Growth and development of young male inflorescences of oil palm (Elaeis guineensis Jacq.) In tissue culture system: The effect of 2,4-Dichlorophenoxyacetic Acid

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Abstract. An effort in developing a standard protocol for rapid clonal propagation of oil palm through somatic embryogenesis was established with the aim to determine the effect of various concentrations of growth regulators on the development of embryogenic callus from immature inflorescences as explants source. Immature male inflorescences were obtained from 4 – 5 years old oil palm planted in Teaching Farm, Faculty of Agriculture University of Jambi. The inflorescences were taken from leaf axils between 8th and 15th leaf from the top (the upper most exposed leaf). Following surface sterilization with 70% alcohol, the inflorescences were cut into segments of approximately 5 mm long, and cultured on solid MS medium supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) at 10, 20, 30, 40, 50, 60, 70 and 80 ppm respectively. Cultures were kept in culture room with photoperiod of 16 hour per day for 8 weeks. The results indicated that the application of 2,4-D in culture medium was proven to be crucial in accelerating callus formation as well as increasing the percentage of from young male inflorescence of oil palm forming callus. Among the treatments tested, the application of 70 ppm 2,4-D was the best concentration in initiating callus formation on young male inflorescence explants of oil palm plants.

1. Introduction
Oil palm (Elaeis guineensis Jacq.) is an oleaginous plant with high economic value as a source of oil: in food industry, cosmetics, health products, and bio-fuel or bio-diesel. Data from Statista (2018) estimated that in 2017/2018 palm oil production will account for 34.27% from the total world vegetable oil production. While soybean oil occupies the second position (28.77%) and followed by canola oil (14.73%) and sunflower seed oil (9.28%).

Among the world's palm oil producers, Indonesia is the largest palm oil producing country. In 2017 Indonesia's palm oil production was 38.17 million tons of Crude Palm Oil (CPO), an increase of 18% compared to 2016 of 32.52 million tons of CPO. By 2020, Indonesia’s palm oil production is targeted of 40 million tons of CPO (Indonesia Investments, 2018). In order to realize this production target of 40 million tons of CPO, well-planned strategic steps are needed in the form of replanting and land expansion.

Efforts to increase production, both through replanting and land expansion require large amounts of plant material. Provision of seedlings through generative propagation is faced with genetic variation within progeny as the result of cross-pollination. Meanwhile, clonal propagation in oil palm can not be
done conventionally because they are monocots and cannot be propagated through cutting, grafting or other vegetative ways.

An alternative technology for the multiplication of oil palm seeds is tissue culture technique through somatic embryogenesis (Zulkarnain et al., 2015). One of the advantages of this technique is that its capability to produce large number of relatively uniform plants in a relatively short time (Zulkarnain, 2009). Cell growth and differentiation in somatic embryogenesis is influenced by many factors within in vitro environment. Among these are explant sources that are used as culture materials (Steinmacher et al., 2007). One source of explants that can be used in oil palm tissue culture is immature inflorescence (male flowers) which can be obtained easily without damaging the plant or reducing its production (Jayanthi et al., 2015).

The success of plant propagation through tissue culture has been demonstrated in various plantation crops such as cocoa (Traore et al., 2003), dates (Aslam and Khan, 2009), sugar cane (Yadav et al., 2012) and coffee (Ahmed et al., 2013). In oil palm, this technique has also been applied with various levels of success (Zamzuri et al., 2007; Jayanthi et al., 2015; Corrêa et al., 2016). However, the study of the in vitro culture of oil palm male flowers is still very limited (Choo, 1990; Jayanthi et al., 2015). This is partly due to constraints in determining the level of maturity of the right flower to be used as explant material. According to Choo (1990), young male flowers isolated at the right level of maturity are excellent sources of explants for callus initiation.

This study aims to determine the effect of various concentrations of dichlorophenoxyacetic acid (2,4-D) on the development of embryogenic callus from immature inflorescences in oil palm tissue culture. This research was an effort to develop a standard procedure for rapid palm oil propagation through somatic embryogenesis.

2. Materials and methods
2.1 Plant material

Plant tissues that will be used as a source of explants are the young male flowers taken from plants grown in farmer’s field in Pematang Gajah Village, Muaro Jambi Regency. Three inflorescences from the leaf axil between 11th and 15th were taken (Figure 1) and put into a sterile polyethylene bag then taken to the laboratory. The outer spathes were discarded, and the inflorescences which were still wrapped in inner spathes were sterilized by dipping them in 70% ethanol for 5 minutes, then dried in aseptic conditions. Next the inner spathes were removed and the male flower was taken and cut into pieces measuring approximately 1 mm and used as explants. Each inflorescence was a replication, and therefore in this trial there were three replications. Explants were cultured on MS medium solidified with Agar in culture flask with a density of 3 explants per flask.

