Callus induction of mountain papaya endosperm (Vasconcellea pubescens A. DC) with different combination of 2,4-Dichlorophenoxyacetic acid and Kinetin

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Abstract. Endosperm as a result of double fertilization in Angiospermae shows high level chromosomes and polyploidy. It is also considered as dead tissue that unable to be generated to form plantlet. The aim of this research is to determine the effect of kinetin and 2,4-Dichlorophenoxyacetic acid (2,4-D) in induction of callus formation of mountain papaya. This research used a factorial randomized block design with 18 groups, 1 fruit was used for 1 experimental group. Culture using Murashige and Skog (MS) media with combination of three level of kinetin (0, 2, 4 mgL⁻¹) and six level of 2,4-D (0, 1, 2, 3, 4, 5 mgL⁻¹). Maximum endosperm callus induction (0.88%) was achieved from endosperm explant cultured on MS medium fortified with 2.0 mgL⁻¹ Kinetin and 4.0 mgL⁻¹ 2,4-D. The fastest day induction (24.66 day) was observed with 5.0 mg L⁻¹ 2,4-D. The maximum number of browning (0,10) was induced by 2.0 mgL⁻¹ Kinetin and 5.0 mgL⁻¹ 2,4-D. Combination Kinetin and 2,4-D proved could induce callus formation from mountain papaya endosperm.

1. Introduction
Mountain papaya (Vasconcellea pubescens A. DC) belongs to the family Caricaceae. The distribution of mountain papaya covers Chile, Ecuador, Mexico, and along the Andes Mountains. In Indonesia, mountain papaya can only be found in Bali, Bromo and the Dieng Plateau [1]. Mountain papaya can be used as an ingredient in sweets [2], jams, syrups, sauces, pie fillings, pickles, jelly, and preservatives. It is also used for the treatment of arterial sclerosis, treatment of skin infections, anthelmintic [3], cancer sores, constipation and amoebic dysentery [4]. Mountain papaya contains the enzyme papain which can using for proteolitic [5]. Mountain papaya leaves contain flavonoids that can be used as pain relievers and have the potential as cancer chemotherapy materials [6].

The low productivity of Mountain papaya Dieng can be improved by using micropropagation techniques, with the triploid callus induction technique from mountain papaya endosperm. Endosperm culture is an alternative technique to produce triploid plants directly, only through one step [7]. The superiority of endosperm culture can produce triploid plants that have seedless or seedling but sterile [8]. The success of endosperm culture is influenced by the age of the endosperm, the formulation of the media, the germination of the resulting embryo, the age of the culture [8], and endogenous hormones and growth regulators (PGRs) added to the growing media. PGRs has an important role in controlling
biological processes in plant tissues. The effectiveness of PGRs depends on the concentration of endogenous hormones that interact with the given exogeneous hormones [9].

2,4-Dichlorophenoxyacetic Acid is a systemic herbicide for broadleaf plants [10] which can also be a growth regulator at low concentrations [11]. Kinetin is one of the cytokinins that is widely used for shoot propagation because it has the ability to stimulate the formation of shoots with a high concentration (more than 1 mg/L) which is not easily damaged during media sterilization [12]. The concentration of PGRs in a balanced amount between cytokinin and auxin stimulates explants to form callus [13].

Callus growth of young sandalwood seed explants with a combination treatment of 1 ppm 2,4-D (Dichlorophenoxyacetic Acid) and 1 ppm Kinetin resulted in 23.81% [14]. Adult endosperm of kiwi has cultured on MS medium with the addition of 2 ppm 2,4-D and 5 ppm Kinetin to induce callus as much as 80% [15]. The addition of 0.01 ppm 2,4-D in white mulberry endosperm culture resulted in callus multiplication of 92% [16].

Endosperm callus induction in mountain papaya plants in order to obtain triploid callus that has the potential to have better growth characteristics and yields has never been done, therefore it is necessary to conduct research on triploid mountain papaya callus induction with the addition of 2,4-D and Kinetin.

