

In vitro somatic embryogenesis of superior clones of robusta coffee from Lampung, Indonesia: Effect of genotypes and callus induction media

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Abstract. Hapsoro D, Hamiranti R, Yusnita Y. 2020. In vitro somatic embryogenesis of superior clones of robusta coffee from Lampung, Indonesia: Effect of genotypes and callus induction media. Biodiversitas 21: 3811-3817. This study aimed to investigate the effects of genotypes and primary callus induction media on somatic embryogenesis of superior robusta coffee clones of Lampung. Leaf explants of clones Tugusari, Komari, Tugino, and Wanto were cultured on two types of primary callus induction media (PCIM). PCIM1 consisted of half-strength MS salts, 30 gL⁻¹ sucrose, added with (mgL⁻¹) 0.1 thiamine-HCl, 0.5 nicotinic acids, 0.5 pyridoxine-HCl, 100 Myo-inositol, 200 ascorbic acids, 150 citric acids, and 1 benzyl adenine. PCIM2 consisted of NPCM salts, 30 gL⁻¹ sucrose, added with (mgL⁻¹) 15 thiamine-HCl, 1 nicotinic acid, 1 pyridoxine-HCl, 2 glycines, 130 Myo-inositol, 200 ascorbic acids, 150 citric acids, 1 2,4-dichlorophenoxyacetic acid, and 2 thidiazuron. The highest percentage (100%) of primary callus formation was found in Komari and Wanto clones. PCIM2 resulted in more primary calli than PCIM1. When subcultured to embryogenic callus induction medium, primary calli of clone Komari and Wanto developed into a high percentage of embryogenic calli, while those of the other two turned brown and died. PCIM2-derived primary calli developed into more embryogenic calli. When subcultured on somatic embryo (SE) regeneration medium, these calli underwent the formation of SE of various stages. When subcultured to plant regeneration medium, these SEs developed into plantlets.

Keywords: 2.4 D, *Coffea canephora*, culture, regeneration, thidiazuron

INTRODUCTION

Robusta coffee (*Coffea canephora* Pierre ex Frohner) from Lampung, Indonesia, is well-known as one of the Indonesian specialty coffees which have distinctive taste and flavor. This is due to its low acidity and a range of dark- chocolate, nutty, woody, and thick-bitter taste (<http://indonesiacoffees.com>). However, the average yield of coffee in Lampung is very low (722 kg ha⁻¹) (Directorate General of Estate Crop 2018) compared to that in Brazil (1,800 kg ha⁻¹) (USDA 2018). One of the main constraints is the limited availability of high-quality planting materials.

Robusta coffee plantations in Lampung are dominated by small-holder plantations that use seedlings as planting materials. Since robusta coffee is a cross-pollinating plant, these seed-derived planting materials led to genetically non-uniform plant populations due to genetic segregation. This could result in low productivity. Other planting materials are old coffee trees as rootstocks grafted with local high-yielding clones as scions. These agricultural practices often end up with variable success in terms of individual plant yield. This is because not only is robusta coffee a cross-pollinating, but also a self-incompatible plant (Anim-Kwapong et al. 2010; Nowak et al. 2011; Moraes et al. 2018; da Silva et al. 2019). A high yield could be achieved by growing multi-clonal planting materials arranged in alternate rows (Wintgens and Zamarripa 2009). This would need a large number of

clonally-propagated planting materials of several productive clones. One way to fulfill the needs is by use of in vitro propagation through somatic embryogenesis. In vitro somatic embryogenesis is a serial developmental process by which a somatic cell of explants undergo de-differentiation into stem cells followed by re-differentiation into embryos under suitable conditions (Guan et al. 2016; Guerra et al. 2016). This pattern of in vitro plant regeneration has been widely used as a powerful biotechnological tool for the clonal propagation of various important plants (Ducos et al. 2007; Mazri and Mezziani 2015; Guerra et al. 2016; Egertsdotter. 2019; Meira et al. 2019). Besides, somatic embryogenesis is also applicable for synthetic seed production (Cheruvathur et al. 2012), germplasm conservation, and cryopreservation (Guan et al. 2016). Coffee plants regenerated through in vitro somatic embryogenesis were found to be true-to-type and non-significantly different from those produced by cuttings (Ducos et al. 2003).

