OPTIMIZATION OF THE GROWTH REGULATOR CONCENTRATION FOR THE INDUCTION OF SOMATIC EMBRYOGENESIS OF LIBERICA COFFEE (*Coffea liberica* L. cv. Liberica Tungkal Composite)

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Abstract. The liberica coffee (*Coffea liberica* L. cv. Liberica Tungkal Composite) is a type of coffee that has been widely developed in the lowland areas of Jambi Province, Indonesia specifically in West Tanjung Jabung and East Tanjung Jabung Regencies. However, the mother tree of liberica coffee is old and attacked by many diseases, so that a fast seed propagation technique, tissue culture is an efficient plant propagation technique. This study aims to examine the interaction of auxin and cytokinin concentrations that are most optimal for the induction of somatic embryogenesis of liberica coffee. The experiment used a completely randomized design factorial pattern with 2 factors, the first factor was auxin 2,4-D and the second factor was cytokinin 2-iP treatment. The results revealed that application of different dosage of growth regulators 2,4-D and 2-iP in the forming of somatic embryogenesis of liberica coffee showed an interaction with the observational variables of span time for callus proliferation, percentage of explant forming callus, and callus size. The balanced application of growth regulators 2,4-D and 2-iP was able to induce the formation of somatic embryogenesis from liberica coffee immature leaf explants. The most optimal interaction for the development of embryo stages from globular, heart and torpedo is 5 µM 2,4-D + 5 µM 2-iP treatment.

Key words: 2,4-Dichlorophenoxyacetic acid; 2-isopentenyladenine; liberica coffee; somatic embryogenesis.

INTRODUCTION

Liberica coffee is suitable for cultivation in the lowlands, which is 2 m above sea level, and can live and grow well on peatlands, thus liberica coffee is well known as peat-specific coffee [23]. In Indonesia, liberica coffee can be found planted in Sumatera and Kalimantan. Liberica coffee has a distinctive jackfruit flavor, so that in some areas this coffee is called jackfruit coffee. Because of its taste, liberica coffee has a high selling value. Therefore, in the liberica coffee-producing areas, the Indonesian government continues to strive to intensify 300 hectares of liberica coffee per year [16]. In Jambi Province, liberica coffee, known as The Tungkal Composite of liberica coffee has been granted Geographical Indication Certification by the Directorate General of Intellectual Property of the Ministry of Law and Human Rights of the Republic of Indonesia. However, the current challenge pertains to the aging mother trees of liberica coffee which are utilized for plant propagation and are prone to diseases. Hence, there is a requirement for rapid and healthy seed propagation technology.

Somatic embryogenesis is a method to obtain clonal seedlings in large quantities, quickly and disease free. Somatic embryogenesis techniques have been widely studied and used for *in vitro* plant propagation, because the number of propagules produced is unlimited and the time required is faster [30]. Also added by Ibrahim and Hartati [18] that plant propagation using somatic embryogenesis techniques will produce plants that have a strong root system (taproot), because it is the result of the growth and development of a bipolar embryo, which has two poles where there is one part that will become shoots and part of it will become roots.

Somatic embryogenesis through callus occurs through 4 stages, namely primary callus induction, embryogenic callus induction from primary callus and somatic embryo initiation, somatic embryo maturation and plant regeneration from somatic embryos [13]. The success of coffee plant somatic embryogenesis has been widely reported, namely arabica coffee [1, 2, 12, 17, 38] canephora coffee [9, 14, 15], while information on the successful induction of somatic embryogenesis from liberica coffee is still limited. Embryogenic callus induction from *C. liberica* cv. Arruminensis has been carried out by Ardiyani [4], while Lizawati *et al.* [24], has succeeded in inducing the formation of forming callus from explants of immature leaves of *C. liberica* cv. Liberica Tungkal Composite, but with a low success of 56.67%.

