Pineapple callus induction from Sipahutar North Sumatera Indonesia (*Ananas comosus* L.) with bud as a source explant

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**Abstract**. The preservation and development of Sipahutar pineapple is very important to do. One of the most effective ways is with tissue culture techniques. The aim of this research is to know the effect of 2,4-Dichlorofenoxyacetic acid (2,4-D), kinetin, and interaction of both Plant Growth Regulator (PGR) to callus induction on pineapple (*Ananas comosus* L.) from Sipahutar Sumatera Utara Indonesia. The design of this study used a Factorial Completely Randomized Design, namely 2,4-D with 3 doses of treatment (0, 1, 2 ppm) as the first and the second factor of kinetin with 3 doses (0, 0.5, 1 ppm). The observation process was conducted for 28 days and 35 days after induction. The parameters observed were the formation time of callus, callus color, callus biomass, and the height of callus pile. The results showed that 2,4-D, kinetin and interaction of 2,4-D and kinetin significantly influenced the time of callus formation, callus biomass, and significant effect on the height of callus pile. The time of callus formation is the fastest on day 8 after induction. The best of callus color is whitish green comes from 2,4-D 0 ppm and kinetin 1 ppm treatment. The highest biomass is 1.69 grams. The highest stack height of callus was 2.32 cm.

1. **Introduction**

Pineapple (*Ananas comosus* L.) is a plant that has long been known by the people [1], and one of the leading fruit commodities in Indonesia. Pineapple is very popular and has great economic meaning [2], and mostly canned, made jams, jellies, and fruit juices [3]. Almost all regions of Indonesia are pineapple-producing regions because they are supported by an appropriate tropical climate [4]. Indonesia is the fifth largest producer after Brazil, Thailand, Philippines and China [5]. For the Southeast Asian region, Indonesia is among the third largest pineapple producers after the Philippines and Thailand with a contribution of around 23% [4].
Pineapple production in Indonesia in 2015 reached 1,729,603 tons. Some pineapple production centers include Lampung (534,775 tons), North Sumatra (223,128 tons), East Java (171,304 tons), Jambi (142,845 tons), West Java (187,555 tons) and Central Java (201,039 tons). Sipahutar pineapple was grown by farmers in the Sipahutar area, North Tapanuli, North Sumatra, Indonesia. This pineapple has the advantage that it tastes more sweet, water is low content, denser texture, yellow color and liked by the community. This fruit is one of the leading horticultural crops commodities in North Tapanuli.

The problem in pineapple cultivation in Indonesia is there are no producers that can supply quality pineapple seeds in large quantities. On the other hand, a number of issues related to food quality and safety are the cause of the inadequate contribution of Indonesia’s fresh pineapple in international trade, so we need a solution that can overcome these problems. One alternative technology choice that can solve this problem is through tissue culture techniques. Tissue culture is a technique of isolating and maintaining even pieces of plant tissue that are removed from their natural environment, then grown on an appropriate artificial media and aseptic conditions. These parts then multiply and regenerate into complete plants again. Tissue culture technique is an alternative to solve the problem of low productivity of pineapple plants that have not been able to meet market demand. This technology has been widely used for the procurement of uniform seeds and the quality is guaranteed, especially in a variety of horticultural crops.

The success in the tissue culture method depends on the media used. In addition to the media that determine success in tissue culture, there are several factors that determine the success of tissue culture systems including nutrient composition and balance of growth regulators (GRS). Research on induction of pineapple callus from Sipahutar North Sumatra Indonesia (Ananas comosus L.) with buds as a source of explants has never been done. So that this study was conducted with the aim of knowing the effect of variations in the combination of 2,4-dichlorophenoxyacetate (2,4-D) and Kinetin on callus induction in pineapple (Ananas comosus L.), to know which GRS is more responsive by using pineapple hump explants and the appearance of good callus warrants in which composition.

2. Materials and Methods

2.1. 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 YAHDI Tissue Culture Laboratory. The sample in this research was 1 cm bud with the addition of PGR 2,4D and Kinetin treatment.

2.2. Research Design
The study design uses factorial completely randomized design (CRD) with 9 treatment combinations with 4 replications, with the combination as shown in Table 1.

| No | 2,4-D (ppm) | Kinetin (ppm) |
|----|-------------|---------------|
|    |             | 0  | 0.5 | 1   |
| 1  | 0           | 2,4-D₀ K₀ | 2,4-D₀ K₀,₅ | 2,4-D₀ K₁ |
| 2  | 1           | 2,4-D₁ K₀ | 2,4-D₁ K₀,₅ | 2,4-D₁ K₁ |
| 3  | 2           | 2,4-D₂ K₀ | 2,4-D₂ K₀,₅ | 2,4-D₂ K₁ |

2.3. Procedure
2.3.1. Sterilizing Tools and Materials
All tools and materials are washed with detergent and running water thoroughly, then sterilized using an autoclave at 1210°C (for 1 hour, pressure 17.5 psi). Pineapple shoots in vitro plants are taken from the tubers and used as a source of explants.