![Figure 1. Stock plant of oil palm (Elaeis guineensis Jacq.) aged of 5 – 6 years old used as explant (young male inflorescences) sources.](image)
2.2 *Culture medium*
Culture medium used was MS composition (Murashige and Skoog, 1962) supplemented with vitamins, myo-inositol and sucrose 3% (w/v) and with or without activated charcoal. The pH of the medium was set at 5.8 ± 0.02 before being compacted with 0.8% (w/v) agar. The medium was sterilized in an autoclave at 1.06 kg cm$^{-2}$ and a temperature of 121°C for 15 minutes.

2.3 *Growth regulators tested*
At this stage eight different levels of 2,4-dichlorophenoxyacetic acid (2,4-D) were tested: 10, 20, 30, 40, 50, 60, 70 and 80 ppm. Each treatment was repeated 3 times, and within each replication there were 3 explants cultured.

Cultures were maintained in dark conditions at temperature of 27 ± 1°C for 16 weeks without subculture. Observations were carried out on the percentage of explants forming callus, time required from culture initiation to callus proliferation, and characteristics of callus (color and structure).

2.4 *Data analysis*
Data were analyzed using Descriptive Statistic, and standard errors calculated were using Microsoft Excel (Microsoft-Corporation, 2000). Qualitative data (color and structure of callus) are presented in the form of images to support quantitative data.

3. **Results and discussion**
Observations were carried out starting from 7 days after culture initiation, and continued until 60 days after culture initiation. The observation was made on the following parameters: percentage of explant forming callus, time required until first callus proliferation was seen, and characteristics of callus (color and structure).

The application of 70 ppm 2,4-D resulted in the highest percentage of explant forming callus (66.67% on the average) (Table 1). In addition, 2,4-D at 70 ppm was also found to produce the fastest callus proliferation rate (averagely 9.56 days after culture initiation) (Table 2). Proliferated callus on the surface of explants treated with 2,4-D at different concentration showed relatively similar in their characteristics, which were initially white and friable, and later turned into creamy or yellowish creamy in color, with structures dominated by friable to compact (Table 3).

| Table 1. The effect different concentrations of 2,4-D on the percentage of explant forming callus on young male inflorescences explants of oil palm. |
|-----------------|-----------------|
| 2,4-D concentration (ppm) | Total explant forming callus (%) |
|-----------------|-----------------|
| 10 | 55,56 ± 11,11 |
| 20 | 33,33 ± 19,25 |
| 30 | 33,33 ± 19,25 |
| 40 | 44,45 ± 22,22 |
| 50 | 55,56 ± 11,11 |
| 60 | 44,44 ± 29,40 |
| 70 | 66,67 ± 19,25 |
| 80 | 55,55 ± 22,22 |

± standard error
Table 2. The effect different concentrations of 2,4-D on time required for callus proliferation on the surface of young male inflorescences explants of oil palm.

| 2,4-D concentration (ppm) | Time of callus formation (day after culture initiation) |
|---------------------------|--------------------------------------------------------|
| 10                        | 11.78 ± 2.78                                           |
| 20                        | 13.46 ± 0.89                                           |
| 30                        | 11.00 ± 0.29                                           |
| 40                        | 12.28 ± 1.36                                           |
| 50                        | 10.83 ± 1.25                                           |
| 60                        | 10.06 ± 0.55                                           |
| 70                        | 9.56 ± 0.71                                            |
| 80                        | 9.70 ± 2.30                                            |

± standard error

Table 3. The effect different concentrations of 2,4-D on the characteristics of callus formed on the surface of young male inflorescences explants of oil palm.

| 2,4-D concentration (ppm) | Callus color                          | Callus structure     |
|---------------------------|---------------------------------------|----------------------|
| 10                        | white then turned into creamy         | friable to compact   |
| 20                        | white then turned into creamy         | friable to compact   |
| 30                        | white then turned into creamy         | friable to compact   |
| 40                        | white then turned into creamy         | friable to compact   |
| 50                        | white then turned into creamy         | friable to compact   |
| 60                        | white then turned into creamy         | friable to compact   |
| 70                        | white then turned into creamy         | friable to compact   |
| 80                        | white then turned into creamy         | friable to compact   |