One of the important stages in the success of somatic embryogenesis is the formation of embryogenic callus, which is largely determined by the type and concentration of growth regulators used. The cytokinin growth regulators that are often used in the induction of somatic embryogenesis are benzyladenine (BA), 2-isopentenyladenine (2-iP), and thidiazuron (TDZ), while the auxins that are commonly used are 2,4-dichlorophenoxyacetic acid (2,4-D). Ibrahim *et al.* [18] reported that the addition of 2,4-D and TDZ to *in vitro* culture media on arabica coffee leaf explants was able to induce somatic embryogenesis, but the effect was still varied for each type of arabica coffee clone used. Research conducted by Lizawati *et al.* [24] found that the best combination for inducing callus proliferation from young leaves of liberica coffee were 1.0 mg·L^{-1} 2,4-D + 1.0 mg·L^{-1} 2-iP and 0.5 mg·L^{-1} 2,4-D + 2.0 mg·L^{-1} 2-iP although the percentage of callus formation is still low. Therefore research is needed to find optimal media formulations to induce embryogenic callus formation from Jambi superior

tungkal composite liberica coffee and its proliferation in order to obtain good callus. competent to be regenerated to form somatic embryos. This study aimed to examine the interaction of auxin and cytokinin concentrations that are most optimal for the induction of somatic embryogenesis of *Coffea liberica* L. cv. Liberica Tungkal Composite.

MATERIALS AND METHOD

Plant materials and planting explants

The planting material used was immature leaf explants of liberica coffee Tungkal composite that has been fully opened. The explants were washed using a liquid detergent, rinsed with running water, followed by soaking in a solution of 2.5 g/L Benlox and Agrept fungicide for 30 min, then rinsed with running water. Furthermore, in the laminar air flow cabinet (LAFC) the explants were soaked and shaken in 5.25% NaOCl bleach solution with a concentration of 10% for 10 minutes, then rinsed 3 times with sterile water, then dipped in 70% ethanol for 3 seconds and then rinsed 3 times with sterile water.

The cut explants measuring 1 x 1 cm were cultured on the basic medium of Murashige and Skoog 1962 [27] which had been added with plant growth regulators (PGR) according to the treatment, the pH of the culture medium was set on a scale of 5.6-5.8. Cultures were stored in a dark room for 16 weeks at 24 ± 3 °C with 60% humidity, irradiated for 16 hours, light intensity 13.5-20.25 $\mu mol \cdot m^{-2} \cdot s^{-1}$.

Experimental design

The experiment used a completely randomized design (CRD) with 2 factors, the first factor was auxin 2,4-dichlorophenoxyacetic acid (2,4-D) with concentrations of (5, 10, 15 and 20 µM and the second factor was cytokinin 2-isopentenyladenine (2-iP) with concentrations of (5, 10, 15 and 20 µM). The experiment applied each treatment with 4 replications where each bottle contained one explant and each experimental unit consisted of 4 bottles. Cultures were maintained for 4 months and subcultured on new media every month with the same treatment.

Observations were made by monitoring the growth and development of the explants every day for the variable span time for callus proliferation, percentage of explant forming callus, callus structure, callus color (observed using references in the Munsell Color Chart for Plant Tissue book [8], callus size and the percentage of somatic embryo formation were observed in age 16 weeks after culture (WAC).

Data analysis

Observational data were analyzed statistically using analysis of variance (ANOVA) with a significant level of α = 5%. If there is a significant effect, a further test is carried out by employing the least significant difference (LSD) with a significant level of α = 5%. Parameters that cannot be measured quantitatively, were qualitatively observed, and descriptively analyzed.

RESULTS

Span time for callus proliferation

All of the media formulations used were able to induce the formation of callus from explants of young leaves of liberica coffee, but have different time on the callus appearance for each treatment. Callus formation begins with swelling on the explant surface. The leaf explants began to swell 7 days after culture (DAC) then the explant incisions were wavy (swelling), followed by the formation of callus on the edges of the leaves or on the leaf veins. Leaf veins are areas that distribute food to all parts of the leaf surface so that cells found near the leaf bones can divide and form calluses.

