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Regeneration and development of *Coffea arabica* L. plants through indirect somatic embryogenesis

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ABSTRACT

Micropropagation of AS2K clones Arabica coffee (*Coffea arabica* L.) was attempted through indirect somatic embryogenesis by using ten different parts of the leaf such as shoot, first leaf base, second leaf base, third leaf base, first leaf middle, second leaf middle, third leaf middle, first leaf tip, second leaf tip, and third leaf tip. The influence of the part of leaf explants, combination of plant growth regulator (PGRs) in the induction of embryogenic callus and regeneration of embryo somatic were studied. Furthermore, the various protocols to induce regeneration of somatic embryo into plantlet through different step of subculture and the use of various germination medium were demonstrated. The morphological characteristics and histological analysis of embryogenic callus and embryo development were observed. In this experiment, it was observed that the M5 medium supplemented with 1 mg/L 2,4-D, 1 mg/L BAP and 4 mg/L 2-ip was closely associated with third leaf tip explants for induction of embryogenic callus. The maximum number of globular, heart-shape, torpedo and cotyledones (18, 4, 12, 4, respectively) were achieved on ERM6 medium containing 2 mg/L BAP without activated charcoal on 90th day for regeneration of embryo somatic. The length of roots is the most influence paramater on plantlet regeneration, and the 17th protocol which used B medium, large embryos and twice phase of subculture from liquid medium to solid medium is the best protocol for plantlet regeneration. The protocol developed could be useful highly for large-scale micropropagation in these commercially important Arabica coffee clones.

Key words: Indirect somatic embryogenesis; 24-D; BAP; histology; anatomy.

1 INTRODUCTION

Coffee is one of the two major commodities in the world. The agriculture of coffee provides livelihood to more than 80 million people and more than 11 million hectares are cultivated around the world annually (Loyola-Vargas et al., 2016). Arabica coffee (*Coffea arabica* L.) is one of the most commercial powerful crops for more than 80 countries and accommodates more than 10.2 millions hectares of land in the tropical and subtropical region of the world, mainly in Latin America, Africa, and Asia (Mishra; Slater, 2012) and it is highly valuable for beverage consumption universally. Arabica coffee is the most extensively commercially cultivated species in the world (Gatica; Arrieta; Espinoza, 2007). It is favorable beverage quality with low caffeine and chlorogenic acid content (Ogutu et al., 2022). Most Arabica coffee is more resistant to the most fungal diseases, including leaf rust and coffee berry disease compare to the Robusta coffee (*Coffea canephora*) (Silva et al., 2006).

Conventional breeding of coffee is difficult because of the long duration of processes involved in selection, hybridization, progeny evaluation, backcrossing before seeds are set (Etienne et al., 2018). Somatic embryogenesis (SE) is a plant biotechnology approach to produce somatic embryos that is extensively used for plant regeneration, micropropagation, and genetic improvement through stable genetic transformation and genome editing (Valencia-Lozano et al., 2021). SE is a method in which somatic cells can dedifferentiate into totipotent cells within certain procedures and regenerate their establishment toward the embryogenic cycle when prompted (Fehér; Pasternak; Dudits, 2003; Jiménez, 2005; Karami et al., 2009; Guan et al., 2016; Aguilar-Hernández; Loyola-Vargas, 2018). A somatic embryo is a bipolar formation that undergoes from a somatic cell without the need for vascular contact to the original cell, nearly identical to a zygotic embryo (Von Arnold et al., 2002).

SE has been effectively conquered on an industrial level to multiply at large scale F1 Hybrids of *C.arabica* species (Bobadilla Landey et al., 2013). There have been several reports on somatic embryogenesis in *C. arabica* (Campos; Panis; Carpentier, 2017; Nic Can et al., 2015; Bartos et al., 2018; De Almeida, 2020; Etienne et al., 2018). In coffee, this process has been achieved via direct somatic embryogenesis from pro-embryogenic cells directly formed on leaf explant tissue i or by indirect somatic embryogenesis (ISE) via the previous formation of a friable embryogenic callus before the development of somatic embryogenic structure initiated from the callus (Molina et al., 2002). ISE in coffee comprises a sequence of steps including callus induction and proliferation, and embryo development, as well as germination and regeneration into plants (van Boxtel; Berthouly, 1996). Numerous strategies and procedures have been evaluated with varying levels of success

using synthetic auxin (2,4-D) as an inducer of ISE (Etienne et al., 2013; Loyola-Vargas et al, 2016; Campos; Panis; Carpentier, 2017; Georget et al., 2017; Freitas et al., 2019; Valencia-Lozano et al., 2019; Awada et al., 2020; Nic-Can; Loyola-Vargas, 2016).