The regeneration of plants through in vitro indirect somatic embryogenesis consists of several steps, i.e., primary callus induction, embryogenic callus establishment, somatic embryo (SE) development, plant regeneration of somatic embryos (Yusnita and Hapsoro, 2011). In vitro somatic embryogenesis of both robusta and arabica coffee has been reported (Samson et al. 2006; Ducos et al. 2007; Ahmed et al. 2013; Etienne et al. 2013; Ardiyani et al. 2017; Ibrahim et al. 2017; Muniswamy et al. 2017). However, it is well-documented that in vitro somatic embryogenesis is

highly affected by genotypes of mother plants; it means that different genotypes show different responses to the same treatment (Naranjo et al 2016; Campos et al. 2017). Hence, it is necessary to find a reproducible protocol of somatic embryogenesis of robusta coffee clones of Lampung. Among superior Lampung robusta coffee clones are Tugino, Wanto, Komari, and Tugusari (Ramadiana et al. 2018). In addition to genotypes, the primary callus induction medium was reported to be crucial for in vitro somatic embryogenesis of coffee (Samson et al. 2006). The present study was conducted with the objectives of investigating the effects of genotypes and primary callus induction medium on in vitro somatic embryogenesis and regeneration of plants of Lampung robusta coffee.

MATERIALS AND METHODS

Plant materials and sterilization

Two-year-old plants grown from orthotropic stem cuttings were used as sources of explants. The youngest fully expanded leaves were harvested from the second or third leaf pairs position from the apex. The leaves were taken from four selected Lampung robusta coffee clones (Tugino, Wanto, Komari, and Tugusari). Leaves were thoroughly washed under running tap water, soaked in fungicide solution 0.2% (Mancozeb 80%) for 15 minutes, dipped in ethanol 70% for five seconds, shaken with NaOCl 0.5% solution plus 1-2 drops of Tween-20 for 10 minutes in a laminar airflow hood, followed by three rinses with sterile distilled water.

Culture steps

This experiment consisted of four steps of in vitro culture, namely (1) primary callus induction, (2) embryogenic callus establishment, (3) somatic embryo development, and (4) plant regeneration of somatic embryos (SE). All cultures of steps 1, 2, and 3 were incubated in a dark room, while that of step 4 was incubated under illumination with continuous cool-white fluorescent light of approximately 2000 lux. The temperature of the culture room was set at $25\pm 2^\circ\text{C}$.

Primary callus induction

Leaf segments (0.5 cm x 0.5 cm) in size with the main vein in the middle part were planted on two different primary callus induction media (PCIM). PCIM1 consisted of 1/2MS salts (Murashige and Skoog, 1962), 30 g L⁻¹ sucrose, added with (mgL⁻¹) 0.1 thiamine-HCl, 0.5 nicotinic acids, 0.5 pyridoxine-HCl, 100 Myo-inositol, 200 ascorbic acids, 150 citric acids, and 1 benzyl adenine (BA). PCIM2 consisted of NPCM salts (Samson et al. 2006), 30 g L⁻¹ sucrose added with (mgL⁻¹) 15 thiamine-HCl, 1 nicotinic acid, 1 pyridoxine-HCl, 2 glycines, 130 Myo-inositol, 200 ascorbic acid, 150 citric acids, 1 2,4-dichlorophenoxyacetic acid, and 2 thidiazuron. The media were solidified with 7% agar powder before being autoclaved. After 4 weeks in culture, percentage of explants forming calli was recorded and primary calli were scored as described in Tabel 1.

Embryogenic callus establishment

After 1-passage culture (4 weeks) on PCIM, the primary calli were subcultured to embryogenic callus induction medium (ECIM) for three consecutive passages with 4-week intervals. After the first passage on ECIM, callus clumps were separated from explant tissues and subcultured to the same fresh medium. ECIM consisted of modified MS salts, 1 mg L⁻¹ 2,4-D, 4 mg L⁻¹ BA, 30 g L⁻¹ sucrose, 200 mg L⁻¹ Myo-inositol, 20 mg L⁻¹ thiamine-HCl, 20 mg L⁻¹ glycine, 40 mg L⁻¹ cysteine, 22 mg L⁻¹ adenine, 800 mg L⁻¹ malt extract and 200 mg L⁻¹ casein hydrolysate (Samson et al. 2006). The percentage of cultures forming embryogenic calli was observed at the end of the third passage on ECIM.

