Micropropagation of Red Ginger (Zingiber officinale Rosc. Var. Rubrum) Using Several Types of Cytokinins

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Abstract. One of the medicinal plants that is widely cultivated is the red ginger (Zingiber officinale Rosc. Var. Rubrum). The plant contains an active compound gingerol that is used as an ingredient for various treatments such as cough and flu. To meet the demand of medicinal and industrial raw materials, quality ginger seeds are needed. One alternative to producing seeds is to use tissue culture technology. This study aimed to obtain the best type and concentration of cytokinins in increasing the multiplication of red ginger shoots in vitro. This study used a factorial Completely Randomized Design (CRD) with 2 factors, namely the type of cytokinins (BAP, thidiazuron, zeatin, kinetin, and 2ip) and cytokinin concentrations (0, 0.1, and 1 ppm). The results showed that 1 ppm thidiazuron treatment produced the highest number of shoots and the highest shoot length in the first subculture. The responses in the second subculture showed that shoots from thidiazuron, 2ip, and BAP treatment media produced the highest number of shoots, roots, and leaves compared to kinetin and zeatin. Multiplication continued until the sixth subculture, and the best multiplication was found on shoots from 2ip treatment.

Keyword: BAP, 2ip, thidiazuron, multiplication, medicine, subculture

1. Introduction
Red ginger (Zingiber officinale Rosc. Var. Rubrum) is one of 3 varieties of ginger (emprit ginger, elephant ginger, and red ginger). Red ginger is widely used for spices and medicines. Gingerol and shogaol are the main ingredients of flavonoids in ginger. These compounds have antioxidant effects that can prevent the presence of free radicals in the body [1].

The benefits of red ginger include carminative (fart releasing), seizure reliever, anti-hardening of blood vessels, sweat laxative, anti-inflammatory, anti-microbial and parasitic, anti-pyretic, anti-rheumatism, and stimulates the release of gastric juice and bile [2]. Red ginger is also effective in reducing the intensity of joint pain in the elderly [3], reducing uric acid levels [4], and lowering cholesterol levels [5].
Red ginger has been used in various beverage products [6, 7], meat canning industry, confectionery, tobacco processing, and soap making [8]. The current high demand for red ginger has not been matched by an increase in red ginger production. The harvested area for red ginger in 2017 to 2018 has decreased by 3.32% [9]. One of the factors causing the decline in the production is the difficulty in getting a source of red ginger seeds.

One way to get quality seeds from superior breed stock is by using tissue culture technology. An important stage in tissue culture technology is the multiplication stage. The more shoots that are produced, it is hoped that the more ginger seeds will be produced. In principle, multiplication media requires nutrients, vitamins, energy sources, and growth regulators. Types of growth regulators to induce shoots are cytokinins. The addition of cytokinins in tissue culture has been shown to increase shoot multiplication. Cytokinins are one of the growth regulators (ZPT) that play an important role in regulating cell division and influencing shoot differentiation [10]. Each plant has a different response ability to growth regulators, so the right type of cytokinins is needed to obtain optimal shoot multiplication [4].

Several earlier studies on tissue culture of red ginger have reported that the combination of BAP 4 ppm and NAA 3 ppm is the medium that forms the most shoots [11]. According to another study, the accumulation of endogenous auxins from ginger is sufficient for in vitro propagation so that it does not require additional auxins [12]. The addition of the right concentration and type of cytokinins can support the production of red ginger in large quantities and in short time, and suppress the occurrence of somaclonal variations. This study aims to obtain the best type of cytokinins and concentrations in increasing the multiplication of red ginger shoots in vitro.

2. Materials and Methods

2.1. Plant Material

The plant material used as explants was red ginger shoots from Sukabumi, West Java, Indonesia. Red ginger rhizome was moisturized to produce buds. The buds were isolated and sterilized using a 70% alcohol solution, sodium hypochlorite, providone iodine, fungicides, bactericides, and antibiotics. Sterile bud explants were used as the source material in this study.

