Induction of kopyor coconut embryogenic callus using 2.4-D and TDZ

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Abstract. This research was conducted to obtain an effective method to produce true to type coconut kopyor seeds and determine the effect of 2,4-D with or without TDZ in inducing callus to produce somatic embryos. This research was conducted in the Lampung State Polytechnic laboratory with concentrations of 2,4-D 0, 2.5, 5, 7.5, and 10 mg.L⁻¹ with or without the addition of 0.5 mg.L⁻¹. Further analysis of the data used the 5% LSD test. The parameters observed in this study were: percentage of callus explants, callus diameter, callus weight. The results showed that increasing the concentration of 2,4-D and 7.5 mg. L⁻¹ could increase the percentage of embryogenic callus, callus diameter, and callus weight and the addition of 0.5 TDZ concentration to the 2,4-D medium resulted in a decrease in parameters. The best 2,4-D concentration to induce callus embryogenic in kopyor embryos is 7.5 mg. L⁻¹. The use of 2,4-D for embryogenic callus formation can accelerate callus growth and obtain kopyor coconut seeds true to type

1. Introduction
Coconut (cocos nucifera L) is one of Indonesia's main crop, grown on 3 million ha and can produce 19.5 million tons of oil per year. However, about 96% of coconut farmers are still small farmers who cultivate less than 0.5 ha of land. As a result, marginalized coconut farmers has an annual revenue of less than the US $ 600 [1]. One approach might be to reduce poverty among the coconut farmers are planting certain crops with a higher market value. Among the best natural resources in Indonesia, mutant coconut so-called elite kopyor is promisingly. This mutant type is still rare and the price of fruit kopyor relatively much higher than ordinary coconut. The unique characteristics of the fruit of the coconut kopyor are that of the soft endosperm or solid meat. Kopyor coconut, is a natural coconut mutant that produces soft endosperm and tableware which is very rare in Indonesia. The tree is only found in Java and Sumatra [2].

Genes that control kopyor mutant character is a single recessive gene, designated as the 'k'. Fruit kopyor has kk homozygous embryos and triploid endosperm. On the other hand, regular coconut can have KK homozygous embryos and KKK or triploid endosperm, in contrast to Kopyor, Kk heterozygous triploid embryo, and endosperm KKK or Kkk. Fruit kopyor (with kk embryo and endosperm kkk) will not germinate naturally that is attributed by endosperm can not support normal embryo development. As a result, kopyor fruit is rotten and embryos are dead. Normal coconut fruit (with the zygotic embryo and endosperm KKK KKK) to be normal coconut seedlings germinate. However, the seed will never produce fruit kopyor. Besides that, Normal fruit (with embryos and KKK or KkkKk) can produce normal coconuts and coconut fruit kopyor. In addition to producing fruit
kopyor, farmers who cultivate coconut kopyor heterozygote Kk will produce palm seedlings kopyor heterozygote which are also traded. So in terms of quantity, kopyor cultivated coconut farmers are currently only able to produce between 10-30% kopyor pieces or in one bunch coconut coconuts there are only 1-3 kopyor. Besides the coconut nursery kopyor still, be done naturally by planting normal oil produced from trees that can produce fruit kopyor. Fruit kopyor can not be grown naturally. That difficulty also makes coconut kopyor as an expensive natural commodity market price. In addition to produce fruit kopyor, farmers who cultivate coconut kopyor heterozygote Kk will produce palm seedlings kopyor heterozygote that can be traded also.

Embryo culture is the only alternative to get the coconut seedlings kopyor true to type. Embryo culture is a component of embryo culture in vitro fertilization in which embryos produced were allowed to grow for some time in an artificial medium to obtain seedlings [3]. However, this technique produces only one nursery of the embryo explants cultured, so alternative in vitro methods should be done to increase the rate of plantlets to raise seedlings obtained. The development of a reliable propagation clone such as somatic embryogenesis will quickly provide embryos for kopyor coconut. From the coconut embryos produced kopyor will get a lot of somatic embryos which then can be kopyor coconut seedlings.

Somatic embryogenesis in coconut plant kopyor can be done with indirect somatic embryogenesis that occurs from disorganized tissue (callus) which further developed into embryos. There are some reports of oil through the multiplication of somatic embryos formed from the callus [4][5] [6]. Some researchers claim, callus can be produced from a variety source such as flowers and leaves [7][8] plumula [6], and the embryo [7][5]. Stages of embryonic development require the support of the growing media, the appropriate media will encourage the development of the embryo while the less appropriate media will support the embryo in inducing all stages of embryonic development. Callus initiation required the presence of auxin like 2.4-D is usually combined with TDZ. Although much successful somatic embryogeneses have been done in Indonesia for palm normal, but for coconut research kopyor not much to report. The combination of the concentration of various plant-growth regulators can cause a different response. The study was conducted to optimize the induction callus from explants of coconut kopyor.