Somatic embryo development and plantlet regeneration

The embryogenic calli grown on ECIM were selected and then cultured for 16 weeks on the somatic embryo regeneration medium (SERM). Subcultures were done every 4 weeks. SERM consisted of modified MS salts, 1 mgL⁻¹ BA, 30 gL⁻¹ sucrose, 200 mgL⁻¹ Myo-inositol, 10 mgL⁻¹ thiamine-HCl, 1 mgL⁻¹ nicotinic acid, 1 mgL⁻¹ pyridoxine-HCl, 2 mg L⁻¹ glycine, 14.7 mg L⁻¹ adenine, 400 mg L⁻¹ malt extract, and 400 mg L⁻¹ casein hydrolysate (Samson et al. 2006). Scanning electron microscopy (SEM) was used for photography of embryogenic callus at the end of the third and fourth passage in this medium. Somatic embryos formed during the third and fourth passage on ECIM were selected and transferred into plant regeneration medium (PRM) for two consecutive passages. The composition of PRM was the same as that of SERM except that the SERM contained 1 mgL⁻¹ BA.

Media preparation

All components of media were solubilized and the media was set at pH 5.8 before being mixed with 7 g L⁻¹ agar powder. The media mixture was boiled and dispensed into 250-ml culture vessels, 30 ml per vessel. All of the vessels containing media were autoclaved for 15 minutes at a temperature of 121°C and pressure of 1.2 kg/cm².

Experimental design

This experiment was conducted in Plant Science Laboratory University of Lampung, from August 2018 to July 2019. The experiment was carried out in a completely randomized design with three replicates. The treatments were factorially arranged, consisting of two factors, i.e. primary callus induction media (PCIM1 and PCIM2) and clones (Tugino, Wanto, Komari, and Tugusari). Each experimental unit consisted of three culture vessels, 5 explants per vessel.

RESULTS AND DISCUSSION

All of the explants cultured on primary callus induction media (PCIM) appeared expanding during the second week of culture. In the third week, primary calli started to form at the cut edges of explants and continued to proliferate. Table 1 showed that at the end of the fourth week, clones

Komari and Wanto produced more calli per explant than Tugusari and Tugino in both PCIM1 and PCIM2. They also showed a higher percentage of callus formation (100%) than the other two clones in both media. These data indicate that clones Komari and Wanto are more responsive in terms of callus formation than Tugusari and Tugino. The significance of genotype effect on callus induction ability in this experiment was in agreement with those reported by previous studies with *Coffea arabica* (Irene et al. 2019), barley (Han et al. 2011), lettuce (Mohebodini et al. 2011), and cocoa (Quainoo and Dwomo 2012).

Concerning the effects of media, Table 1 showed that PCIM2 resulted in higher callus formation than PCIM1. This might be attributable to the use of 2,4-D and TDZ in PCIM2. It has been well documented that 2,4-D in combination with TDZ induced the formation of callus and somatic embryos (Samson et al. 2006; Quainoo and Dwomo 2012; Ibrahim et al. 2013). The use of BA was also intended to induce callus formation in coffee (Ducos et al. 2007), even though in many cases it is not for callus induction. All of the calli were compact, semi-transparent, and white-yellowish in color (Figure 1).

Table 2 showed that the primary calli of Komari and Wanto produced a high percentage of embryogenic calli when cultured on ECIM, while those of Tugusari and Tugino produced no embryogenic calli. At the end of the second passage, most of the PCIM-derived calli of Komari and Wanto continued to proliferate to produce embryogenic calli, while those of Tugusari and Tugino did not proliferate, became slimy (soft and transparent) and eventually turned brown and died at the end of the third passage. This indicates that as for Tugusari and Wanto, the PCIMs did not provide the condition for their cells to become embryogenic competent. Other reports also showed that genotypes of mother plants also affected the formation of embryogenic calli (Quainoo and Dwomo 2012; Naranjo et al. 2016; Campos et al. 2017; Mwaniki et al. 2019; Narváez et al. 2019).