The analysis of Table 1 indicated that there was an interaction between the different dosage of growth regulators (2,4-D and 2-iP) and the time of callus appearance (19-23 DAC). The d4p2 treatment (20 µM 2,4-D with 10 µM 2-iP) produced the fastest callus appearance time of 19.81 DAC but was not significantly different from the d1p1 treatment (5.0 µM 2,4-D + 5 µM 2-iP); d1p1 (10 µM 2,4-D + 5 µM 2-iP); d2p3 (10 µM 2,4-D + 15 µM 2-iP); d2p4 (10 µM 2,4-D + 20 µM 2-iP) and d3p4 (15 µM 2,4-D + 20 µM 2-iP). The same result was reported by Damayanti *et al.* [9] that callus induction from Todolo Toraja coffee leaf explants on average begins to form at the age of 17-23 days after culture. The results showed that the use of 2,4-D (5-10 µM) combined with 2-iP (5-20 µM) was the best treatment for the initiation of callus formation.

Percentage of explant forming callus

The concentration of 2,4-D and the concentration of 2-iP indicated significant interaction on the percentage of explants forming callus capability. The average percentage of explants that form callus is 75-100%. Table 2 indicated that the highest percentage of 100% callus formation was found in d1p1 (5 µM 2,4-D + 5 µM 2-iP); d3p1 (15 µM 2,4-D + 5 µM 2-iP) and d4p1 (5 µM 2,4-D + 20 µM 2-iP), while the lowest treatment was found in d2p3 (10 µM 2,4-D + 15 µM 2-iP) with a

Table 1. The effect of different concentrations of 2,4-D and 2-iP on the time of callus appearance from young leaf explants of liberica coffee Tungkal composite (Days After Culture)

2,4-D (µM)	2-iP (5 µM)	2-iP (10 µM)	2-iP (15 µM)	2-iP (20 µM)
5	23.44 (B) b	26.31 (A) a	25.56 (A) a	26.13 (A) a
10	22.06 (B) b	26.13 (A) a	23.63 (A) b	22.81 (A) b
15	27.69 (A) a	23.69 (B) b	26.00 (A) a	23.25 (A) b
20	27.19 (A) a	19.81 (B) b	25.38 (A) a	24.38 (A) a

Note: Numbers followed by the same lowercase letters (rows) and uppercase letters (columns) are not significantly different in the LSD test level α = 5%.

callus percentage of 75%, this indicated that differences in the concentrations of growth regulators used showed different response of explants cultured *in vitro* as expected (Table 2).

Callus structure

One of the markers applied to determine the quality of a callus is callus structure. The results of visual investigation of the callus structure formed on each explant did not show any differences between treatments of various concentrations of 2,4-D and 2-iP, generally the callus formed was predominantly friable and partly compact in structure. Compact callus has a dense and hard texture, which is composed of small cells that are very tightly packed, while friable callus has a soft texture and is composed of cells with many intercellular spaces. Callus with a friable and compact structure was found in the d1p1 treatment (5 µM 2,4-D + 5 µM 2-iP); d2p2 (10 µM 2,4-D + 10 µM 2-iP); and d1p4 (5 µM 2,4-D + 20 µM 2-iP) while the other treatments resulted in a friable callus structure (Table 3).

Callus color

An indicator of explant growth in *in vitro* culture in the form of callus color that describes the visual appearance of the callus. So that it can be seen whether a callus still has cells that are actively dividing or have died. Based on visual observations of the callus color formed on liberica coffee leaf explants at the age of 16 weeks after culture, it showed that the callus appeared in various different colors (Table 4).

