The effect of activated charcoal dose and benzyl amino purine concentration on the growth of orchid plantlets in Murashige and Skoog media in vitro

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Abstract. Indonesia has a high variety of orchids. In vitro propagation of orchids was strongly influenced by the composition of the medium used. This study aims to conduct the effect of activated charcoal and BAP concentration on the growth of orchid subcultures in Murashige and Skoog Media. The experimental design used was a Completely Randomized Design (CRD) with two factors. Benzyl Amino Purine concentration (0 ml/l, 0.25 ml/l, 0.5 ml/l, and 0.75 ml/l) and 0 g/l, 1 g/l, and 1.5 g/l. The results showed that BAP concentrations up to 0.75 ml/l at the age of 8 mst had a significant effect on root length, but did not significantly affect the number of leaves, root number, and plantlet height. The activated charcoal up to 1.5 g/l does not have a significant effect on the number of leaves and number of sprouts at the age of 2-7 mst, but at the age of 8 mst was significantly affect the number of roots, root length, and plantlet height.

Keyword: Tissue culture, BAP, activated charcoal, orchid seeds, in vitro

1. Introduction
Orchid is an ornamental plant that has a high aesthetic value. The unique shape and color of the flower is the main attraction that attracts many people. Besides of its high aesthetic value, orchids also have high economic value compared to another ornamental plants. The variety of colors and shapes of orchid flowers is an important factor in orchid plants, when it becomes more unique and rare, thus the economic value is higher. Therefore, the cultivation of orchid plants is a good effort to increase society income [1-2]. However, it should be known that there are some challenges and obstacles to fulfill the orchid needs, especially the cultivation process. The orchid breeding can be done vegetatively or generatively. Vegetative propagation of vegetation is considered to be less effective because the number of tillers produced is limited, while generative breeding is inhibited because the orchid seeds are difficult to germinate. The problems of the difficulty of breeding orchids conventionally, make culture techniques as an alternative to large-scale orchid propagation. The use of this technique can provide the large quantities of orchid seedlings and can be done in a relatively short time with guaranteed quality [3-5].
In tissue culture, the role of growth regulators greatly helps to accelerate the formation of the callus because the growth substances are the important factors in influencing the differentiation process in all phases of growth and development. Thus, the knowledge of growth regulating substances will greatly affect the smoothness and success of tissue culture work [6-7]. Benzyl Amino Purines (BAP) are cytokines. Cytokines are plant hormones derived from adenine to stimulate cell division and mitotic differentiation, and it was synthesized and translocated through xylem vessels [8-9]. BAP application is to stimulate the growth of sprout in tissue culture or in plants, but it is often nonoptimal for mature plants.

Cytokines have adenine-like structures that promote cell division and have other similar functions to kinetin [10-11]. In vitro orchid culture usually uses media that was supplemented with activated charcoal or carbon that can absorb toxic in media or inhibitor compounds, stabilizing the pH of the media, measuring growth by reducing the amount of light entering the media, and demonstrating morphogenesis. In addition, activated charcoal can reduce the browning of the media because the high heating during the sterilization process. The addition of activated charcoal can help the growth and development of culture because it adsorbs toxic compounds contained in the media [12-13]. From the description, the authors were interested in conducting research on the effect of activated charcoal dose and BAP concentration on the growth of orchid plantlets in Murashige and Skoog media in vitro.

2. Methods
The research was conducted at the UPT Tissue Culture Laboratory, BBI Gedung Johor Dinas Pertanian, Sumatera Utara. The study was conducted on January 6th to March 6th 2018. Orchid plantlet used was 4-month-old plantlets with MS medium, sucrose / sugar, agar, BAP, activated charcoal and distilled water. The sterilization materials were NaOCl2, 96% alcohol and detergent. The equipment used was autoclave, laminar air flow cabinet (LAFC), pH-meter, gas stove, culture bottle, 100 cc beaker, 500cc beaker, drop pipette, hand-sprayer, 500 cc sterilizing bottle, funnel, growth rack, sterilization oven, analytical scales, measuring cups, erlenmeyer, petridis dishes, test tubes, air conditioners, aluminum foils, refrigerators, calculators, stationery, and fluorescent lamps.