2.2. Media Preparation

The culture medium used was the basic MS medium [13]. The treatment medium consisted of MS base medium added with growth regulators for the cytokinin group. The types of cytokinins added to the treatment medium were BAP, thidiazuron, zeatin, kinetin, and 2ip. The concentrations of growth regulators added were 0 (control), 0.1 ppm, and 1 ppm. MS medium was also prepared without the addition of growth regulators (MS0). MS0 media was prepared for planting the second to the sixth subcultures. A tube was used for culture container with a medium volume of 12 mL for each tube. The media was sterilized at 121 °C for 20 minutes. After having cooled to room temperature, the media were stored in a room without light until use.

2.3. Planting and subculture

Sterile red ginger explants were selected based on their size (uniform explant material). Subsequently, explants were planted in the treatment medium. Each treatment was planted 5 buds (replicates). The explants that had been planted in the treatment medium were incubated in a culture room with a light intensity of about 1500 lux for a long spraying time of 16 hours, at a temperature of 24-25 °C. The first culture explants were incubated for 6 weeks. Then, the explants in the second subculture were planted on media without growth regulators (MS0) and incubated for 4 weeks. Subcultures were then carried out repeatedly every 6 weeks, until the sixth subculture in MS0 media.

2.4. Observation and analysis

The experimental design used was factorial Completely Randomized Design (CRD) with two factors. The first factor was a type of cytokinin, in which 5 cytokinins were used (BAP, thidiazuron (TDZ), kinetin, zeatin, and 2ip). The second factor was the variation of cytokinin concentrations consisting of
3 levels (0, 0.1, and 1 ppm) and each treatment was repeated 5 times. Observations at the first subculture stage on the treatment media were conducted at 6 WAP (Week After Planting). Observation parameters were the number of shoots, shoot length, and the number of roots. The second subculture observation on media without a growth regulator (MS0) was observed at 4 WAP. The parameters of the observations carried out were the percentage of viability, sprouting, roots, and leafy shoots. Observations in the sixth subculture on media without growth regulators (MS0) were carried out at 6 WAP. The parameters observed were the number of shoots and shoot height. The data obtained from all observation parameters at the first subculture stage in the treatment media were tested by Analysis of Variance (ANOVA) with Two-way Analysis of Variance followed by DMRT test at the 5% level. The second and sixth subculture data were analyzed descriptively.

3. Results and Discussion

3.1. First subculture of red ginger explant in treatment media

In general, red ginger explants formed and experienced root and shoot lengthening starting from the age of 1 week after planting (WAP). The results of statistical analysis showed that the interaction between types and cytokinin concentrations only had an effect (sig <0.05) on shoot length. Thidiazuron treatment resulted in a higher number of shoots and the highest shoot length compared to other cytokinins at the 6th week after planting (Table 1).

This showed that the growth regulator thidiazuron affected not only cell division but also cell elongation. According previous study [14], thidiazuron is a synthetic phenylurea type cytokinin which has a better ability to induce shoots than other cytokines such as zeatin, BAP, and kinetin.

The growth regulator 2ip was the second type of cytokinin that showed the highest average number of shoots and the highest shoot length. The addition of 2ip as much as 1 ppm was a treatment with the third highest average number of shoots after thidiazuron 1 ppm and thidiazuron 0.1 ppm. Previous study showed that 2ip produced the highest number of shoots and shoot lengths compared to BAP, kinetin, and thidiazuron in the Sophora tonkinensis multiplication [15].