Callus size

The results showed that there was a significant interaction between the application of various concentration levels of 2,4-D and 2-iP concentrations on the callus size of composite stump liberica coffee leaf explants. Our experiment revealed that application of d4p4 (20 µM 2,4-D + 20 µM 2-iP) resulted in the largest callus size of 1.96 cm, followed by d1p3 treatment (5.0 µM 2,4-D + 15 µM 2-iP), d1p4 (5 µM 2,4-D + 20 µM 2-iP), d2p2 (10 µM 2,4-D + 10 µM 2-iP), and d1p1 (5 µM 2,4-D + 5 µM 2-iP), while the smallest callus size was found in the d2p3 treatment (10 µM 2,4-D + 15 µM 2-iP) which was 1.24 cm (Table 5).

Percentage of embryo somatic formation

The results revealed that there was a significant interaction between the application of various concentration levels of 2,4-D and 2-iP on the percentage of somatic embryo formation. Based on Table 6, it can be seen that d1p1 (5 µM 2,4-D + 5 µM 2-iP) and d2p2 (10 µM 2,4-D + 10 µM 2-iP) treatments gave the highest percentage of somatic embryo formation, namely 18.75% was followed by d3p3 (15 µM 2,4-D + 15 µM 2-iP) and d4p4 (20 µM 2,4-D + 20

Table 2. Effect of different concentrations of 2,4-D and 2-iP on the percentage of callus forming from explants of young leaves of liberica coffee Tungkal composite (16 weeks after culture)

2,4-D (µM)	2-iP (5 µM)	2-iP (10 µM)	2-iP (15 µM)	2-iP (20 µM)
5	100.00 (A) a	81.25 (B) b	100.00 (A) a	100.00 (A) a
10	93.75 (B) b	100.00 (A) a	75.00 (C) c	87.50 (B) b
15	100.00 (A) a	87.50 (B) b	87.50 (B) b	87.50 (B) b
20	100.00 (A) a	75.00 (B) b	93.75 (B) b	100.00 (A) a

Note: Numbers followed by the same lowercase letters (rows) and uppercase letters (columns) are not significantly different in the LSD test level α = 5%.

Table 3. Callus structure formed on young leaf explants of Tungkal composite Liberica coffee cultured on medium with various concentration levels of 2,4-D and 2-iP (16 weeks after culture)

2,4-D (µM)	2-iP (5.0 µM)	2-iP (10 µM)	2-iP (15 µM)	2-iP (20 µM)
5	Friable and compact with predominance of friable structure	Friable structure	Friable structure	Friable and compact with predominance of friable structure
10	Friable structure	Friable and compact with predominance of friable structure	Friable structure	Friable structure
15	Friable structure	Friable structure	Friable structure	Friable structure
20	Friable structure	Friable structure	Friable structure	Friable and compact with predominance of friable structure

Table 4. Callus color appeared on young leaf explants of liberica coffee Tungkal composite cultured on medium with various concentration levels of 2,4-D and 2-iP (16 weeks after culture)

2,4-D (µM)	2-iP (5.0 µM)	2-iP (10 µM)	2-iP (15 µM)	2-iP (20 µM)
5	Green Yellowish White	Brownish white Brownish green	White Yellowish	Brownish white Brownish
10	Brownish Yellow	White Yellowish	White Green Brownish	Green Brownish
15	Green Yellowish	White Green	Green White Yellowish	Yellowish Brownish
20	Green Yellowish Brownish	White Brownish Yellow	Yellowish Brownish	White Yellowish

Note: The color of the callus is determined based on direct observation by using Munsell Color Plant.

