Evaluation of arabica coffee propagation using cell suspension culture

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Abstract. Arabica coffee is highly produced in Indonesia, thus need to be propagated using a powerful approach like cell culture in somatic embryogenesis for providing seedlings. The study aimed to evaluate the use of cell cultures in somatic embryogenesis on several Arabica coffee genotypes. The embryogenic calli (200 mg) were subcultured into solid and liquid regeneration media (half concentration of Murashige and Skoog [MS] added with 2 mg/L of kinetine, 35 g/L of sucrose, 400 mg/L of casein hydrolysate, and mall extract). For the somatic embryos germination, MS media was used by adding it with 0.3 mg/L of BAP and 40 g/L of sucrose. The solid media was added with 2.5 g/L of phytagel. This study was used Factorial Completely Randomized Design (CRD) (genotype and media solidity) with 10 replications.

The results showed that production of the number of somatic embryos is highly depend on the genotype. The size of somatic embryos, fresh weight, and root length on the torpedo and germination phases in liquid media was higher than solid media. Using liquid media at germination phase isn’t recommended, because it can resulted hyperhidricity.

Keywords : Embryogenesis somatic, hyperhydricity, liqluid culture, plant genotype, solid culture

1. Introduction
Coffee propagation using somatic embryogenesis techniques is an important method in providing seedlings in large quantities, especially for new varieties generated through crosses or composites. This technique has been used in mass production of coffee seedlings [1]. Using liquid media proved more efficient to provide better results compared with solid media.

Cell aggregate in the form of embryogenic callus is the material required in cell culture. Embryogenic callus suspended in liquid media contains various nutrients and compounds needed for optimal growth, thus causing cells to be differentiated or undifferentiated. Cell suspension is placed on top of the shaker so that the cell aggregates are spread evenly in liquid media, and gas exchange occurs. Cells will continue to grow until one of the factors becomes a barrier that slowers the cell growth. Initially, cells are subcultured in media containing hormones that will activate specific growth [2,3].

Plant cell cultures cauld be utilized for biochemical studies of cell physiology, plant propagation, metabolism, protoplast fusion, transformation, cryopreservation, and producing secondary metabolites [4]. In some studies the use of cell culture in plant propagation is easier and time-efficient [5,6].
The use of liquid media in vitro culture not only reduces production costs, but also can streamline the multiplication method because it can be applied to mass multiplication using a shaker or bioreactor. The use of cell suspension cultures allows constant regulation of conditions at each phase of development of the curvature [7,8]. The use of liquid media in coffee embryogenesis has been reported by Ettienne since 2005 [9]. Previous studies showed that each type of coffee has a varied response to the growing media composition in use [9,10], therefore using a different genotype requires new study. Considering wide production of Arabica coffee to meet consumer’s demand, its multiplication to provide large seedlings should be put in highly effort using this cell culture method. This study aimed to evaluate the use of cell cultures in somatic embryogenesis of some particular Arabica Coffee genotypes.

2. Materials and methods

The study had been conducted from March 2016 to October 2017 in the Tissue Culture Laboratory, Indonesian Industrial and Beverage Crops Research Institute (IIBCRI). Two Arabica coffee varieties, i.e. Sigarar Utang and Andung Sari 2 (AS2K) and one local superior of Arabica coffee from Garut Residency, i.e. Arabica Yellow Garut (AGK) grown in the glass house culture collections were used as plant materials.

2.1. Explant sterilization

Young leaves of the three Arabica coffee genotypes were cleaned under running water for ± 10 minutes, soaked for one hour in 0.1% mixture solution consisted of fungicide (0.2% mankozeb) and bactericide (15% streptomycin sulphate and 1.5% oxytetracycline). The leaves were rinsed with water and put into a laminar air flow. The samples were then sterilized by dipping for 15 minutes in 50% alcohol followed with 0.25% and 0.35% sodium hypochlorite solutions. Finally, the leaves were rinsed 3 times with sterile aquades. The treated leaves were cut to small pieces (± 10 mm x 10 mm) and then put into the treatment media.

2.2. Callus initiation

Half concentration of Murashige and Skoog (MS) medium added with sucrose 30 g L⁻¹, polyvinyl polypyrrolidon (PVPP) 250 mg L⁻¹, phytagel 2.5 g L⁻¹ was used as a basal medium for calli induction. For the calli induction of AGK Arabica coffee cultivar, the basal medium ada enriched with 2,4-D 4.52 μM +2-iP 24.65 μM, whereas the AS2K and Sigarar Utang followed the method of Ibrahim [10], i.e 2.4-D 4.52 μM + 2-iP 19.72 μM and 2,4- D 4.52 μM + 14.79 μM, respectively. The calli induction media were sterilized using an autoclave at 121°C and 1.5 psi for 20 minutes. The calli formed the explants were subcultured to an advanced callus induction media containing a half-concentration MS media added with casein hydrolyzate 200 mg L⁻¹, malt extract 800 mg L⁻¹, 2,4-D 4.52 μM, BAP 17.76 μM, sucrose 30 g L⁻¹, and phytagel 2.5 g L⁻¹. The cultures were then incubated in a dark room at ± 25 °C.