Table 1. Average number of shoots, shoot length, and number of roots in the first subculture

| Type of Cytokinin | Concentration (ppm) | Number of Shoot | Shoot Length | Number of Roots |
|-------------------|---------------------|----------------|--------------|----------------|
|                   | 0                   | 0.2<sup>a</sup> | 0.04<sup>a</sup> | 0.4<sup>ab</sup> |
| BAP               | 0.1                 | 1.4<sup>abcd</sup> | 0.72<sup>ab</sup> | 0.6<sup>ab</sup> |
|                   | 1                   | 1.2<sup>abc</sup> | 0.70<sup>ab</sup> | 0.4<sup>ab</sup> |
| Thidiazuron       | 0                   | 0.2<sup>a</sup>  | 0.04<sup>a</sup> | 0.4<sup>ab</sup> |
|                   | 0.1                 | 2.0<sup>cd</sup> | 1.58<sup>b</sup> | 2.4<sup>b</sup> |
|                   | 1                   | 2.8<sup>d</sup>  | 2.96<sup>c</sup> | 0.4<sup>ab</sup> |
| Zeatin            | 0                   | 0.2<sup>a</sup>  | 0.04<sup>a</sup> | 0.4<sup>ab</sup> |
|                   | 0.1                 | 0.6<sup>bc</sup> | 0.3<sup>a</sup>  | 1.4<sup>ab</sup> |
|                   | 1                   | 0.4<sup>ab</sup> | 0.08<sup>a</sup> | 0<sup>a</sup>  |
| Kinetin           | 0                   | 0.2<sup>a</sup>  | 0.04<sup>a</sup> | 0.4<sup>ab</sup> |
|                   | 0.1                 | 0.8<sup>abc</sup>| 0.34<sup>a</sup> | 1.0<sup>ab</sup> |
|                   | 1                   | 0.4<sup>ab</sup> | 0.12<sup>a</sup> | 0<sup>a</sup>  |
| 2ip               | 0                   | 0.2<sup>a</sup>  | 0.04<sup>a</sup> | 0.4<sup>ab</sup> |
|                   | 0.1                 | 1.8<sup>abcd</sup>| 0.58<sup>ab</sup> | 1.0<sup>ab</sup> |
|                   | 1                   | 1.6<sup>abcd</sup>| 0.94<sup>ab</sup> | 1.8<sup>ab</sup> |

Note: The numbers followed by the same letter in the same column show no significant difference based on the DMRT test at the 95% confidence level.
BAP was the third type of cytokinin that showed the highest average number of shoots and the highest shoot height in the sixth week after planting. BAP was thought to be able to form shoots faster and more if the concentration was higher or in combination with other growth regulators. BAP was demonstrated in previous study to be capable to form shoots and leaves faster, the number of shoots and leaves was higher after being combined with IAA [16]. According to earlier finding [17], BAP with high concentration reduced shoot length. BAP does not function to stimulate shoot height but has a more role in cell division [18].

Kinetin and zeatin caused no significant changes compared to the other two growth regulators. Previous research found that the use of kinetin was effective for the multiplication of ginger shoots but must be combined with other cytokinins, namely BA [19]. Another study also showed that 0.1 ppm zeatin was able to produce the highest number of shoots in turmeric explants when combined with 1 ppm NAA [20]. The results showed that the increase in cytokinin concentrations could increase the number of shoots produced. Thus, the higher the concentration of cytokinins given to the plant, the higher the number of shoot [21]. Thus, it was suggested that the endogenous cytokinin content of red ginger was very low so that for the induction of the shoots, additional exogenous cytokinins were needed.

All treatments were able to form roots except at 1 ppm kinetin. Media without the addition of cytokinins is better in root formation [22]. This is because cytokinins can inhibit endogenous auxin biosynthesis in forming roots. However, it was different in red ginger culture, because the roots appeared in all treatments. Roots continued to grow in the medium without cytokinins or in the medium with cytokinins. This is because cytokinins function to stimulate shoot growth and can stimulate root growth when combined with exogenous auxins. This situation occurs presumably due to the high content of endogenous auxin ginger in the explants.

All treatments as shown in Table 1 produced new shoots with different numbers. The height of the bud was not visible in all treatments, the height of the shoot was still very short. To maximize growth and see the response that arose, each shoot produced was separated and further sub-cultured to the media without growth regulator MS0. This was done to ascertain whether the stunted growth in the red ginger culture in the first subculture was affected by the added growth regulator, so it required neutral conditions to trigger its growth.