2.3.2. Media Making
The media used in this study were Murashige and Skoog (MS) media with Growth Regulatory Substances (GRS) 2,4-D and Kinetin according to treatment. The initial stage of making MS media is to make stock solutions of C, D, E, F, and vitamins, weighed macro nutrients such as NH4NO3, KNO3, myo-inositol, sucrose, and agar according to the amount listed in the composition of MS 1 Liter media making. Subsequently, all the ingredients are mixed into a glass beaker, then pipette micro nutrients and add sterile distilled water to a volume of 1 L. Then stir until homogeneous, 2,4-D and kinetin added according to the treatment, stir until homogeneous. Then the acidity test of the culture media was carried out by measuring the pH of the media solution, add gelatin then heat while stirring until it boils. Then pour into the culture bottles that have been sterilized beforehand, close the lid and label it. Then the media is autoclaved for 20 minutes.

2.3.3. Callus Induction
The parts taken are tubers, then placed one by one into a petri dish then cut into explants with a size of 1 cm and put into bottles according to the dose that has been made. After finishing everything is placed in the culture room.

2.3.4. Maintenance
Bottles that have been filled with explants are placed on the culture rack in the temperature range of 180 – 220C and the irradiation time is around 16 hours a day.

2.3.5. Observation Parameters
a. Time of Callus Formation. Observations were made once every two days until 28 days after induction (28 DAI).
   b. Callus Color. The color of the callus was observed after the formation of the callus from the explant source until the 28th day of observation. Determination of callus color based on Andaryani (2011): Score 1 = Chocolate; Score 2 = Brownish yellow; Score 3 = Brownish white; Score 4 = Greenish White; Score 5 = Brownish Green; Score 6 = Yellowish Green; Score 7 = Leucorrhoea green; Score 8 = Green.
   c. Callus Biomass. Weighed after the end of observation (28 DAI) using analytical scales
   d. Callus Pile Height. Callus pile height was measured at the end of observation (28 DAI) using a ruler.

2.3.6. Data analysis technique
This research uses factorial completely randomized design (CRD) model with the following formula;
\[
Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \epsilon_{ijk}
\]
\(Y_{ijk}\) = the results of observations on the k-test which was treated with 2.4 D and kinetin
\(\mu\) = General midpoint
\(\alpha_i\) = The effect of 2,4-D concentration at i level
\(\beta_j\) = Effect of kinetin concentration at the jth level
\((\alpha\beta)_{ij}\) = The interaction effect of 2,4-D treatment at i level and jth kinetin treatment
\(\epsilon_{ijk}\) = Effect of an error receiving 2,4-D treatment at the i-th level and kinetin treatment at the j-level at the k-th test

3. Result and Discussion
3.1. Time of Callus Formation
Observation results obtained from the influence of GRS 2,4-D and Kinetin on the time of callus formation can be seen in table 2 below.

| Treatment | Days of induction |
|-----------|------------------|
| 2,4D0 K0  | 18               |

Table 2. Time of callus formation
Based on the results of the study, the time of callus formation can be seen that the callus was induced in almost all treatments. The callus that appeared most quickly in the treatment was 2,4-D 1 ppm treatment, where the time of callus formation was on the eighth day after induction (8 DAI). In the treatment 2,4-D0K0.5 (2,4-D 0 ppm, kinetin 0.5 ppm) can also initiate callus formation in a fast time, ie on the eighth day after induction (8 DAI).

Seen from the results of this study it is known that these two growth regulators (2,4-D and Kinetin) play an important role in the process of callus formation. Where at certain concentrations (not exceeding 1 ppm) 2,4-D growth regulators or kinetin will be able to accelerate callus formation. But on the other hand if both combinations of growth regulators are given together, in high concentrations, it will prolong the formation of callus. In accordance with existing theories, said that the factors that determine the success of tissue culture systems include nutrient composition and balance of growth regulators [13]. Callus induction is preceded by thickening of the explant in the section of the cut and the area that is injured [14], and widening to the surface of the explant [15].