Table 1. Percentage of explant forming primary callus from each clone on two different primary callus induction medium (PCIM) after four weeks in culture.

Primary callus induction medium *)	Clones	Percentage of callus formation	Score of calli **)
PCIM1	Tugusari	57.8 ± 8.0	+
	Komari	100.0 ± 0.0	++
	Tugino	48.9 ± 8.0	+
	Wanto	100.0 ± 0.0	+++
PCIM2	Tugusari	82.22 ± 5.9	+
	Komari	100 ± 0.0	++++
	Tugino	64.44 ± 4.4	+
	Wanto	100 ± 0.0	++++

*) PCIM₁: Half-strength MS salts + 1 mg L⁻¹ BA. PCIM₂: NPCM salts + 1 mgL⁻¹ 2,4-D and 2 mgL⁻¹ TDZ. **) -: No callus formation, +: Callus covered < 25% of explant cutting edge, ++: Callus covered 25% to < 50% of explant cutting edges, +++: Callus covered 50 to 75% of explant cutting edges, ++++: Callus covered >75% of explant cutting edges

Table 2. Embryogenic callus formation on embryogenic callus induction medium (ECIM) after three consecutive passages with 4 weeks interval.

Medium for primary callus induction	Clones	Clumps of primary callus forming embryogenic callus on ECIM (%)
PCIM1	Tugusari	0.0
	Komari	75.0
	Tugino	0.0
	Wanto	65.5
PCIM2	Tugusari	0.0
	Komari	82.9
	Tugino	0.0
	Wanto	80.0

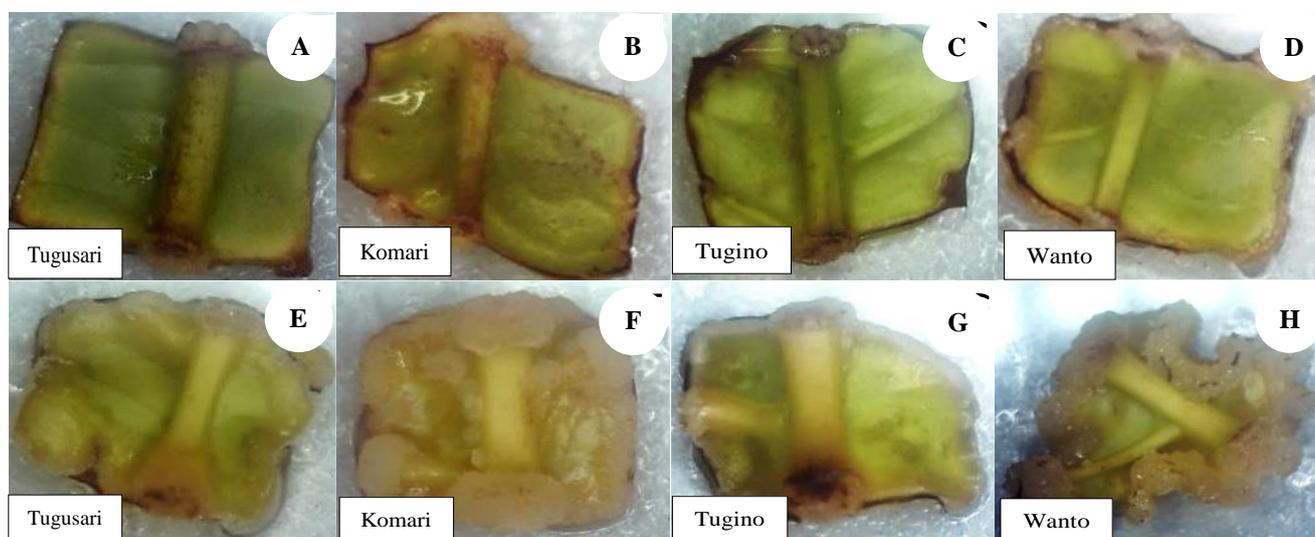


Figure 1. Primary calli formed on leaf explants from four clones (Tugusari, Komari, Tugino, and Wanto) cultured for four weeks on primary callus induction medium (PCIM). A-D: cultures on PCIM1. E-H: cultures on PCIM2