2. Material and Method

2.1材料

The research was conducted in plant tissue culture laboratory, Department of Plant Crops, Politeknik Negeri Lampung from April 2019 until October 2019. The tools used in the manufacture of the media is a measuring cup, beaker glass, tweezers, magnetic stirrer, the autoclave, pipettes, pH meters, analytical balance, the culture bottles, plastic, rubber bands, paper labels, camera, and stationery. While the tools used in planting are tweezers, scalpel, petri dish, LAFC, Bunsen lamp. Before it is used for planting. tweezers, scalpel, and Petrides autoclave sterilized for 1 hour at a temperature of 121°C. Materials used are coconut embryos kopyor, MS stock solution (consisting of macro and micro salts, and vitamins), sugar, alcohol, spritus, rubber, plastics, labels, TDZ, and 2.4-D. Kopyor coconut embryos are used both inside and in types of early maturing types obtained from South Lampung regency.

The experiment was conducted using a completely randomized design with treatments arranged various concentrations of 2.4-D are 0, 2.5, 5, 7.5 and 10 mg.L-1 with or without the addition of 0.5 mg.L-1 TDZ. Each treatment was repeated three times with a single experimental unit consists of 3 bottles of cultures, each containing one explant. Sub-culture into new media with the same treatment done every 4 weeks. The data obtained by doing an analysis of variance followed by a Least Significant Difference test (LSD 5%). Implementation of the trial includes the preparation of equipment and materials, culture media preparation, planting explant and culture conditions.
2.2. Preparation Tools and Materials

Before it is used for planting, tweezers, scalpel, cotton, and petridish sterilized by autoclave at a temperature of 121°C with a pressure of 1.2 atm for 60 minutes. The explants used are derived from coconut embryos kopyor aged 11 months. After kopyor coconut fruit peeled and sliced, then the embryo is isolated from a certain part of the endosperm, coconut, which is just part one of three 'eyes' of the coconut. But too often the case embryos already in place properly due to the nature of the kopyor endosperm, so that the embryo must be sought in the endosperm were destroyed. Isolation is done by using a tablespoon by taking an embryo with a partial quote endosperm. Once isolated, embryos were then washed with running water and washed quickly with 95% ethanol to remove lipid attached. In a laminar airflow cabinet (LAF), the embryo is isolated from the endosperm, then put in a sterile glass beaker filled with a solution of sodium hipoklorida (5%) for 15 minutes. Subsequently, the solution was discarded and replaced with sterile distilled water 4 times and embryos ready to be used for subsequent experiments [9]. Planting sterile explant and subcultures performed in LAFC under aseptic conditions.

2.3. Making the Media Induction Callus

The culture medium used was Murashige and Skoog (1962) [10], which is enriched with vitamins, mio inositol 100 mg.L⁻¹, sucrose 30 g.L⁻¹. Further regulate media pH to 5.8 if the pH is less than 5.8 then by the addition of KOH 1 N whereas if the pH is more than 5.8 then given HCL 1 N, after the pH to 5.8 was added 8 g.L⁻¹ of agar powder so then the media was cooked to a boil and then pour the media into culture bottles of 30 ml per bottle. Close the bottle containing the media with clear plastic and then tied up with rubber and sterilized by autoclaving at 121°C with a pressure of 1.2 atm for 15 minutes.

2.4. Making the Media Induction Callus

Planting experiments were carried out with embryos sliced into 2 parts and haustorium discarded. Sliced embryos grown on MS medium containing 2.4-D (0, 2.5 mg.L⁻¹, 5 mg.L⁻¹, 7.5 mg.L⁻¹, 10 mg.L⁻¹) 3 g.L⁻¹ activated carbon, 30 g.L⁻¹ sucrose, 8 so g.L⁻¹, and vitamins. A bottle that had contained the explants sealed using plastic and tied with a rubber band. Culture bottles are then placed on the shelves of culture in the dark for four weeks at room temperature 25°C and every 4 weeks explants were subcultured with embryogenic callus proliferation media.