2.3. Evaluation of arabica coffee propagation using solid and liquid medium

The embryogenic callus was subcultured into a half concentration MS medium containing vitamin B5 (modified), sucrose 30 g L⁻¹, and Kinetin 9.30 μM as a growth promotion (Fig 1A). The cultures were incubated in a dark room at 25 °C (± 1°C). The liquid calli culture were shaked at a speed of 100 rpm (Fig 1B). The torpedo phase somatic embryos formed were then germinated on the MS media enriched with BAP 0.3 mg / l based on the formulation from Etienne in 2005 [11]. The cultured bottles were irradiated in 1000-1500 lux for 16 hours in temperature 25°C and relative humidity ± 60%.

The experiment was designed in a complete randomized, 2 factors. The first factor was genotypes and the second was calli media densities (with and without phytagel 2.5 g L⁻¹). The treatments were repeated 10 times. The number of germinations formed and morphological performances were
observed. The collected data were analysed using Duncan significant difference at 5% significant level.

Figure 1. A. Embryogenic callus performance of Arabica coffee. B. Embryogenic callus using liquid media in the shaker with a speed of 100 rpm.

3. Results and discussion

Embryogenic callus formations of three Arabica coffee genotypes were first seen in the liquid induction media 4 months after treatments. The somatic embryos were indicated with the formations of a globular phase then elongated months and torpedo phase after 6 months. The embryogenic calli formations in the solid media was slightly slower than in the liquid, i.e. the elongated and torpedo phase were formed after 6 months and 8 months, respectively (Figure 2).

The faster formation and development of the somatic embryos of Arabica coffee in liquid media presumably because the embryogenic cells and tissues absorbed more nutrients as the results of having surface areas than those grown in the solid media. The stirring and shaking of the embryonic cultures in the liquid media enabled the cells to separate and blend in the media. Also, the stirring increases aeration in cell suspension cultures, especially it is important in the submerged calli cultures [12,13].

Figure 2. Performance of somatic embryogenesis in liquid and solid media A. Pro embryonic phase in liquid media. B. Globular phase in liquid media. C. Globular phase on solid media. D. The elongated phase in liquid media. E. The elongated phase on solid media. F. Torpedo phase in liquid media.

Statistical analysis indicated that there was no interaction between the Arabica coffee varieties and media densities treatments. The calli medium treatments significantly induced different number of
somatic embryos of Arabica coffee tested. The highest number of torpedo and germinated somatic embryos types were produced in AS2K, followed by AGK and Sigarar Utang varietis (Table 1). The study showed that AS2K Arabica coffee variety was more responsive than the two varieties tested. This result was similar to previous report in other Arabica coffee genotype [14] and date palm [15].

Table 1. Number of somatic embryos of torpedo and germination phase of Arabica coffee varieties formed in regeneration and germination media

| Varieties                  | Number of Somatic Embryos |          |          |
|----------------------------|---------------------------|----------|----------|
|                            | Torpedo Phase             | Germinated Phase |
| Andungsari 2 K (AS2K)      | 79.95 a                   | 72.05 a  |
| Arabika Garut Kuning (AGK)| 74.10 b                   | 67.95 a  |
| Sigarar Utang              | 68.65 c                   | 72.65 b  |

The effects of liquid media in inducing the torpedo and germination calli somatic embryo phase are not significantly different (Table 2). This is most likely due to the different compositions of the liquid and solid media. The previous study showed that calli and somatic embryos of Arabica coffee in the calli liquid media is better than on the solid media [16,17].

Table 2. Number of somatic embryos on torpedo and germination phase of Arabica coffee varieties formed in solid and liquid media.

| Media Density | Number of Somatic Embryos |          |          |
|---------------|---------------------------|----------|----------|
|               | Torpedo Phase             | Germination Phase |
| Solid         | 73.47                      | 65.80    |
| Liquid        | 75.00                      | 60.30    |

Statistical analysis revealed that there is no interaction between varieties treatment and media density on the fresh weight and length of the somatic embryo at the torpedo phase. In contrast to the number of somatic embryos of the torpedo and germination phase which significantly showed a real interaction between varieties, the parameters of the fresh weight and length of the somatic embryo of the torpedo phase were not significant (Table 3). In the treatment of media density, the parameters of the fresh weight and length of the somatic embryo showed a significant difference (Table 4). Cultures grown into liquid media appeared to have heavier fresh weight compared with solid cultures, as well as the length of the somatic embryo torpedo phase. The size of secondary somatic embryos of Arabica coffee globular, oblong, torpedo and germination phase that are regenerated on semi-solid media, the size is also larger compared to solid media, but not classified as abnormal [18].