Table 2 also showed that when subcultured to ECIM, primary calli from PCIM2 resulted in a higher percentage of embryogenic calli than those from PCIM1. This suggested that calli from PCIM2 were more embryogenic competent than those from PCIM1. This might be due to the use of auxin (2,4-D) in PCIM2 compared to the use of cytokinin (BA) in PCIM1. Nic-Can and Loyola-Vargas (2016) mentioned the essentials of 2,4-D for the initiation of somatic embryogenesis. Méndez-Hernández (2019) capitalized on the importance of adding auxins in culture media for the induction of embryogenic calli which could undergo somatic embryogenesis. Somatic embryogenesis of various plant species used an auxin alone or in combination with a cytokinin for callus induction (Verma et al. 2011; Ahmed et al. 2013; Al-Hussaini et al. 2015; Zhang et al. 2016; Guillou et al. 2018; Yang et al. 2018; Swamy et al. 2019; Bhusare et al. 2020). At the end of the second passages, most of the PCIM1-derived callus of Komari and Wanto continued to proliferate and underwent changes in color, becoming white and opaque in appearance (Figure 2.A-C), while those originated from PCIM2 formed more friable calli, and gradually change its color from white to yellowish or slightly brown (Figure 2.D-F).

Scanning electron microscopy (SEM) of calli showed that after eight weeks on ECIM, part of the cells appeared as a spherical form (Figure 3a) arranged in clusters. These morphological characteristics were also reported by Padua et al. (2014) in *Coffea arabica* cv. 'Catiguá MG2. Recent review papers also mentioned the same feature of cell embryogenic (Campos et al. 2017). Padua et al. (2014) reported that the embryogenic cells were small (24x21µm). We found embryogenic cells in *Coffea canephora* even smaller than that (approximately 10 µm in diameter) and much smaller when microscopy was conducted at an earlier stage of embryogenesis. Our data showed that at the end of

the second passage (8 weeks on culture), the embryogenic cells were less than 2 µm (Figure 3.A). These cells were getting more in number and larger in size when the calli were subcultured to the same fresh medium for another passage (Figure 3.B). By comparing the micrograph of Figure 3.A and 3.B, from the end of the second passage (8 weeks in culture) to the end of the third passage (12 weeks in culture) on ECIM medium, the embryogenic cells were getting larger from approximately less than 2 µm in diameter (Figure 3.A) to approximately 10 µm in diameter (Figure 3.B). The appearance of embryogenic cell clusters and the increase in cell number on ECIM could indicate that embryogenic cells sporadically appeared, then divided. Subsequently, cell division occurred when the cells were very small (Figure 3.A), then they enlarged (Figure 3b). It is interesting to know whether the large cells could undergo cell division or developed into somatic embryos at the globular stage. It is also interesting to know whether, in robusta coffee, somatic embryos are one-cell or multi-cell origin. This information would hopefully complement with SEM of somatic embryo development in robusta coffee from globular to cotyledonary stage reported by Nic-Can et al. (2013).

After 12 weeks on ECIM, the embryogenic calli were transferred to the somatic embryo regeneration medium (SERM) for four consecutive passages. On this medium most of the embryogenic calli continued to grow and develop, becoming more friable and yellowish-brown in color. During the third and fourth passages on this medium, most of the embryogenic calli developed to form asynchronous structures of somatic embryos (SEs), i.e. in globular (G), heart-shaped (HS), torpedo (T), and cotyledonary (C) stage (Figures 4 and 5). This might be due to the use of a semi-solid medium. Unlike a liquid medium, this medium might cause non-uniform uptake of nutrients and plant growth regulators.