2.5. Embryogenic callus induction of 2.4-D with the addition of 0.5 mg.L⁻¹ TDZ

Implantation to induce embryogenic callus on the treatment of 2.4-D with the addition of 0.5 mg.L⁻¹ TDZ, together with callus induction treatment using 2, 4-D. Sliced embryos grown in medium 2, 4-D (0, 2.5 mg.L⁻¹, 5 mg.L⁻¹, 7.5 mg.L⁻¹, 10 mg.L⁻¹) supplemented with TDZ 0.5 mg.L⁻¹ in every media treatment of 2.4-D. Storage is done in a dark culture room and every 1 month does subculture with embryogenic callus proliferation media. Observations were made after the culture was 12 weeks after explant are grown on media treatment.

3. Results and Discussion

Development of embryogenesis techniques has several specific stages, ie cells and embryogenic callus induction, the stage of embryonic development, germination, and hardening. At this stage of embryogenic callus induction explant isolation and cultivation done in the growing medium. Embryogenic callus induction culture generally was grown on a medium containing auxin that has a strong activity or high concentrations. Embryogenic callus induced by 2.4-D at concentrations of 0, 2.5, 5, 7.5 and 10 mg.L⁻¹ and longitudinally model of cleavage embryos. At this stage of somatic embryo formation, PGR TDZ 0.5 mg.L⁻¹ in combination with 2.4-D 0, 2.5, 5, 7.5 and 10 mg.L⁻¹ is used to induce the formation of somatic embryos. MS medium supplemented with 2.4-D 2.5-7.5 mg.L⁻¹
generally produce better growth, demonstrated by the high percentage of embryos that form a callus, callus wider diameter, and weight of the heaviest callus compared to the media with the addition of TDZ or without the administration of 2.4-D.

3.1. Percentage of embryogenic
Increasing concentrations of 2.4-D in the treatment of the media composition of 2.5 mg.L\(^{-1}\) to 7.5 mg.L\(^{-1}\) affect embryogenic callus percentage increase 56.92% - 91.62% and the percentage of embryogenic callus decreased by administering 2.4-D 10 mg.L\(^{-1}\) (Fig. 1). However, not all explants were cultured to form a callus. In the treatment without giving 2.4-D explants showed only thickening and does not develop into callus although cultured in the long term. The addition of 2.4-D in the culture medium will stimulate cell division and enlargement of the explant so that it can stimulate the formation and growth of callus and improve the natural chemical compounds flavonoids. It is also delivered by [11], which states that 2.4-D can cause elongation of cells, tissue swelling, and callus

According to [12] [2], growth regulators 2.4-D is often used to induce embryogenic callus in a lot of palm trees. According to [13] it also can initiate gene activation to differentiation and increase embryogenic cells through repeated cell division. [14], said that the formation of callus from leaf explants of ramin prove that the higher the concentration of 2.4-D is used, the faster callus induction occurs because 2.4-D more easily diffuse into the plant tissues as a result of the incision so that 2.4-D is added will help to stimulate the endogenous auxin stimulates cell division or cell especially around the area of the wound.

![Figure 1. The average percentage of embryogenic callus (%) per explant cultures kopyor coconut aged 12 MST in response to the concentration of 2.4-D and TDZ. The median value, followed by the same letter are not significantly different with LSD at 5%](image)

The highest percentage of explants on 2.4-D treatment with the addition of 0.5 TDZ only can the treatment of 7.5 mg L\(^{-1}\) with add 0.5 mg L\(^{-1}\) TDZ, but not the higher the percentage of explants callus the highest in the treatment of 2.4-D 7 mg.L\(^{-1}\) without TDZ is 91.68%. According to [15] embryogenic callus growth requires a high concentration of auxin and cytokinin low. According to [16] in monocot plants require auxin range of 2 ppm-5 ppm. Embryogenic callus of ginger belonging monocot plant is not too affected by the level of cytokinin (BAP) which adds on media by the statement of [17], to induce callus monocot plant cytokinins are not a very important role. According to [18] exogenous hormones will increase osmotic pressure, increase protein synthesis and cell permeability to water, and soften the cell wall followed by a decrease in cell wall pressure so that water can enter the cell and cells will become enlarged and elongated. Callus grown on MS medium with different concentrations of 2.4-D indicates the level of proliferation that is different. At the beginning of the planting, the size
of the weight and diameter of the callus on each treatment was relatively flat at around 0.2 mg (weight) and 0.5 cm (diameter). In the second week after planting, the development of the embryo is still small. Until week 4 appears that embryos undergo proliferation which is characterized by shape, color, and size of the callus growing and widening. At the age of 12 weeks after planting, diameter and weight of callus for each different treatment. Differences in embryogenic and no embryogenic callus can be seen in Fig.2.