2. Materials and methods

2.1. Plant material and culture condition
Unripe fruit obtained from Dieng Plateau, Central Java, Indonesia with altitude 1700-2000 meters above sea level. The fruit was taken directly and wrapped in a plastic bag and tightly tied. The seeds were separated by cutting the flesh of the fruit. The collected seeds were surface sterilized using 2.5 mgL⁻¹ NaOCl solution and 6 drops of tween 20, shaken for 7 minutes. The seeds were then washed three times in sterile distilled water.

After surface sterilisation, the soft seed coat was detached by using scalpel. Each seed was cut to remove the embryo. Furthermore, endosperm of mountain papaya was planted on suitable medium.

This experiment design was a factorial randomized block design using combination of two different plant growth regulators: six level of 2,4-Dichlorophenoxyacetic acid (2,4-D; at 0.0; 1.0; 2.0; 3.0; 4.0; and 5) mgL⁻¹ combine with three level of N-6-furfuryladenine (Kinetin; at 0.0; 2.0; and 4.0) mgL⁻¹. Experiment carried out with thrice repetition using 90 endosperm per treatment, on solid Murashige and Skoog (MS) medium. All media contained 30 mgL⁻¹ sucrose and 7 mgL⁻¹ agars, at pH 5.8.

2.2. Variable
There are 5 variables observed, with 3 quantitative variables and 2 qualitative variables. Quantitative variables included the day of callus formation, the percentage of callus formation, and the percentage of browning explants (the equation used is in formulas (1) and (2)). While the qualitative variables were callus colour (assessment of Munsel Colour Chart for Plant Tissue) and callus texture.

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\text{Percentage of Callus Formation} = \frac{\sum \text{callus form}}{\sum \text{total callus}} \quad (1)
\]

\[
\text{Percentage Brown Callus} = \frac{\sum \text{brown callus}}{\sum \text{total callus}} \quad (2)
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2.3. Data analysis
Data obtained from observations during the experiment will be tabulated, then analysed by Kruskal-Wallis test using SPSS.
3. Results and discussion
In this study, the PGRs 2,4-D and Kinetin were used either singly (Table 1.) or in combination (Table 2.) to induce the formation of callus endosperm *V. pubescens*.

**Table 1.** Effect of single 2,4-D and Kinetin on callus induction from endosperm of *V. pubescens*.

| MS + Hormone (mg L⁻¹) | Day Formation | Percentage (%) of callus formation | Percentage (%) of Brown Callus | Callus Texture | Callus Colour Dominant |
|-----------------------|---------------|-----------------------------------|-------------------------------|----------------|-----------------------|
| Control               | NR            | NR                                | 0.08                          | Crumbly        | NR                    |
| 1.0 D                 | 28.71         | 0.12                              | 0.07                          | Crumbly        | 8/4                   |
| 2.0 D                 | 31.85         | 0.24                              | 0.02                          | Crumbly        | 8/4                   |
| 3.0 D                 | 25.00         | 0.12                              | 0.03                          | Crumbly        | 8/6                   |
| 4.0 D                 | 30.50         | 0.10                              | 0.07                          | Crumbly        | 8/6                   |
| 5.0 D                 | 24.66         | 0.06                              | 0.03                          | Crumbly        | 7/4                   |
| 2.0 K                 | 34.00         | 0.02                              | 0.01                          | Crumbly        | 8/2                   |
| 4.0 K                 | NR            | NR                                | NR                            | Crumbly        | NR                    |