The culture bottle used was first cleaned with detergent, and then it was soaked with clorox which was mixed with water for ± 3 hours. Then the bottles were oven at 150 oC for ± 4 hours. LAFC was sterilized first with 96% alcohol, 96% alcohol was sprayed to LAFC and then ultraviolet (UV) for ± 60 minutes. Bottles containing MS media were put into the autoclave and then locked by regulating the temperature of 121 °C and pressures of 15-17.5 psi (pounds per Square) for 1 hour. After the pressure drops to 0 psi, the lock was opened and the pan containing the media was removed. Then, sterilizing the work environment by sweeping the inner surface of the LAFC sprayed with 96% alcohol and then ultraviolet for ± 6 hours.

Solvent of stock A was made by weighing 165 g of NH4NO3 and dissolving it in 1000 cc of distilled water, while solvent of stock B was made by weighing 190 g of KNO3 and dissolving it in 1000 of distilled water. Then, solvent of stock C was made by weighing 170 g K2HPO4, 6.2 g H3BO3, 0.25 g Na2MoO4.2H2O, 0.025 g COCl2.6H2O, 0.83 g KI and dissolving it in 1000 cc distilled water. Solvent of stock D was made by weighing 44 g of CaCl2.2H2O and dissolving it in 1000 cc of distilled water. Solvent of stock E was made by weighing 37 g MgSO4.7H2O, 2.23 g MnSO4.4H2O, 0.86 g ZnSO4.7H2O, 0.0025 g CuSO4.5H2O and dissolving it in 1000 cc of distilled water. Solvent of stock F was made by weighing 3.73 g Na2EDTA, 2.78 g FeSO4.7H2O and dissolving it in 1000 cc of distilled water. Solvent of stock E was made by weighing 37 g MgSO4.7H2O, 2.23 g MnSO4.4H2O, 0.86 g ZnSO4.7H2O, 0.0025 g CuSO4.5H2O and dissolving it in 1000 cc of distilled water. Solvent of stock F was made by weighing 3.73 g Na2EDTA, 2.78 g FeSO4.7H2O and dissolving it in 1000 cc of distilled water. Solvent of stock F was made by weighing 3.73 g Na2EDTA, 2.78 g FeSO4.7H2O and dissolving it in 1000 cc of distilled water. The media which was prepared should be weighted with the number of combination treatments, namely 12 combinations and from the combination 36 units were obtained. Next, add 15 g of agar, 30 g of sugar, and BAP and activated charcoal into each treatment. Then, the media was heated with hot plate until boiling. After boiling, the pH of the medium was measured with litmus paper and weighted between 5.6-5.8. If the pH was under the pH, thus add 0.1 N NaOH. Next, if the pH was up to the pH, then add HCl 0.1 N. Next, the media was poured into culture bottles and filled with ± 20 ml of media. Then the bottle was closed tightly with aluminum foil.

After that, the media was sterilized by autoclave at 121°C and 15 psi for 15 minutes. Media that had been sterile, then stored in a culture room and left for 3 days, if there is no contamination, the
media can be used. From the culture bottles, the large and high orchid plantlets were taken and then it was transferred to MS media bottles. Furthermore, closed tightly with a culture tube and strip in order to minimize the contamination. The hands should be sprayed with 70% alcohol before touching the LAFC. After it was done, then turned off the bunsen quickly and remove the materials and tools from LAFC. After that, the LAFC was sprayed again with 70% alcohol. When it was done, all the bottles containing the Orchid plantlets were placed in the culture culture room.

To avoid the contamination, the culture room should be sprayed with alcohol every day. Then LAFC was turned off and the door was closed. To avoid contamination of the inoculated plants, the culture room and culture bottle were sterilized with 96 alcohol with hand sprayer every day. The bottles that contaminated were immediately removed from the culture room, so it would not spread to another bottles. The parameters observed were the percentage of living plantlets, number of leaves, number of sprouts, number of roots, root length, and plantlet height. The flowchart of the research can be seen in Figure 1 below.