Figure 2. Embryo kopyor do not embryogenic callus, a. explant which does not form a callus, b. no embryogenic callus explant, c. Explant kopyor only enlarged, d. Creamy and compact embryogenic callus

The appearance of kopyor coconut embryo structure using microscopic SEM shows that the location of the kopyor coconut embryo is right at the top of the embryo. The appearance of kopyor coconut embryo structure can be seen in Fig 3.

Figure 3. The appearance of kopyor coconut embryo structure using microscopic SEM

3.2. Callus diameter
The separation of the middle value with BNT 5% against the average diameter of callus per explants in vitro culture of coconut kopyor aged 22 MST in response to the concentration of 2.4-D and TDZ showed that there are significant differences between the explants on media with no or additions TDZ 0.5 mg.L⁻¹ (fig. 4). Increasing concentrations of 2.4-D 2.5 mg.L⁻¹ to 7.5 mg.L⁻¹ significantly increases the callus be 1.17 to 1.47 cm diameter. The addition of 10 mg.L⁻¹2.4-D can produce callus diameter that is narrower than the treatment of 7.5 mg.L⁻¹2.4-D, but better than in control media (0.43 cm). The addition of TDZ 0.5 mg.L⁻¹ in the medium 2.4-D also produces callus narrower diameter than other treatments (0.47 to 0.83 cm). The resulting callus size at each different treatment media. This is caused by the network's ability to absorb water and nutrients is different is the ability to hold the process of diffusion, osmosis, cell turgor pressure. Increasing the diameter of the callus showed a growth process.

According to [19], auxin can increase osmotic pressure, protein synthesis and cell permeability to water. This causes the water can get into the cell so that the callus volume increases. With an increase in protein synthesis, it can be used as a power source in growth. In particular, plant cells expand by taking water without synthesizing new cytoplasmic components. The expansion of cells with this
mechanism is characterized by debilitating auxin area of the cell wall, thus encouraging the expansion of cell turgor pressure. The water that flows into the cells accumulates in a large central vacuole so that the cell cytosol expand without increasing its volume. According to [20] the expansion could result in an increase of 10 to 100 times the size of the plant cells during development.

![Callus diameter (cm)](image)

**Figure 4.** The average callus diameter (cm) per explant cultures kopyor coconut aged 12 MST in response to the concentration of 2.4-D and TDZ. The median value, followed by the same letter are not significantly different with LSD at 5%

### 3.3. Weight of callus

Growth is a permanent increase in the size of organisms or parts of plants which are the result of an increase in the number and size of cells. Growth characterized by weight gain irreversible, so that measures can represent a callus fresh weight callus growth variables are derived from coconut embryos kopyor. Fresh weight physiologically consists of two content that is water and carbohydrates. A large callus fresh weight was due to the high water content.

The LSD 0.05 on average fresh weight of callus kopyor in media MS in response to the addition of 2.4-D and or without TDZ is presented in Fig. 5. The addition of TDZ on MS medium with 2.4-D does not give a positive response against the weight of the callus. The highest callus weight is achieved by treatment of 2.4-D 7.5 mg.L\(^{-1}\) (2.76), but the treatment of 2.4-D with the addition of TDZ 0.5 mg.L\(^{-1}\) gives a lower weighting than without TDZ. The addition of 2.4-D because the 2.4-D role is to encourage the process of morphogenesis callus, callus induction and can influence the genetic stability of the plant cell. The callus that formed in this treatment, is influenced by the presence of auxin and cytokinin either endogenous or exogenous. The use of auxin and cytokinin with prompt and appropriate comparison would support the growth of callus in vitro. According to research [21] the optimal concentration of 2.4-D for callus induction coconut was 100 μM and 125 μM.
Figure 5. The average weight of callus (g) per explant cultures kopyor coconut aged 12 MST in response to the concentration of 2.4-D and TDZ. The median value, followed by the same letter are not significantly different with LSD at 5%.

4. Conclusion
The increasing of concentrations of 2.4-D and 7.5 mg.L$^{-1}$ can increase the percentage of embryogenic callus, callus diameter, and weighing callus. Increasing concentrations of 2.4-D from 2.5 mg.L$^{-1}$ to 7.5 mg.L$^{-1}$ added the percentage of embryogenic callus, callus diameter, and weighing callus but adds a percentage of embryogenic callus, callus diameter, and weighing callus will decrease if the concentration of 2.4-D were 7.5 mg.L$^{-1}$. The addition of TDZ concentration of 0.5 to the medium 2.4-D resulted in a decrease in the percentage of embryogenic callus, callus diameter is narrower and lower weight on embryonic callus kopyor coconut. The concentration of 2.4-D best to induce callus on coconut embryo kopyor is 7.5 mg.L$^{-1}$.

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