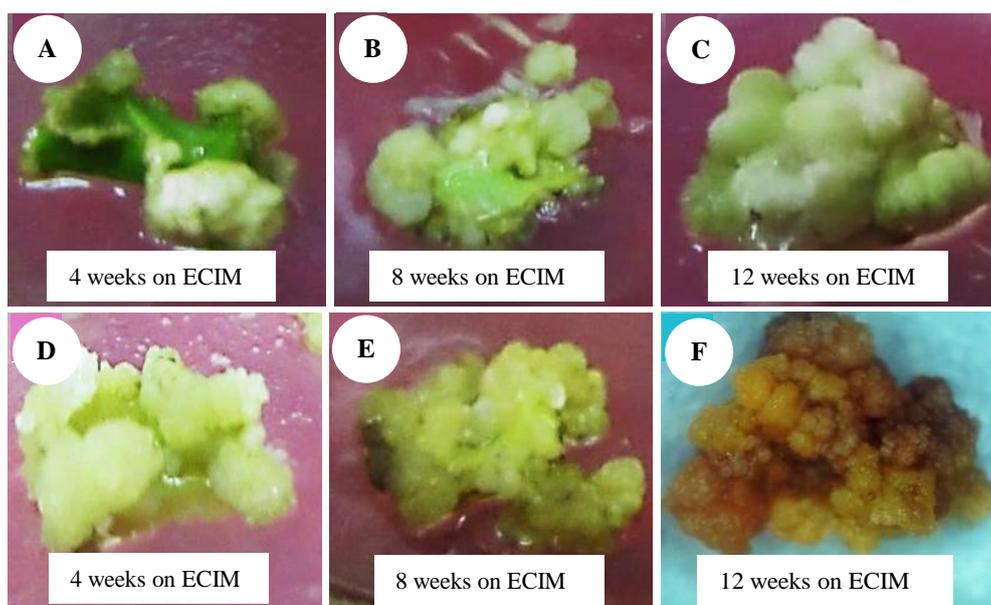


Figure 2. Callus cultures of the coffee plant of clone Komari on ECIM medium for 4, 8, and 12 weeks. A, B, and C were cultures derived from the PCIM1 medium, while D, E, and F were those derived from the PCIM2 medium. PCIM1 and PCIM2: primary callus induction medium. ECIM: embryogenic callus induction medium

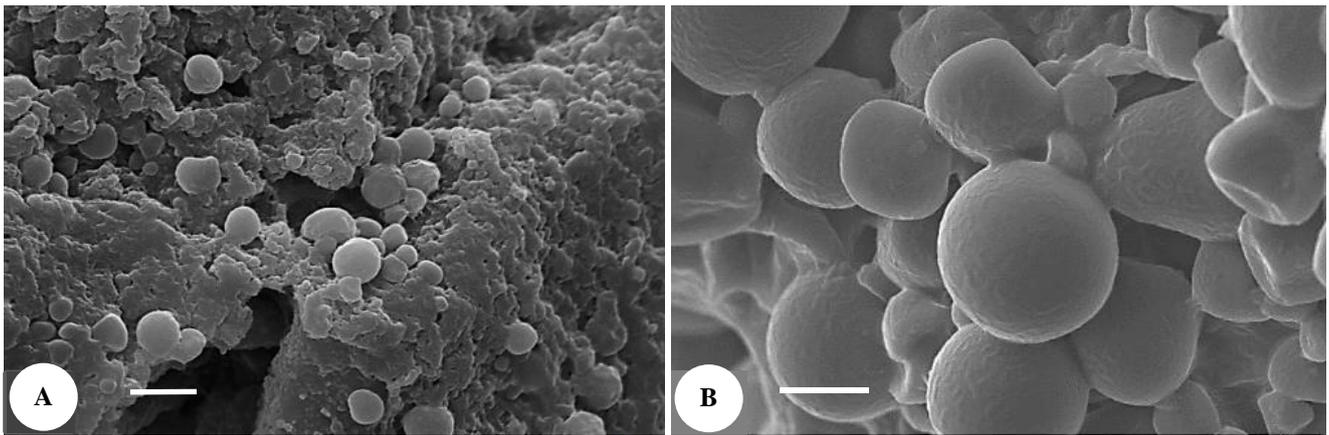


Figure 3. Scanning electron microscopy of calli cultured on an embryogenic callus induction medium (ECIM). A. 8 weeks in culture, with spherical cells (approximately 1 µm in diameter) arranged in clusters. Bar = 2 µm; 15,000 x magnification. B. 12 weeks in culture, with spherical cells of approximately 10 µm in diameter covering a large part of calli. Bar = 10 µm; 1500 x magnification

