Optimization of growth regulators to induce callus in chili [Capsicum annuum] cv. Berangkai

R Yunita1*, F R M Bagus2, B Nova3, F N Rosadi1, and J Jamsari1,2
1Department of Agrotechnology, Andalas University, Padang, West Sumatera, 25136, Indonesia
2Postgraduate Biotechnology Program, Andalas University, Padang, West Sumatera, 25136, Indonesia
3Graduate Program of Agriculture, Faculty of Agriculture, Andalas University, Padang, West Sumatera, 25136, Indonesia
Email: yunitaroza@agr.unand.ac.id

Abstract. The imbalance between demand and yield production causes the price of chili to rise sharply in the market at a particular time. One of the factors driving chili productivity to decrease is the attack of Geminivirus. Geminivirus that attacks chili plant is Pepper Yellow Leaf Curl Virus [PepYLCSV], and causing failure in chili production. Therefore, it is necessary to develop chili varieties that are resistant to PepYLCSV by applying a combination strategy of genetic engineering and a tissue culture approach. However, the morphogenetic ability of chilies to regenerate into complete plants is low in developing this strategy. Optimizing growth regulators in the media of callus induction can increase the morphogenetic response of chilies. This research aimed to obtain the optimal composition of callus induction media. The results showed that the use of the media composition of 2.5 mg/L NAA + 2.5 mg/L IAA and 3.5 mg/L 2.4 D + 0.25 mg/L TDZ could initiate callus formation on the eighth day after induction. Both media compositions have a callus growth percentage of about 100%, which has a friable and yellowish-white callus morphology.

Keywords: auxins, Berangkai, callus, cytokinins, plant growth regulators

1. Introduction
The demand for chilies for household consumption, industrial raw materials, and pharmaceutical raw materials is increasing, but chili production is limited. The imbalance between demand and availability causes chili's price to rise sharply in the market at a specific time. One of the limitations of chili production is the attack of the Geminivirus, which causes Pepper Yellow Leaf Curl Disease [PepYLCSV]. Various efforts to control this disease's development such as the use of chemicals and technical improvements in cultivation shown insignificant results. Therefore, other efforts are needed, such as developing chili varieties that are resistant to PepYLCSV through genetic engineering and tissue culture techniques.

Tissue culture techniques are increasingly playing an essential role in biotechnology since the new plants can be obtained in larger quantities and free from disease-causing pathogens. Callus induction is a crucial stage in tissue culture activity because it will determine the source of planting material to form new individuals and will be used as material for genetic transformation. Liu et al. [1] stated that callus induction is a powerful tool for regenerating plants.
Several factors influence callus induction, such as the composition and concentration of growth regulators in the media. The success rate of tissue culture is determined by the composition design and type of growth regulators in the media [2]. Growth regulators play a critical role in cell growth and differentiation [3]. In tissue culture activities, auxins and cytokinins can induce callus formation and cell differentiation to form new organs [4], [5], [6], [7], [8]. Auxins and cytokinins were the most studied and used hormones in callus formation and subsequent organ regeneration. So, in this study, we used several composition and concentration of growth regulators that obtained to induce callus formation of chili cv. Berangkai.

2. Materials and methods
The research was conducted at the Biotechnology Laboratory, Faculty of Agriculture, Andalas University. The main materials used are seeds of chili plants cv. Berangkai, Instant MS media, sucrose, agarose, NAA, BAP, IAA, NAA, and Thidiazuron. This study used an experimental design method with a completely randomized design [CRD] consisting of 5 levels of treatment, namely: MIK 1 [4.0 mg/L BAP + 0.5 mg/L IAA], MIK 2 [3.5 mg/L 2,4D + 3.5 mg/L AgNO3 + 3 mg/L Tryptophan], MIK 3 [2.5 mg/L NAA + 2.5 mg/L BAP], MIK 4 [0.2 mg/L TDZ + 0.1 mg/L IAA], and MIK 5 [3.5 mg/L 2.4 D + 0.25 mg/L TDZ]. Each treatment was repeated six times.

The chili seeds were sterilized using a fungicide 0.2 g/L for 30 minutes, then rinsed using sterile distilled water. Furthermore, the seeds were sterilized using NaOCl then rinsed with sterile distilled water and drained on a clean tissue. Moreover, the seeds were germinated in MS medium supplemented with 3 g/L sucrose and 8 g/L agar. The hypocotyl from 28 days after germination plants were used as the explant. Explants were cut to a size of 0.5-1 cm, then planted in various compositions of callus induction media. The observed variables were the explants' age forming callus, explants forming callus percentage, callus color, and callus texture.

3. Results and discussion
3.1. Seed germination
Chili cv. Berangkai seeds were germinated on MS medium began to sprout on the seventh day and reached complete growth on the 28th day. The seed germination percentage reached 91% of the total germinated seeds. This indicates that the composition of the MS medium can increase seed germination. Seed germination is influenced by two factors, namely genetic and environmental factors [10]. The genetic factors affect the availability of protein, carbohydrates, nutrients, and fats in the seed embryo [11]. Enzyme activity will also affect the cell wall's elasticity and porosity, which will affect the growth process and water absorption [12]. Simultaneously, environmental factors that affect germination include the composition of media, light, and growth regulators [13].