NR = no response; D = 2,4-D; K = Kinetine

**Table 2.** Effect of combined 2,4-D and Kinetin supplementation on callus induction from endosperm of *V. pubescens*.

| MS + Hormone (mg L⁻¹) | Day Formation | Percentage (%) of callus formation | Percentage (%) of Brown Explants | Callus Texture | Callus Colour Dominant |
|-----------------------|---------------|-----------------------------------|----------------------------------|----------------|-----------------------|
| Control               | NR            | NR                                | 0.08                            | Crumbly        | 8/2                   |
| 2.0 K + 1.0 D         | 31.00         | 0.03                              | NR                              | Crumbly        | 8/4                   |
| 2.0 K + 2.0 D         | 25.00         | 0.03                              | 0.04                            | Crumbly        | 8/4                   |
| 2.0 K + 3.0 D         | 30.50         | 0.04                              | 0.03                            | Crumbly        | 8/2                   |
| 2.0 K + 4.0 D         | 35.00         | 0.88                              | 0.06                            | Crumbly        | 8/2                   |
| 2.0 K + 5.0 D         | 27.87         | 0.44                              | 0.10                            | Crumbly        | 8/4                   |
| 4.0 K + 1.0 D         | 31.50         | 0.03                              | 0.01                            | Crumbly        | 8/2                   |
| 4.0 K + 2.0 D         | 31.25         | 0.05                              | 0.03                            | Crumbly        | 8/4                   |
| 4.0 K + 3.0 D         | 26.83         | 0.11                              | 0.05                            | Crumbly        | 8/4                   |
| 4.0 K + 4.0 D         | 40.75         | 0.04                              | 0.04                            | Crumbly        | 8/6                   |
| 4.0 K + 5.0 D         | 27.50         | 0.05                              | 0.02                            | Crumbly        | 8/4                   |

NR = no response; D = 2,4-D; K = Kinetine

**Table 3.** The results of the significance of the Kruskal Wallis analysis from quantitative variables.

| Treatment | Day Formation | Percentage Callus Formation | Percentage Browning Explants |
|-----------|---------------|-----------------------------|------------------------------|
|           | Asymp. sig    | sig/no                      | Asymp. sig                   | sig/no          |
| K         | 0.00          | *                           | 0.00                         | *               | 0.464          | **           |
| D         | 0.29          | **                          | 0.09                         | **              | 0.906          | **           |
| K*D       | 0.00          | *                           | 0.00                         | *               | 0.412          | **           |

* = significant; ** = not significant
3.1. Day formation of mountain papaya endosperm callus (V. pubescens)
Observations of callus formation time were carried out every two days after explants were planted on the growing media. The fastest callus formation day average was K0D5 treatment with 24.66 days after planting, while the longest callus formation day was K4D4 treatment with 40.75 days after planting. Meanwhile, K0D0 (control) and K4D0 treatments did not experience callus growth until the last day of observation. The results of the Kruskal Wallis showed that the treatment of Kinetin and combine treatment are significant.

K0D5 treatment was the best treatment to induce endosperm callus of mountain papaya, this was due to the addition of 2,4-D to the media. The addition of 2,4-D in culture media will stimulate cell division and enlargement in explants so that it can stimulate callus growth [17]. Swelling in explants is the initial stage of callus formation which indicates the presence of cell activity in explants. The process of swelling in the explant tissue is followed by the start of callus growth marked by the presence of undifferentiated tissue [18].

3.2. Percentage of endosperm callus formation of mountain papaya (V. pubescens)
The success of callus growth was expressed by the percentage of the number of explants in forming callus at the observation 8 weeks after planting. The calculation of the percentage of callus endosperm Vasconcellea pubescens formed by calculating the callused mountain papaya explants divided by the number of explants planted and then multiplied by 100%.

The highest average callus percentage was K0D2 treatment with a percentage of 0.24%, while the smallest percentage of callus formation was K2D0 treatment with 0.02%. Meanwhile, K0D0 (control) and K4D0 treatments did not experience callus growth until the last day of observation.

The percentage of callus formation in the K0D2 treatment obtained the highest results compared to other treatment combinations. Of the 18 treatment combinations, there were 16 treatments that succeeded in inducing callus because the combination of 2,4-D and Kinetin given could balance the exogenous and endogenous concentrations in explants. A balanced concentration is known to stimulate callus induction in explants [13].