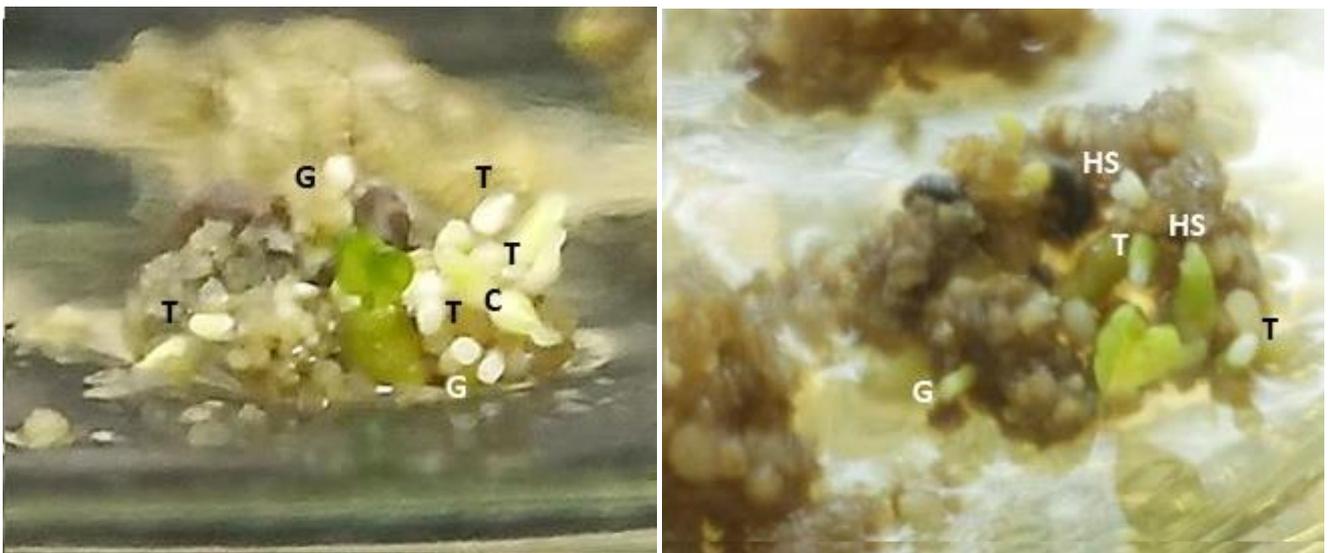


Figure 4. A synchronous formation of somatic embryos of various stages, i.e., globular (G), heart-shaped (HS), torpedo (T), and cotyledonary (C) after 12 weeks on somatic embryo regeneration medium (SERM)

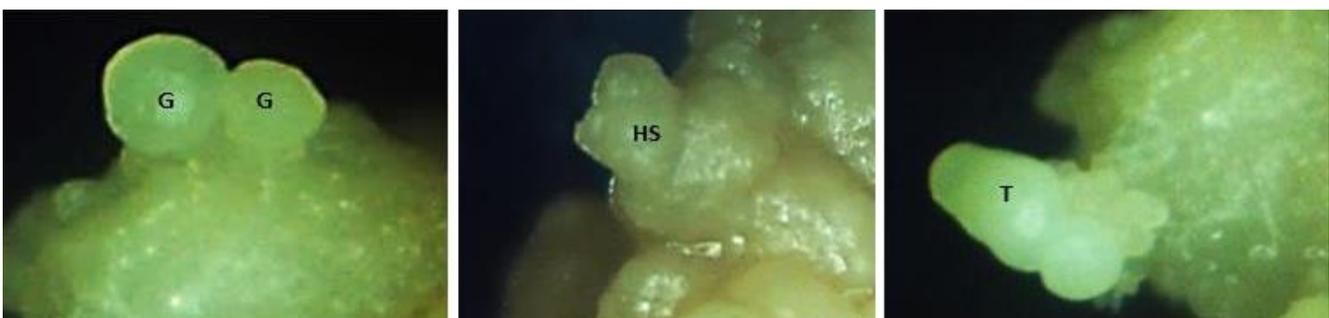


Figure 5. Various stages of somatic embryos, i.e., globular (G), heart-shaped (HS), and torpedo (T) somatic embryos