The longest callus formation was found in the treatment 2,4-D0K0.5, where the callus only appeared after the eighteenth day was induced. In treatment 2,4-D0K0.5 (2,4-D 0 ppm Kinetin 0.5 ppm) and 1,4-D2K0.5 (2.4-D 2 ppm Kinetin 0.5 ppm) have the same effect on callus formation, where the new callus is formed after 13 days induced.

From the results of the analysis of variance analysis, the effect of 2,4-D administration and kinetin give a very significant effect (α> 0.01) for the time of callus formation, where H0 is rejected with the provisions if F count> F table at 99% confidence level (Table 3).

Table 3. Analysis of Variance (ANAVA) effect of Interaction between 2,4-D and Kinetin on the Time of Formation of Age 28 DAI

| Effect of Variance | Degrees of Freedom | Number of Squares | Middle Squared | Fcount | Ftable 0.05 | Ftable 0.01 |
|--------------------|--------------------|--------------------|----------------|--------|-------------|-------------|
| Main Influence     |                    |                    |                |        |             |             |
| Factor A           | 2                  | 118.16             | 59.08          | 738.50** | 3.35        | 5.49        |
| Factor B           | 2                  | 21.50              | 10.75          | 134.37** | 3.35        | 5.49        |
| 2 Factor Interactions | 4                  | 206.84             | 51.71          | 646.37** | 2.73        | 4.11        |
| Error              | 27                 | 2.25               | 0.08           |         |             |             |
| Total              | 35                 | 209.09             |                |         |             |             |

Based on the calculation results of the analysis of variance for 2,4-D GRS on callus biomass obtained F count = 78 and F table (0.01) = 5.49. Thus the effect of 2,4-D on callus biomass has a very significant effect, and for the calculation results of the analysis of variance GRS kinetin obtained F count = 62 and F table (0.01) = 5.49. So the effect of kinetin on callus biomass is very significant. Similarly, for the results of the analysis of variance analysis on the interactions of GRS 2,4-D and kinetin obtained F count = 646.37 and F table (0.01) = 4.11 thus the effect of the interaction of GRS 2,4-D and kinetin has a very significant effect with respect to the time of callus formation. For this reason, it is necessary to carry out further tests of average differences or test the treatment hypotheses that are significantly different or very real from the DMRT test. The DMRT test showed the fastest callus formation time at the GRS 2,4-D0K1 treatment (2,4-D 1 ppm and kinetin 1 ppm) showed the time of callus formation on the 8th day.
2,4-D (2,4-Dichlorophenoxyacetic acid) is a growth regulator which has better properties compared to other types of synthetic auxin, because it is more easily absorbed by plants, not easily decomposed by heating in the sterilization process and also functions to trigger morphogenic [16].

3.2. Callus Color
The color of the callus is also an indicator of callus growth. The white callus is an embryogenic tissue that does not yet contain chloroplasts, but has a high starch content [17]. Based on observations of the treatment of GRS 2,4-D and produce a variety of callus colors. The GRS 2,4-D$_0$K$_0$ (2,4-D 0 ppm and kinetin 0 ppm) results in a green callus (average score 8). The 2,4-D$_0$K$_{0,5}$ (2,4-D 0 ppm and kinetin 0.5 ppm) treatment had a greenish-white callus color (mean score 4).

**Table 4. Pineapple Callus Color Score Age 28 DAI**

| Combination of Treatment and Color  | Average Score |
|------------------------------------|---------------|
| 2,4D$_0$ K$_0$ 8 (green)           |               |
| 2,4D$_0$ K$_{0,5}$ 4 (greenish white) |               |
| 2,4D$_0$ K$_1$ 7 (Whitish green)   |               |
| 2,4D$_1$ K$_0$ 2 (Brownish yellow) |               |
| 2,4D$_1$ K$_{0,5}$ 2 (Brownish yellow) |           |
| 2,4D$_1$ K$_1$ 5 (Brownish green)  |               |
| 2,4D$_2$ K$_0$ 3 (Brownish white)  |               |
| 2,4D$_2$ K$_{0,5}$ 5 (Brownish Green) |             |
| 2,4D$_2$ K$_1$ 3 (Brownish white)  |               |

The best of Callus color is whitish green comes from 2,4-D 0 ppm and kinetin 1 ppm treatment K$_1$(average score 7). Treatment with 2,4-D and Kinetin interactions produced callus brownish yellow (average score 2). In 2,4-D$_1$K$_{0,5}$ treatment, 2,4-D$_1$K$_1$ and in 2,4-D$_2$K$_{0,5}$ treatment produced brownish green callus colors (average score 5), then for treatment 2,4-D$_2$K$_0$ and 2,4-D$_2$K$_1$ produce brownish white callus colors. The color of the callus that leads to green is a good callus because of its high cell division activity, this is indicated by the high absorption of color [18]. This callus color difference is due to changes in pigmentation [19].