Table 3. Somatic embryo fresh weight and length on torpedo phase of three Arabica coffee varieties

| Varieties                  | Somatic Embryo Torpedo Phase |          |          |
|----------------------------|------------------------------|----------|----------|
|                            | Fresh weight (gram)          | Length (cm) |
| Andungsari 2 K (AS2K)      | 0.041                        | 0.40     |
| Arabika Garut Kuning (AGK)| 0.043                        | 0.44     |
| Sigarar Utang              | 0.039                        | 0.45     |

Table 4. Somatic embryo fresh weight and length on torpedo phase on two media density

| Media Density | Somatic Embryo Torpedo Phase |          |          |
|---------------|------------------------------|----------|----------|
|               | Fresh weight (gram)          | Length (cm) |
| Solid         | 0.033 b                      | 0.36 b   |
| Liquid        | 0.048 a                      | 0.50 a   |
The lack of interaction was also observed in the treatment of varieties and media densities in the height and length parameters of the germination roots. The height and length of the germination roots were not evident between varieties, but were proved in the density of the media (Tables 5 and 6). The height and length of the sprouts of the Sigarar Utang variety appeared higher than the other two varieties. In the liquid media, the height and length of the root appeared higher than in the solid media.

Table 5. Somatic embryo height and root length on germination phase of three Arabica coffee varieties

| Varieties                  | Somatic Embryo Germination Phase |          |          |
|----------------------------|--------------------------------|----------|----------|
|                            | Height (cm)                    | Root length (cm) |
| Andungsari 2 K (AS2K)      | 1.15                           | 0.40     |
| Arabika Garut Kuning (AGK)| 1.17                           | 0.44     |
| Sigarar Utang              | 1.18                           | 0.45     |

Table 6. Somatic embryo height and root length on germination phase on two media density

| Media Density | Somatic Embryo Germination Phase |          |
|---------------|---------------------------------|----------|
|               | Height (cm)                      | Length of Root (cm) |
| Solid         | 1.00 b                           | 0.50 b   |
| Liquid        | 1.33 a                           | 0.36 a   |

Morphological observations in the somatic embryo phase of the germination showed symptoms of hyperhydricity in the resulting germinatin. The cotyledons appear pale green, while the roots are transparent white (Figure 3). Hyperhydricity or also known as vitrification is a change in morphology, anatomy and physiology that occurs in plant tissue culture. The main symptom of hyperhydricity is the plants being succulent, characterized by lack of chlorophyll and high water content. Symptoms of verrification in liquid media were also reported by Pancaningtyas in 2013 [19], who conducted research on the development of somatic cocoa embryos on cocoa plants. The use of semi-solid media in Arabica coffee germination media reported by Ibrahim et al in 2017 [16] also experienced symptoms of mild hyperhydricity.

Figure 3. Performance of Arabica coffee germination from cell culture grown on liquid media. A. The cotyledons is greenish white. B. Transparent roots indicating symptoms of hyperhydricity.
Germination affected by hyperhydricity may be difficult to develop into a normal plantlet, it will grow with pale green leaves, with stems and roots that are succulent. According to Franck et al. (2004)[20], hyperhydricity is characterized by irregular stomata location, underdeveloped cell walls, large intracellular space in the mesophyll cell layer, reduced cuticle layer and number of palisade cells. This condition is certainly not expected to occur in germinations or plantlets produced, because it can cause the acclimation process to fail.

This study showed that the use of liquid media as germination media can cause hyperhydricity. Hence it is advised to use liquid culture only on regeneration media to avoid the symptoms of hyperhydricity in Arabica coffee propagation through somatic embryogenesis, while germination media may use solid media, or use a temporary immersion system (TIS). The use of a temporary immersion system (TIS) was conducted using RITA in coffee propagation using somatic embryogenesis has also been carried out by Albarran et al., (2005), Ibrahim et al., (2017) and Etienne (2018)[21,16,9]. The use of solid media on germination phase is expected to reduce the symptoms of hyperhydricity, whereas to avoid the occurrence of hyperhydricity in liquid culture is to reduce immersion and subculture in liquid media [19].

4. Conclusions
Liquid media can accelerate the formation of somatic embryos. The number of somatic embryos produced is influenced by the Arabica coffee genotype used. Fresh weight, length of somatic embryo at torpedo stage, germination height and root length using liquid media were higher compared to solid media. The time for formatting somatic embryos in the liquid media was shorter than in the solid media. The use of liquid media in the germination stage of somatic embryogenesis can cause hyperhydricity. To avoid hyperhydricity, it is recommended to use solid media or temporary immersion system (TIS) on Arabica coffee germination media.

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