3.2. The age of the explants to form callus [days]
The success of explants in forming callus is an indicator of cell growth in in-vitro activity. Callus growth begins with a swelling process at the end of the incision on the explant. After that, a white bulge will form a collection of amorphous material and over time will spread over the entire surface of the explants.
Figure 1. Effect of composition and concentration of growth regulators on the age of explant to form a callus

Figure 1 showed that the use of various auxin and cytokinin compositions and concentrations affected the age of Callus forming explants. MIK 3 and MIK 5 can induce Callus on the eighth day after culture, faster than other treatments, which take eleven to twelve days after induction. This indicates that the MIK 3 and MIK 5 treatments are optimal to induce callus of chili cv. Berangkai.

The availability of auxins in optimal concentrations will cause increased enzyme activity. This causes the loosening of the explants cell wall so that they can spur faster growth [14]. NAA's biochemical conversion could cause callus initiation to form faster. With an optimal auxin concentration, it can increase propagation, enzyme activity, and rRNA transcription, thereby accelerating callus's growth [16].

3.3. Percentage of explants forming callus [%]
Base on figure 2, MIK 3 and MIK 5 treatments can induce callus up to 100 percent. This shows that the concentration and composition of the regulators between auxin and cytokinins are balanced in the media so that cell division and elongation are optimal. This is following the statement by Skoog and Miller [17], that if the auxin and cytokinin concentration is balanced, it will induce callus formation. This study's results are also in line with research obtained by Kumar et al. [14], 2.5 mg/L NAA + 2.5 mg/L BAP on MS Basal media can induce Callus up to 100 percent in chili plants.

Figure 2. Effect of composition and concentration of growth regulators on callus induction frequency.

The callus is formed due to cell division activity, which is very active in developing a rare collection of cells. Callus formation is initiated due to an explant cut in contact with the media and plant growth
regulators to activate cell metabolism [18]. The success of callus induction depends on the nutrients in the media, growth regulators, and other organic substances [14]. Mahadi et al. [19] also stated that the callus formation percentage was also influenced by MS media composition, which contained essential elements such as micro and macronutrients, sucrose, iron, and vitamins. The balance and reactions of growth regulators on the media will affect morphogenesis and in vitro growth [20].

3.4. Callus Color

In table 1, the composition and concentration of growth regulators gave different responses to callus color. The variation of callus' color can be seen in figure 3. MIK 3 and MIK 5 treatments resulted in a yellowish-white callus color, while other treatments produced a greenish-white callus color. This may be due to the explant's response to the type and concentration of growth regulators added to the media. Sari et al. [13] state that explants type, growth regulators concentration, and growth regulators variations will produce color variations in the callus formed. Afshari et al. [21] also added that light and pigmentation also influenced callus color.

The presence of chlorophyll content influences the greenish color of the Callus. The average greenish-white Callus was induced by high cytokinin [BAP and TDZ] concentrations. When cytokinin concentration is high and exposed to light, it will play a role in chlorophyll initiation [22][13]. Conversely, the yellowish-white callus is induced in higher auxin [2.4 D] concentration, which causes chlorophyll formation process inhibition in the Callus. Sari et al. [13] also stated that the yellow color in Callus could occur due to chlorophyll degradation.

Table 1. Effect of composition and concentration of growth regulators on callus color and callus texture

| Treatment | Callus color   | Callus texture |
|-----------|----------------|---------------|
| MIK 1     | Greenish white | Compact       |
| MIK 2     | Greenish white | Compact       |
| MIK 3     | yellowish-white| Friable       |
| MIK 4     | Greenish white | Compact       |
| MIK 5     | yellowish-white| Friable       |

Figure 3. The variation of callus color. a. Yellowish-white callus. b. Greenish-white callus.

3.5. Callus Texture

In Table 1, the composition and concentration of growth regulators gave different responses to the callus texture. The variation of callus’ color can be seen in figure 4. MIK 3 and MIK 5 could produce callus with a friable texture, while other treatments resulted in a compact texture. The friable texture is considered good because it is embryogenic, which can form organs, especially shoots.

According to Sari et al. [13], a friable callus is formed due to the growth of cells with a smaller size later [13]. As a result of interacting with auxin, the cells lose their interaction, making it easier to
separate. Besides, the friable callus' water content was higher because the cell walls were not yet lignified. This made the cell clusters separate easily. In contrast to friable callus, compact callus has a more compact texture. The cells are dense and difficult to separate from one another. Callus texture can be used to describe the potential for regeneration to form the root and shoot organs [23]. Friable callus has a high potential to develop shoots than compact callus [13].

Figure 4. The variation of callus texture. a. Friable callus. b. Compact callus.

4. Conclusion
MIK 3 and MIK 5 treatments were able to induce callus eight days after induction with callus formation is reached 100 %, has a friable texture, and yellowish-white color.

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