![Figure 1. Research flow chart.](image)

3. Results and Discussion

The treatment of BAP concentration had no significant effect on the number of plantlet leaf at the ages 2-7 weeks, as shown in Table 1 below. At the age of 8 weeks, the highest number of leaves was found in treatment B3. The number of leaves between treatments B0, B1 and B2 was not too different. In the treatment of active charcoal, it was not give significant effect on the number of leaves. At the age of 8 mst, the highest number of leaves was found on A1 treatment. In the given of activated charcoal, it had no significant effect on leaf sprouts. At the age of 8 mst, the highest number of sprout was found in A2 treatment.

| Treatment | 2 mst | 3 mst | 4 mst | 5 mst | 6 mst | 7 mst | 8 mst |
|-----------|-------|-------|-------|-------|-------|-------|-------|
| B0        | 0.00  | 0.67  | 1.33  | 1.56  | 2.11  | 2.78a | 3.00a |
| B1        | 0.00  | 0.67  | 1.44  | 2.11  | 2.56  | 3.22ab| 4.00b |
| B2        | 0.33  | 0.78  | 1.56  | 2.33  | 2.67  | 3.44ab| 4.22b |
| B3        | 0.11  | 1.00  | 1.44  | 2.00  | 2.67  | 4.00b | 4.33b |
| BNJ0.05   | -     | -     | -     | -     | -     | 1.04  | 0.97  |

| Treatment | 2 mst | 3 mst | 4 mst | 5 mst | 6 mst | 7 mst | 8 mst |
|-----------|-------|-------|-------|-------|-------|-------|-------|
| A0        | 0.17  | 0.67  | 1.50  | 2.00  | 2.75  | 3.08  | 3.42a |
| A1        | 0.08  | 0.83  | 1.58  | 2.00  | 2.58  | 3.42  | 4.00ab|
| A2        | 0.08  | 0.83  | 1.25  | 2.00  | 2.17  | 3.58  | 4.25b |
| BNJ0.05   | -     | -     | -     | -     | -     | -     | 0.76  |
The treatment of BAP concentration had no significant effect on the number of roots, but there was a tendency to decrease the number of plantlets by increasing BAP. It was effected by BAP that could inhibit plantlet root growth. In the given of activated charcoal, the highest number of roots was found in A2 treatment. At BAP concentrations the longest plantlets that were found in B0 treatment were significantly different from B3 and B2, but were not significantly different from B1. The root length in treatment B1, B2 and B3 was not real, since cytokines play a crucial role in increasing sprout growth and suppressing root growth. In the given of activated charcoal, the longest root plantlets that were found in A2 treatment were significantly different from A0, but not significantly different from A1. The root length in treatment B1, B2 and B3 was not real, since cytokines play a crucial role in increasing sprout growth and suppressing root growth. In the given of activated charcoal, the longest root plantlets that were found in A2 treatment were significantly different from A0, but not significantly different from A1. In BAP treatment, the effect on plantlets height was not significant, but there was a tendency to increase the plantlets height and BAP concentration. It was thought because of the unbalanced ratio of auxin and cytokines, so that it could affect the growth of plantlets. Average number of roots, length of root and height of plantlet cattleya orchid of treatment of BAP and activated charcoal at 8 weeks can be seen at Table 2, as below.

| Parameter | Treatment | A₀  | A₁  | A₂  | Average |
|-----------|-----------|-----|-----|-----|---------|
| Number of Roots | B₀       | 2.03| 1.77| 2.03| 1.94    |
|            | B₁       | 1.39| 1.81| 2.00| 1.73    |
|            | B₂       | 1.17| 1.86| 2.11| 1.71    |
|            | B₃       | 1.10| 1.76| 1.77| 1.54    |
|            | B₀       | 1.18| 1.30| 1.31| 1.27ab  |
| Root Length | B₁       | 0.81| 1.32| 1.42| 1.18ab  |
|            | B₂       | 0.81| 0.91| 1.29| 1.00a   |
|            | B₃       | 0.77| 1.03| 1.12| 0.97a   |
|            | B₀       | 0.97| 1.10| 1.27| 1.11    |
| High Plantlet | B₁     | 0.73| 1.23| 2.03| 1.33    |
|             | B₂     | 1.03| 1.53| 1.63| 1.40    |
|             | B₃     | 1.10| 1.57| 1.70| 1.46    |