3.2. Second subculture of red ginger explants on media without growth regulators
The red ginger explants that had been induced in vitro for 6 weeks were then removed from the culture tube. The explants that had sprouted were then separated and grouped based on bud class. This grouping was to see the response of shoots that were sub-cultured on MS0 media. The shoots grouped by class (Figure 1) were then sub-cultured to MS0 media and observed for 4 weeks. The results of the second subculture based on shoot class showed that class 3 shoots had the highest percentage in each parameter (Figure 2). This showed that the red ginger shoots separated from the parent explants could survive if they are > 1 cm in size.

**Figure 1.** Morphology of red ginger explants based on shoot class: (a) Class-1 (0.1-0.5 cm); (b) Class-2 (0.6-1 cm); and (c) Class-3 (> 1 cm)
Figure 2. Results of the second subculture of red ginger explants on media without growth regulators at 4 weeks after planting.

Figure 3. Average number of shoots, leaves, and roots formed based on the previous subculture treatment media at 4 weeks after planting.
Class-I shoots were the class with the lowest percentage of life. These results indicated that shoots with a size of 0.1-0.5 cm had not been able to survive when separated from the parent shoots. Shoots with a size of 0.1-0.5 cm mostly still needed a food source from the parent shoots. This could be seen from the change in the color of the explants per week, starting from brownish then blackening to drying. Although some survived, the explants did not develop. This result showed that smaller explant size caused a longer growth time.

The response of shoot growth in the second subculture was still influenced by the previous originating treatment media. Shoots originating from different treatment media in the first subculture showed a different response in the second subculture (Figure 3). The highest number of shoots was produced by explants of red ginger shoots from 0.1 ppm BAP media treatment (Figure 4a), 1 ppm thidiazuron (Figure 4d), and 0.1 ppm 2ip (Figure 4e). The number of shoots that appeared in the second subculture was thought to be due to the presence of an exogenous growth regulator that was given, stored in the culture, and began to be expressed when the media was neutral or without growth regulators. The expression that emerged was the formation of more shoots than the first subculture. The response to the second subculture originating from 0.1 ppm BAP and 1 ppm thidiazuron treatment media produced the highest average number of shoots.

The pre-treatment medium added with 1 ppm thidiazuron produced the most shoots and leaves in the second subculture. This happens because thidiazuron has a more stable structure than other types of cytokinins. Thidiazuron can last longer in plant tissue so that it continues to express itself even though it has been transferred to media without growth regulators [14]. Thidiazuron is a cytokinin that has a higher biological activity than BAP. Thidiazuron is widely used in tissue culture and gives the most optimal multiplication response. Thidiazuron cytokinin application gave a positive response to the multiplication of Vanda Douglas orchid cultures [23], and satoimo culture [24]. Thidiazuron also produced the highest number of leaves in banana microshoot multiplication compared to kinetin, BAP, and 2ip [25]. It was shown that the combination of thidiazuron 10 ppm and 1 ppm NAA could induce Phalaenopsis orchid leaves through somatic embryogenesis and produced many new shoots [26].

![Figure 4](image-url)

**Figure 4.** Red ginger shoots in the second subculture, 4 weeks after planting, the origin of the treatment media: (a) BAP 0.1 ppm (b) BAP 1 ppm (c) thidiazuron 0.1 ppm, (d) thidiazuron 1 ppm (e) 2ip 0.1 ppm, and (f) 2ip 1 ppm.
Red ginger shoots capable of forming roots with the highest average number of roots were those treated with BAP 1 ppm (Figure 4b), thidiazuron 1 ppm (Figure 4d), and thidiazuron 0.1 ppm (Figure 4c). The explants that had formed shoots were mostly able to produce roots, this was thought to be due to the presence of endogenous auxins in the culture of red ginger.

Based on the results of data collection and observations, it was found that in the red ginger culture there was a delay in response when treated with growth regulators and began to respond when conditions were neutral. These results indicated the presence of exogenous hormone storage in the cells, which began to be expressed in subsequent subcultures. The expression of multiple shoots was continuously observed in the sixth subculture.

