23]. Formation of buds in vitro through direct and indirect morphogenesis is highly dependent on the exact type and concentration of organic, inorganic and growth regulating composition.

From several buds that grow there some that experienced browning. This happened because cells are injured and each tissue has a different ability to start the process of forming buds. Cell death on the surface of explants inhibits the speed of tissue to absorb nutrients and these explants cannot survive with in vitro methods. Signs that the regenerated callus can form buds include a change in color from brown or yellow to yellowish white and then greenish. The change in color is a sign of morphogenesis, but growth stops after forming a leaf. The callus must be transferred to organogenesis media with an appropriate PGR ratio in order to form other organs so that they become plantlets. In this study callus cells can develop to form somatic embryos, but not all callus cells are able to develop into somatic embryos. This is due to competition between embryogenic cells to carry out further development.

5. Conclusions
The most common stage of callus regeneration to form buds was 0.5 ppm Kinetin treatment. This shows that the concentration of kinetin 0.5 ppm is the best concentration to stimulate the formation of pineapple buds.

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