3.3. Percentage of endosperm browning explants of mountain papaya (V. pubescens)
Browning in tissue culture is caused by the increased production of phenolic compounds followed by oxidation of phenolic compounds followed by oxidation by the activity of the polyphenol oxidase (PPO) enzyme and its polymerization. Phenylalanine ammonia lyase (PAL) is one of the enzymes in phenylpropanoid which is very influential on the occurrence of browning. One of the main causes of browning in this vitro culture is wound from cutting tissue. Injury occurs when the callus is broken into small pieces or subculture techniques that are not careful [19]. Browning on younger tissue is less than old light tissue [13].

The percentage of callus browning, indicated by a colour change in the culture to chocolate. Observations were made at the end of the observation and calculated by the formula (2). Based on Tables 1 and 2, the percentage of browning callus in total (single and combination PGRs) is 0.080%. the number of callus that experienced browning in this experiment was quite low, presumably this was due to the callus being used was induced from young tissue.

3.4. Endosperm callus colour and texture of mountain papaya (V. pubescens)
Callus colour describes the visual appearance of callus cells so that the level of cell characteristics can be known. Observation of callus colour was carried out visually at the end of the observation. Determination of callus colour is guided by the Munsell Colour Chart for Plant Tissue. The results of the observations showed different results from each treatment.

Different callus colours are caused by differences in pigmentation and genetic origin of explants. Pigmentation can be evenly distributed throughout the callus surface or only partially. Colour
combinations in callus may be observed, namely white, green, brown, brownish white, and greenish white. Callus colour that is getting darker indicates decreasing callus growth. In this study the colour of the callus varied, from off-white (5Y 8/2) to dark (5Y 6/6). It was recorded that from 18 treatments, there were 12 treatments of which had pale white callus (5Y 8/2). White callus is an embryonic tissue that does not yet contain chloroplasts but contains starch grains which are polysaccharide deposits in plants, which gradually grow into a membrane system [20]. Callus that is white or light in colour indicates that the callus growth is in quite good condition.

Faint yellow (5Y 8/4) to yellow (5Y 8/8) also became the dominant endosperm callus colour in the study, there were at least 12 treatments that had callus colours of 5Y 8/4, 5Y 8/6, and 5Y 8/8. Callus that is yellow to greenish yellow is a callus that grows well because the callus is still actively metabolizing in cells. In addition to faded white and yellow, the observations also showed a brownish green colour as found in the K0D4 and K0D5 treatments. In this study, callus green-brown colour coded 5Y 7/4, 5Y 6/4, and 5Y 6/6 where green-brown callus was produced on media containing 2,4-D with a relatively high concentration.

**Figure 1.** Endosperm callus of *V. pubescens* with hue colour 8/6 and its crumbly callus (a&b).

Callus texture is one of the markers of the quality of a callus. Callus texture was observed visually at the end of the observation. All callus obtained were crumbly, meaning that the combination of treatments used induced the formation of callus crumbly, except for K0D0 and K4D0 treatments because no callus growth was found.

The formation of callus with crumb texture is triggered by the presence of endogenous auxin hormone which is produced internally by explants that have grown to form callus. Auxin has a role in the formation of callus crumbly. 2,4-D stimulates cell elongation by increasing the plasticity of the cell wall to become loose, so that water can enter the cell wall by osmosis and the cell undergoes elongation. Therefore, callus crumbly contain a lot of water because they have not undergone cell wall lignification, and between groups of cells are relatively easy to separate. The crumb callus texture experienced rapid cell division than the compact callus texture.

**Figure 2.** *V. pubescens* in the wild (a); *V. pubescens* view with horizontal cut (b); sarcotesta *V. pubescens* (c); a cut of *V. pubescens* seed with the endosperm (d).
4. Conclusions
The combination treatment of ZPT 2,4-D with Kinetin was able to induce the formation of mountain papaya endosperm callus. Callus formation occurs in various ways, both from the percentage of callus formed, the time of callus formation, and the colour of the callus. The callus produced in this study was in the form of callus that was crumbly and potentially embryonic.

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