Table 5. Callus size formed on young leaf explants of liberica coffee Tungkal composite which were cultured on medium with various concentration levels of 2,4-D and 2-iP (16 weeks after culture)

2,4-D (µM)	2-iP (5.0 µM)	2-iP (10 µM)	2-iP (15 µM)	2-iP (20 µM)
5.0	1.73 (A) a	1.44 (B) b	1.83 (A) a	1.81 (A) a
10	1.51 (B) b	1.77 (A) a	1.24 (C) b	1.39 (B) b
15	1.62 (A) a	1.43 (B) b	1.62 (B) a	1.61 (B) a
20	1.66 (A) b	1.28 (C) b	1.57 (B) b	1.96 (A) a

Note: Numbers followed by the same lowercase letters (rows) and uppercase letters (columns) are not significantly different in the LSD test level α = 5%.

Table 6. Percentage of somatic embryos formed in young leaf explants of liberica coffee Tungkal composite cultured on medium with various concentration levels of 2,4-D and 2-iP (16 weeks after culture)

2,4-D (µM)	2-iP (5.0 µM)	2-iP (10 µM)	2-iP (15 µM)	2-iP (20 µM)
5.0	18.75 (A) a	0.00 (B) b	0.00 (B) b	0.00 (B) b
10	0.00 (B) b	18.75 (A) a	0.00 (B) b	0.00 (B) b
15	0.00 (B) b	0.00 (B) b	12.50 (A) a	0.00 (B) b
20	0.00 (B) b	0.00 (B) b	0.00 (B) b	12.50 (A) a

Note: Numbers followed by the same lowercase letters (rows) and uppercase letters (columns) are not significantly different in the LSD test level α = 5%.

µM 2-iP) treatments with a value of 12.50% while the callus in the other treatments failed to form an embryo somatic.

Figure 1 described that the developmental stages of the embryo from globular, heart and elongated embryos to torpedoes found in d1p1 (5 µM 2,4-D + 5 µM 2-iP) and d4p4 (20 µM 2,4-D + 20 µM 2-iP) treatments, while the other treatments up to 16 weeks of age did not lead to the formation of cotyledons and sprouts, that was assumed need more time for the cotyledon stages, germination to the formation of plantlets.

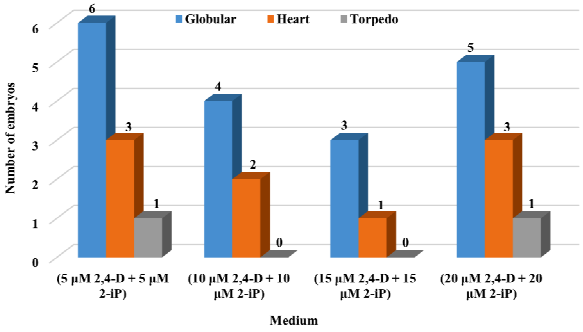

Figure 1. Number of globular, heart and torpedo embryos of liberica coffee Tungkal composite that formed at 16 weeks after culture

DISCUSSION

Experiments with the application of growth regulators 2,4-D and 2-iP at various concentrations had an effect on inducing callus of liberica coffee leaf explants. The treatment of various concentrations of 2,4-D and 2-iP on the variables observed showed an interaction on callus appearance, the percent of explants with callus, the size of the callus, and the percentage of somatic embryos formed as well [7]. This revealed that the growth regulators application of 2,4-D and 2-iP interacted with each other. According to Zulkarnain [40] that the presence of growth regulators are important in regard the response of cultured tissues, especially auxins and cytokinins are factors that become critical points. Therefore, to induce the development of explants as the desired direction, type and concentration of growth regulators applied to the medium is critical point to note, and also the formation of explant development due to the occurrence of callus proliferation from the surface of the explants being cultured [7].