3.3. The sixth subculture of red ginger explants on media without growth regulators

The red ginger culture from the second planting was then carried out with further subcultures. Subcultures were performed every six weeks. In the further subcultures, not all treatments could be sub-cultured due to contamination. The cultures that were saved in the third subculture were those treated with BAP 0.1 ppm, kinetin 0.1 and 1 ppm, thidiazuron 0.1 and 1 ppm, zeatin 0.1 ppm, and 2ip 1 ppm. Subculture was continued until the sixth subculture and was observed. The results of the observation shown in Table 2, which were limited to the number of shoots and shoot height. Observation of shoot height was meant to ensure that the shoots were ready to be acclimatized.

The results of the observation of the sixth subculture showed that the average number of shoots was not the same for each treatment. The average number of shoots from the initial treatment of BAP, kinetin, and thidiazuron at a concentration of 0.1 ppm resulted in 3.7-4.9 shoots per tube except for zeatin treatment. The number of shoots produced from the zeatin treatment resulted in 1-2 fewer shoots per tube. Likewise, the results of observation of shoot height varied. The average shoot height was about 4-9 cm and all of them were rooted except for the addition of zeatin. The low concentration of growth regulators around 0.1 ppm could still induce shoot multiplication until the sixth subculture (Figure 5).

The results of the observation of the number of shoots at the concentration of growth regulators of 1 ppm averaged around 2.67 - 5.5 per tube. The highest average number of cultures produced from the 2ip treatment was about 5.5 shoots per tube with a shoot height of about 8-10 cm (Figure 6). All cultures from treatment with a concentration of 1ppm can produce roots.

| Treatment Media (First Subculture) | Average Number of Shoots (Tubes) | Shoot Height Range (cm) | Status      |
|-----------------------------------|---------------------------------|-------------------------|-------------|
| BAP 0.1                           | 3.7                             | 7-9                     | rooted      |
| Kin 0.1                           | 4.9                             | 4-6                     | rooted      |
| Thidiazuron 0.1                    | 3.9                             | 5-8                     | rooted      |
| Zeatin 0.1                        | 1.5                             | 2-4                     | not rooted  |
| Kinetin 1                         | 4.5                             | 3-6                     | rooted      |
| Thidiazuron 1                     | 2.67                            | 4-6                     | rooted      |
| 2ip 1                             | 5.5                             | 8-10                    | rooted      |
**Figure 5.** Red ginger shoots in the sixth subculture originating from the treatment media containing 0.1 ppm of (a) BAP, (b) kinetin, (c) thidiazuron, and (d) zeatin.

**Figure 6.** Red ginger shoots in the sixth subculture originating from the treatment media containing 0.1 ppm of (a) BAP, (b) kinetin, and (c) thidiazuron.
The result of shoot induction in the sixth subculture still produced quite a lot of shoots. The technique of adding growth regulators to the first subculture media and then neutralized in the subculture could then trigger the emergence of more shoots. This technique could be used for production and could suppress somaclonal variation. Shoot induction generally uses BAP and thidiazuron, but based on the results of observations, other responses could arise from other types of cytokines such as kinetin and 2ip. The best number of shoots and shoot heights in the sixth subculture were obtained from the first subculture media with added cytokinin type 2ip. All plantlets were separated and prepared for acclimatization to test the yield of rhizome production in the field.

4. Conclusion
Based on the research that has been done, it can be concluded that the best type of cytokinin in increasing the multiplication of red ginger shoots in vitro was thidiazuron (TDZ). The best cytokinin concentration in increasing the multiplication of red ginger shoots in vitro was 1 ppm. Interactions between types and cytokinin concentrations only affected shoot length. Multiplication of red ginger shoots appeared in the second to sixth subcultures. The highest number of shoots in the sixth subculture was produced on the original medium treated with the addition of BAP and 2ip.

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