Figure 2a showed that the higher of the concentration of activated charcoal, the number of roots of orchid plantlets would increase and followed the linear regression curve. It was related to the nature of activated charcoal which was a strong absorbent. Based on the research [20-21], the addition of activated charcoal could help the growth and development of culture, depending on the type of culture. The given of activated charcoal in growing media showed the highest number of root growth. It was effected of activated charcoal in growing media that could reduce the light entering the media. Low light intensity could stimulate endogenous growth substances to work actively in the process of root growth and development. Based on the Figure 2b, it can be seen that the higher of the concentration of BAP, thus the length of the roots of the Cattleya orchid plantlets was shorter. It was effected by the cytokines that would suppress the formation of plantlets. Plantlet root growth was strongly influenced by the relatively high presence of auxin ZPT. The condition of ZPT was regulated by a high ratio of auxin from cytokines. High cytokinin concentrations would inhibit plantlet root formation or growth [22]. From the Figure 2c, it can be seen that the higher of the concentration of activated charcoal, the length of the roots of the Cattleya orchid plantlets would increase and followed the linear regression curve. It was effected by the activated charcoal that could reduce the intensity of light, thus it increased the growth of the plantlet root length [23-25]. The bright conditions have a significant effect on the improvement of plantlet regeneration capabilities. Auxin in plant tissues could work actively even in dark conditions, but auxin synthesis occured in a bright conditions. Auxin plays a crucial role in root formation and growth.

From the Figure 2d, it can be seen that the higher of the concentration of activated charcoal, the length of the roots of the Cattleya orchid plantlets would increase and followed the linear regression...
curve. It was effected by the activated charcoal that could reduce the intensity of light. Thus it increased the growth of the plantlet root length, to improve plantlet regeneration capabilities. Auxin in plant tissues could work actively even in the dark, but auxin synthesis occurred in the presence of algae. Auxin plays a crucial role in root formation and growth. Relationship between the given of activated charcoal with the number of roots of Cattleya orchid plantlets can be seen Figure 2.

![Graph](image)

Figure 2. Relationship between the given of activated charcoal with the number of roots of Cattleya orchid plantlets

Figure 2a is the relationship between the given of activated charcoal with the number of roots of Cattleya orchid plantlets at the age of 8 MST. 2b is the relationship between the concentration of BAP with the root length of Cattleya orchid plantlets at the age of 8 MST. 2c is the relationship between the given of activated charcoal with the root length of the Cattleya orchid plantlets at the age of 8 MST. 2d is the relationship between the given of activated charcoal and Cattleya orchid plantlets at the age of 8 MST. The results showed that all plantlets were grown as a whole, both those that were not given growth regulating substances or those that were given growth regulators, as well as MS media that were given activated charcoal or those that were not given activated charcoal. It showed that by using growth regulators, endogenous plant could grow well. The results showed that the treatment of BAP growth regulators had no significant effect on the number of leaves and sprouts at the age of 2-7 MST, but significantly affected at the age of 8 MST, and it was not significantly effect on the number of roots and plantlet height, but significantly affected the root length. The results showed that the given of BAP up to 0.75 ml/l could increase the growth of Cattleya orchid plantlets on MS media in vitro.