In this experiment, callus began to form on the leaf margins or on the leaf veins, where the leaf veins are the food distribution areas to all parts of the leaf surface so that cells near the leaf veins can divide and form calluses. According to Indah and Ermavitalini [20] and Lizawati *et al.* [25], the appearance of callus on the injured part was thought to be due to stimulation of the explant tissue to cover the wound. Callus appearance time in 2,4-D and 2-iP treatment started from 19-23 DAC and the fastest time to produce callus was 19.8 DAC in d4p2 treatment (20 µM 2,4-D with 10 µM 2-iP) but not significantly different from the d1p1 treatment (5.0 µM 2,4-D + 5 µM 2-iP); d1p1 (10 µM 2,4-D + 5 µM 2-iP); d2p3 (10 µM 2,4-D + 15 µM 2-iP); d2p4 (10 µM 2,4-D + 20 µM 2-iP) and d3p4 (15 µM 2,4-D + 20 µM 2-iP). This situation proves that the use of 2,4-D and 2-iP is necessary to stimulate callus proliferation from cultured explants. The same result was reported by Damayanti *et al.* [10] that the average callus induction from Todolo Toraja coffee leaf explants begins to form at the age of 17-23 days after culture. The results showed that the use of 2,4-D (5-10 uM) combined with 2-iP (5-20 µM) was the best treatment for the initiation of callus formation.

The selection of growth regulators is one of the factors that determines the formation of callus in cultured plants. According to Wattimena *et al.* [34], the formation of callus in dicot plants requires a balance between growth regulators auxin and cytokinins. Type of auxin that most often used in callus culture is 2,4-D due to its strong activity to stimulate cell dedifferentiation, suppress organogenesis and maintain callus growth, this is because 2,4-D has a carboxyl group separated by carbon or carbon and oxygen. Meanwhile, 2-iP is a cytokinin that has high activity in stimulating cell division in plant tissue culture, which is known as the process of cytokinesis. Teixeira *et al.*

[30] also informed that the application of growth regulators to *Garcinia brasiliensis in vitro* cultures such as 2,4-D and 2-iP is needed for callus growth because the interaction of these growth regulators is able to respond to increased protein levels and cell mitotic activity.

The success of callus growth is expressed by the percentage of explants in forming callus, the observation for 16 weeks after culture showed that application of 5 µM 2-iP at all concentrations of 2,4-D used produced a significantly higher percentage of leaf callus than the other treatments. Different results were obtained from Ibrahim *et al.*'s research [19], 2,4-D 4.52 µM treatment with the addition of 2-iP 19.72 µM was the best medium for inducing callus from Robusta coffee leaf explants BP 436. According to Zulkarnain *et al.* [41] that the functioning of endogenous growth regulators in explant tissue was affected by the usage of exogenous growth regulators given to the media. Hence, the use of growth regulator given strongly depend on the type and dose applied to produce the best explants development.

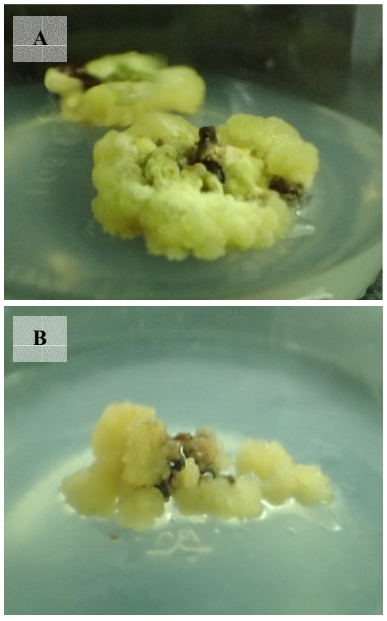

Figure 2. Callus structure formed from young leaf explants of liberica coffee cultured on 2,4-D medium with 2-iP (A) compact callus structure and (B) friable callus structure