The response of explants cultured in the in vitro system is not always the same. It is very much determined by the type of explants, the environmental conditions of the culture, the composition of the medium, and the presence of growth regulators the culture medium. The combination of two or more of these factors is often being a critical factor needed to induce and increase the response of cultured tissue. According to Laslo and Vicaș (2008), the balance of endogenous against exogenous growth regulating substances is very influential on explant growth and development. Winarto et al. (2010) added that the addition of exogenous growth regulators into the medium can affect the performance of endogenous growth regulators in explant tissue. Therefore the type and dosage of the growth regulator given to the medium is very important to be considered in order to induce the development of explants in the desired direction.

One form of explant development that is often found in the in vitro culture system is the occurrence of callus proliferation from the surface of cultured explants. Gamborg dan Shyluk (1981) stated that in vitro callus regeneration was a consequence of random and uneven development of unspecialized cells and the loss of structure of organized cells. In this experiment, the application of 70 ppm 2,4-D was able to increase the number of explants forming callus up to 66.67% on the average. In addition, 2,4-D concentration of 70 ppm was also able to accelerate callus proliferation, so that the callus started to form on the explant surface within 10 days after culture initiation.

The color of the callus was white with friable in structure (Figure 2). The same callus properties was also reported by (Zulkarnain and Lizawati, 2011) on tissue culture of Jatropha curcas treated with 2,4-D, which indicated embryogenic capacity. The finding of this investigation indicates that the callus proliferating on the surface of the explants of young male flower oil palm has the embryogenic potential,
and if they are sub cultured on proper medium, somatic embryos could be produced. However, the development of callus in the reported study has not shown signs of somatic embryo formation. This is presumably due to the limited time for observing further explant development, besides the need for involvement of other factors to induce the emergence of embryogenic properties.

Figure 2. The appearance of white and friable callus (A) which turned into creamy and compact structure (B) on the surface of explant from young male inflorescence of oil palm cultured on medium supplemented with 2,4-D and activated charcoal (bar = 1 mm).

Efforts to generate embryogenic callus can be done by modifying environmental factors, especially the composition of the culture medium, since somatic embryogenesis is chemically controlled by various growth regulators (Tapingkae et al., 2012). The importance of plant growth regulators in somatic embryogenesis had been intensively reviewed by Jiménez (2001), Jiménez (2005), Fehér et al. (2003), Jiménez and Thomas (2005) and Fehér (2008). Among those growth regulators, auxins and cytokinins play important role in determining the embryogenic response. This is probably due to their involvement in cell cycling, division, and differentiation (Fehér et al., 2003). Auxin 2,4-dichlorophenoxyacetic acid (2,4-D), either alone or in combination with cytokinin, has been used for the induction of somatic embryogenesis in several species (Fehér et al., 2003; Ikeda et al., 2006; Raghavan, 2006; Fehér, 2008).

In addition, the mass of callus that has been successfully proliferated needs to be subcultured on new fresh medium. The maintenance of culture on the same medium for a long time will cause nutrient deficiency and water loss due to evapotranspiration in culture flask. Therefore, subculture becomes very important in order to maintain the sustainability of callus growth. For this reason Dodds dan Robert (1985) recommended to subculture callus sizes between 5 – 10 mm or weighing 20 – 100 mg to obtain better growth in new medium. Subcultures can also be done every 28 days (2 - 6 weeks), but the right time to transplant the culture depends on the speed of callus growth.

Thus, the results of this study indicate the magnitude of the opportunity to obtain embryogenic callus from young male flower explants of oil palm with the application of growth regulators, especially 2,4-D at a concentration of 70 ppm. It is expected that through tissue culture techniques efforts to obtain uniform, disease-free and large quantities of oil palm plants can be realized through somatic embryogenesis. Taji et al. (2002) stated that somatic embryogenesis has an important meaning in tissue culture techniques aimed at plant propagation. However, this process is limited by many factors because the somatic embryo will only develop from the embryogenic callus mass, and the time needed to obtain callus with embryogenic properties is sometimes very long. In addition, other factors such as plant hormones, nutrients and environmental conditions must be optimized first so that embryogenesis can take place.
4. Conclusion

Based on the results obtained in this study we conclude that:

a. The application of 2,4-D in culture medium could effectively stimulate callus proliferation on young male inflorescence explants of oil palm.

b. The effective concentration of 2,4-D for callus formation on young male inflorescence explants of oil palm is 70 ppm.

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