It was effected by the cytokines that could increase cytokinesis in plant cells consisted of one hydrophilic group with high specificity (adenine) and one lipophilic group without specificity. Cytokines affected various physiological processes in plant cells. Based on the research [28], the given of BAP growth regulators to a concentration of 3.5 mg/l could increase the percentage of plantlets to grow normally, and increased the number of leaves and length of sprouts, but the given of 3.5 mg could reduce the number of leaves and sprout length. The results showed that the given of BAP could increase the plantlet height growth. Since BAP could stimulate the cell division process to become faster, so that it would spur the growth process of plantlet. The most typical trait associated with cytokines was stimulation of cell division in plant tissue culture. The discovery and separation of kinetin depends on nature and some studies of cytokines emphasize this effect. Non-meristem tissues separated from higher plants for in vitro growth that required cytokinins [29]. The results showed that the treatment of activated charcoal dose had no significant effect on the number of leaves and shoots at 2-7 MST, but had a significant effect at the age of 8 MST, and it had significant effect on the number of
roots, root length and plantlet height. The results of the study showed that the given of acitive charcoal could increase the growth of orchid plantlets. It was effected by the compounds from phenol oxidation that could give toxic to plants and inhibit the growth and differentiation process. To suppress the release of phenol compounds, the culture medium was given an active charcoal compound. But the activated charcoal compounds also absorbed the other compounds that were beneficial for plant growth. Besides that, the given of activated charcoal was not only absorbs toxic compounds, but also absorbed the other organic materials, such as auxins. Activated charcoal could absorb phenol compounds that come out of plant tissues, while it was absorbing other organic materials in the media [30]. The results showed that the given of activated charcoal up to 1.5 g / l could increase the growth of orchid plantlets. It was effected of activated charcoal functions as a nutrient absorber and because it has a very large surface area. In active charcoal tissue culture plays a crucial role in absorbing toxins or inhibitor compounds secreted by plantlets into the media, but it was used to absorb nutrients contained in the media, so that they could not be used optimally by cultured explants. The research finding showed that the interaction between BAP treatment and activated charcoal had no significant effect on all observed parameters. It was effected by BAP for cytokines which play a crucial role in sprout formation in plantlets, while activated charcoal was to absorb compounds that was produced by oxidation of phenol compounds which gave toxic to plants. Plantlet growth was more influenced by the balance of growth regulators auxin and cytokinins in plant tissues.

The number of leaves had a significant positive correlation with the number of sprouts and plantlet height, but was not significantly correlated with the number of roots and root length. The number of sprouts correlated significantly positively with plantlet height, but was not significantly correlated with the number of roots and root length. The number of roots had a significantly positive correlation with root length, but was not significantly correlated with plantlet height. Then, the root length was not significantly correlated with plantlet height. It means that the enhancement of the number of leaves would be followed by the enhancement in the height of the plantlets. The higher of the number of sprouts, the plantlets height would be higher, as shown in Table 3. below

| Parameter | LM | NS | NR | RL | HP |
|-----------|----|----|----|----|----|
| LM        | 1  |    |    |    |    |
| NS        | 0.85* | 1  |    |    |    |
| NR        | 0.04tn | -0.04tn | 1  |    |    |
| RL        | 0.09tn | 0.00tn | 0.84* | 1  |    |
| HP        | 0.70* | 0.61* | 0.53tn | 0.49tn | 1  |

Information:
\[ r_{0.05} = 0.58 \]
NR = Number of Roots
LM = Leaf Amount
NS = Number of Shoots
RL = Root Length
HP = High Plantlet

4. Conclusion
From the results of the study, it can be concluded that: (i) the concentration of BAP up to 0.75 ml/l had no significant effect on the number of leaves and number of sprouts at the age of 2-7 mst, but significantly affected at the age of 8 mst, and significantly affect the root length, but it had no significant effect on the number of roots and plantlet height. The higher of BAP concentration, the
number of leaves, the number of sprouts, and the number of roots that was increased, while the root length was shorter, (ii) the active charcoal dose up to 1.5 g/l did not significantly affect the number of leaves and the number of sprouts at 2-7 mst, but significantly effect on the age of 8 mst. The it was significant effect on the number of roots, root length, and plantlet height. The higher the activated charcoal dose, the number of leaves, number of shoots, root length and plantlets height increased. (iii) the interaction between BAP concentration and activated charcoal dose has no significant effect on all observed parameters.

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