There were 2 types of callus structure produced in this experiment, namely, friable callus structure and compact callus structure, but it was more dominated by crumb callus structure. Visually, the friable callus formed on the Liberica coffee leaf explants shows that the bonds between the cells appear tenuous, are easily separated and when removed with tweezers, the callus breaks easily and some stick to the tweezers (Figure 2). The friable structure of the callus is considered good because it makes it easier to separate into single cells in suspension cultures, besides that it will increase oxygen aeration between cells. Research by Lizawati *et al.* [22] from durian leaf explants also produced friable callus which had a wavy structure characteristic of growing apart into small fragments. Mahadi *et al.* [26] suggested that the friable callus structure has the characteristics of an easily decomposed texture, oxidation of phenolic substances is not easy, and the cells are easy to multiply. The formation of callus with a friable structure is thought to be stimulated by the presence of the endogenous auxin hormone which is produced internally by the explants that form the callus. Meanwhile, compact callus has a texture that is difficult to separate and looks solid. The compact callus structure is caused because the callus undergoes lignification formation so that the callus has a hard structure which is the effect of cytokinins which play a role in nutrient transport.

The color of the callus that appeared varies, namely; yellowish green, brownish green, yellowish white, brownish yellow and brownish. Based on the percentage of callus color, 25% yellowish green callus was obtained, 20% brownish white, 18% brownish green, 15% brownish and followed by 14% yellowish white callus. Table 5 shows that the treatments d1p1 (5 µM 2,4-D + 5 µM 2-iP), d1p3 (5 µM 2,4-D + 15 µM 2-iP), d2p2 (10 µM 2,4-D + 10 µM 2-iP), d3p2 (15 µM 2,4-D + 10 µM 2-iP) and d4p4 (20 µM 2,4-D + 20 µM 2-iP) produced a yellowish-white callus. The results of Ibrahim and Hartati's research [18] reported that the callus of Robusta coffee clone BP 308 which was cultured in 2,4-D treatment media with TDZ also produced callus which was crumbly and yellowish-white in color. The same thing was also reported by Ibrahim *et al.* [19] on the callus of Robusta coffee BP 436 which was cultured on 2,4-D media with 2-iP produced a yellowish white callus, it was suspected that the yellowish white callus was an embryogenic callus. Added by Liang *et al.* [21] and Arimarsetiowati *et al.* [6] that embryogenic callus is characterized by a yellowish callus color and a crumbly texture, this characteristic of callus has the potential to become a somatic embryo. Based on these results, it is expected that callus which is yellowish white can develop to form somatic embryo. Callus browning is thought to be due to the accumulation of phenolic compounds released by leaf explants, thereby inhibiting the process of somatic embryogenesis. Unlike the experimental results of Oktavia *et al.* [29], where somatic embryogenesis can still be formed from brown Arabica coffee leaf explants. It is possible that the phenolic compounds released act as signals for induction of differentiation and another possibility, these compounds also act as chelators that inactivate inhibitory compounds present in embryogenic cultures.

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chelators that inactivate inhibitory compounds present in embryogenic cultures.

The occurrence of callus proliferation from the surface of the explants being cultured found *in vitro* culture systems is a form of explant development. Gamborg and Shyluk [11] informed that *in vitro* callus formation is a result of the random and unequal development of unspecialized cells and loss of organized cell structure. This difference in callus size is influenced by the interaction and balance between growth regulators added to the media and growth hormones produced by plant cells endogenously by cultured cells [33]. The addition of exogenous auxins and cytokinins changed the concentration of endogenous cell growth regulators [31]. The effectiveness of exogenous auxin and cytokine growth regulators depends on the concentration of endogenous hormones in plant tissues. In this study auxin 2,4-D and cytokinin 2-iP were applied as the growth regulators. In regard of callus formation, the function of auxins is cell enlargement, while cytokinins responsible in cell breaking up and forming of chloroplast. Accordingly, combination of auxin 2,4-D and cytokinin 2-iP is assumed to stimulate fast cell development and proliferation.

In this experiment, somatic embryos began to develop towards the formation of globular embryos at the 16th week after culture, this situation took longer compared to the experiment conducted by Ardiyani *et al.* [5] which only took 6 weeks after culture from *C. liberica* clone leaf explants. Arruminensis cultured on MS medium with the addition of growth regulators BAP and 2,4-D. According to Zulkarnain [39], Ajijah and Hartati [3] stated that the ability of explants to totipotency expression highly rely on a amount of variables i.e. explant type, medium composition and the type of growth regulator used.