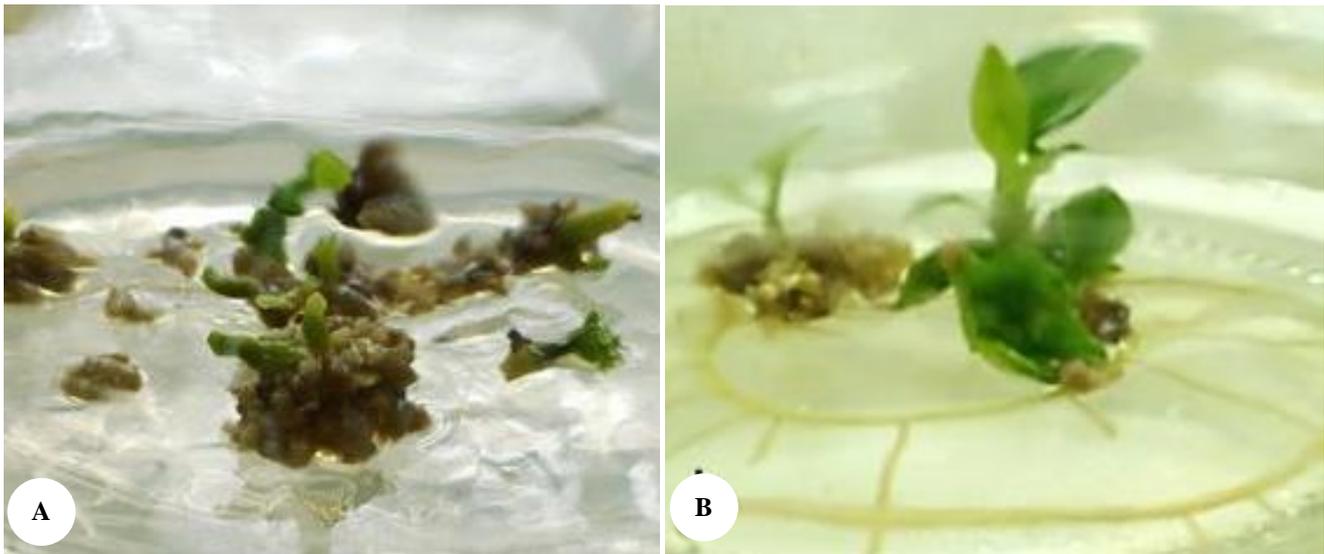


Figure 6. Robusta coffee somatic embryos started to germinate after 4 weeks on plant regeneration medium (PRM) (A), and developed into plantlets after 8 weeks on PRM (B)

When somatic embryos from ECIM were selected and transferred into plant regeneration medium (PRM) for two consecutive passages (8 weeks) they germinated (Figure 6.A) and grew into plantlets (Figure 6.B).

We reported here successful *in vitro* plant regeneration of Lampung robusta coffee from leaf explants through somatic embryogenesis. This regeneration consisted of four stages, i.e. primary callus induction, embryogenic callus establishment, somatic embryo regeneration, and plant regeneration of somatic embryos. Clone Komari and Wanto were very responsive to primary callus induction medium (PCIM) and embryogenic callus induction medium (ECIM). Embryogenic calli of clone Komari developed into somatic embryos and finally to plantlets. PCIM2, which contained 1 mg L⁻¹ g 2,4-D and 2 mg L⁻¹ g TDZ, was more effective than PCIM1, which contained 1 mg L⁻¹ g BA, for primary callus induction. Due to genotype-specific nature of coffee on *in vitro* somatic embryogenesis, this report provides new knowledge on *in vitro* propagation of coffee. This finding would be useful for developing a protocol of *in vitro* propagation of superior local robusta coffee clones through somatic embryogenesis.

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