The green color of the callus is due to the effect of high cytokinin concentrations which influence the formation of chlorophyll [20]. Cytokinins added in the media can inhibit the overhaul of chlorophyll grains because cytokinins are able to activate the process of metabolism in protein synthesis [21]. Callus varying color conditions caused by the presence of pigmentation, light, and plant parts that are used as a source of explants [22].

Callus that has a brownish color range means that the quality is not good. Browning that occurs in the callus due to the metabolism of phenol compounds is toxic, which is often stimulated due to explant sterilization, which inhibits growth or even causes tissue death [23] The browning event is actually a natural event and the process of adaptive changes in plant parts due to physical influences such as stripping and cutting [24]

3.3. Callus Biomass
The results of data analysis showed that the 2,4-D (Dichlorophenoxy Acetic Acid) and Kinetin treatments have a very significant effect on callus biomass. The highest amount of callus biomass was achieved in the treatment of GRS 2,4-D$_0$K$_0$ (2,4-D 0 ppm and kinetin 0 ppm) ie with an average weight of callus bimass of 1.69 gr. the smallest callus biomass in the treatment of 2,4-D$_2$K$_2$, 2,4-D 2 ppm Kinetin 0 ppm), which is weighing 0.26 gr

Observations obtained from the influence of GRS 2,4-D and Kinetin on callus biomass aged 28 DAI can be seen in Figure 1 below;
The resulting biomass is very dependent on the speed of these cells to divide, multiply and continue with the enlargement of the callus [25]. Auxin growth regulator substances and cytokines given at the right ratio can initiate cell division and promote cell growth. The effect of auxin on tissue growth is thought to induce secretion of H+ ions out through the cell wall. Acidification of the cell wall causes K+ to be taken, this uptake reduces the water potential in the cell; As a result, water easily enters the cells and the cells will expand [26,27].

3.4. **Callus Stack Height**

Callus pile height is one of the important variables in observing plant multiplication [24]. Based on research that has been done, it is known that the highest callus stack is found in 2,4-D0K0 treatment with a callus stack height of 2.32 cm. meanwhile the lowest callus stack was found in the 2,4-D2K0,5 treatment, with a callus stack height of 1.07 cm. Based on the results obtained, the highest stack of callus is shown in combination of 2,4-dichlorophenoxy acetic acid at 0 ppm with 0 ppm Kinetin as well. (2,4-D0K0 treatment). The second highest callus stack was found in a combination of 2,4-dichlorophenoxy acetic acid treatment with a concentration of 0 ppm and a kinetin concentration of 1 ppm.

Figure 2 also shows that increasing the ppm concentration of 2,4-Dichlorophenoxyacetic acid will cause a reduction in callus stacks. High concentrations of 2,4-D can inhibit explant growth because it exceeds the optimum concentration for explant growth [16]. in the 2,4-D2K1 treatment, callus height was 1.17 cm. this is the second lowest callus stack after 2,4-D2K0,5 treatment.

The observations obtained from the influence of 2,4-D and Kinetin on callus stack height at the age of 28 DAI can be seen in Figure 2 below;
Figure 2. Average graph height of callus stacks (2,4-D,K0 treatment = 2,4-D 0 ppm K 0 ppm; 2,4-D,K0,5 treatment = 2,4-D 0 ppm K 0,5 ppm; 2,4-D,K1 treatment = 2,4-D 0 ppm K 1 ppm; 2,4-D,K0,5 treatment = 2,4-D 1 ppm K 0,5 ppm; 2,4-D,K1 treatment = 2,4-D 1 ppm K 1 ppm; 2,4-D,K0 treatment = 2,4-D 2 ppm K 0 ppm; 2,4-D,K0,5 treatment = 2,4-D 2 ppm K 0,5 ppm; 2,4-D,K1 treatment = 2,4-D 2 ppm K 1 ppm)

4. Conclusions
In this study, it was found that Dichlorophenoxy Acetic Acid (2,4-D) and Kinetin significantly influenced the time of callus formation, callus color, callus biomass, and significant effect on the height of callus pile. The time of callus formation is the fastest on day 8 after induction, and the best Callus color is whitish green comes from 2,4-D 0 ppm and kinetin 1 ppm treatment. The highest biomass (1.69 gr), and the highest of stack height (2.32 cm), derived from the treatment of 2,4-D 0 ppm and kinetin 0 ppm.

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