Based on Table 6, it can be seen that the application of a given growth regulator greatly influences the success of the formation of somatic embryos, where the balanced use of auxins and cytokinins can induce embryogenic callus to form somatic embryos. Seeing the opportunity in terms of saving the use of growth regulators used, the best media for forming somatic embryos from composite stump liberica coffee leaf explants is MS + 5 µM 2,4-D + 5 µM 2-iP. The same situation was also reported by Yelnitis [35] where the most abundant rattan tohiti somatic embryos were produced in the application of a balanced growth regulator, namely the BA 1.0 mg/L + 2.4-D 1.0 mg/L treatment. This is because exogenous hormones, especially auxins and cytokinins, are able to induce changes in the balance of hormone content in the callus culture thereby stimulating increased somatic embryo formation from the grown embryogenic callus. This exogenous hormone balance can affect the formation of somatic embryos because this hormone balance is needed to activate somatic cell division so that cells can rearrange gene expression and induce cell division towards callus growth and somatic embryogenesis [27].

In this experiment the d1p1 (5 µM 2,4-D + 5 µM 2-iP) and d4p4 (20 µM 2,4-D + 20 µM 2-iP) treatments were able to produce globular, heart and embryos extending to the torpedo but not on the formation of cotyledons and sprouts (Figure 3). According to Purnamaningsih [30]; Lizawati *et al.* [25], the use of the right media can induce the occurrence of all stages of embryo development, on the other hand, in an unsuitable medium, embryonic development is not visible. Furthermore, Yusnita [36] stated that the ability to regenerate plants is often species-specific, meaning that plants of different genotypes will often respond to regeneration of shoots or embryos that are also different. Other factors that also affect the ability of cells to form somatic embryos are closely related to the activity of specific genes that affect the somatic embryogenesis [37, 38]. Application of different dosage of growth regulators 2,4-D and 2-iP in the forming of somatic embryogenesis of *C. liberica* cv. Tungkal composite indicated an interaction with the observed variables when callus appeared, percentage of explants with callus, and callus size. The balanced application of growth regulators 2,4-D and 2-iP was able to induce the formation of somatic embryogenesis from liberica coffee immature leaf explants. The most optimal interaction for the development of embryo stages from globular, heart and torpedo was 5 µM 2,4-D + 5 µM 2-iP treatment.

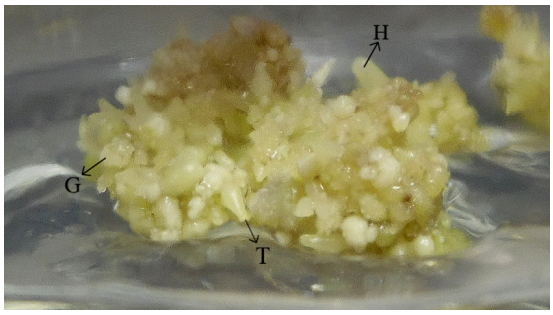

Figure 3. Developmental stages of the embryo from (G) globular, (H) heart and elongated embryo to (T) torpedo

This experimental activity was a contribution in the effort to build the modern clonal propagation of liberica coffee that has been known so far. The important findings of this study are application of various concentrations of growth regulators 2,4-D and 2-iP in the development of somatic embryogenesis of liberica coffee Tungkal composite described an interaction with the observed variables in related to callus appeareance, percent of explants with callus, and callus dimension. The balanced application of growth regulators 2,4-D and 2-iP was able to induce the formation of somatic embryogenesis from composite stump liberica coffee leaf explants. The most optimal interaction for the development of embryo stages from globular, heart and torpedo was 5 µM 2,4-D + 5 µM 2-iP treatment.

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