Therefore, the successful of coffee micropropagation depend on the interaction between explants with plant growth regulator (PGR) that supplemented in the nutrient medium and its concentration level. PGRs have exhibited effects on growth and development in somatic embryogenesis. Cytokinins promotes cell division while auxin promote both cell division and cell growth (Lee et al., 2011). Moreover, somatic embryogenesis depend on the genotype, explant age and developmental stage, and culture condition in solid medium and or liquid medium. The aim of this research was to establish the most reliable, efficient, and reproducible protocol for induction of embryogenic callus of AS2K clone Arabica coffee (*C. arabica L.*) by studying the influence of plant growth regulator *in vitro* culture system at various of media composition from different part of leaf explants for inducing embryogenic callus as well as their regeneration of somatic embryo. We also studied the influence of *in vitro* culture system at various of media composition, subcultures stages and embryo size for high frequency of plantlet conversion.

2 MATERIAL AND METHODS

2.1 Source of plant material, sterilization and explant preparation

Arabica coffee of AS2K clone is the result of individual selection on the population of Catimor ancestry from Brazil with the number C-1662-10-3, selected with the selection number KB II/61/3, then tested clonally and released with the clone name Andungsari 2K. This variety has a high potential productivity of 2,3 tons/Ha, good taste, and leaf rust tolerance (Hulupi, 2016). Mother plants of Arabica coffee of AS2K were established in coffee experimental field of Indonesian Coffee and Cocoa Research Institute at Andung Sari, Bondowoso, East Java, Indonesia. GPS coordinate and elevation of plot were recorded with data showing S07°55'42.7'' and E113°41'30.2'' and 1451 m asl. The leaves (i.e., 100 explants) from the plagiotropic branches were collected from healthy mother plants growing in the field as plant material.

The leaves were washed under running tap water and rinsed with sterile distilled water, followed by surface sterilization in 30% sodium hypochlorite solution (5.25% active chlorine) for 15 min and rinsed thrice using sterile distilled water. The leaf explants were subsequently immersed in 70% (v/v) ethanol for 20-30 s then rinsed thrice with sterile distilled water.

The margin and the mid-rib of each leaf were removed and the remaining leaf tissue was cut into 1.0 cm^2 pieces to be used as explants. The explants were incubated on a half-strength MS medium with the adaxial side down in the culture vessel. These materials were used as source leaf explants for indirect somatic embryogenesis under *in vitro* conditions in a laminar flow chamber.

2.2 Induction of embryogenic callus

To evaluate the effect of five different combinations medium on the induction embryogenic callus, leaf explants were cut by dividing from ten different part of leaf such as shoot, first leaf base, second leaf base, third leaf base, first leaf middle, second leaf middle, third leaf middle, first leaf tip, second leaf tip, and third leaf tip (Figure 1).

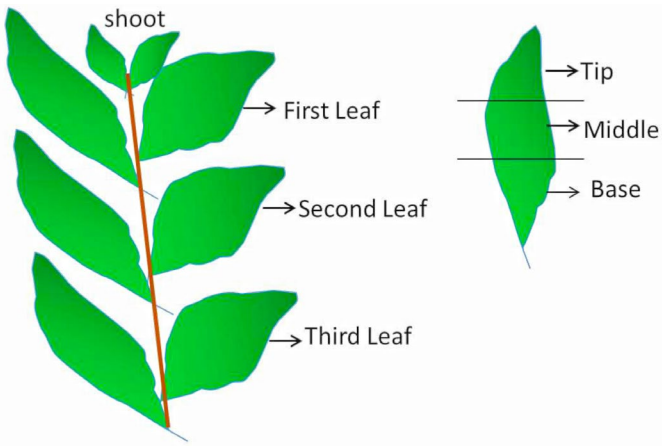


Figure 1: Different parts of leaf tissue *Coffea arabica* L. for induction of embryogenic callus.

Subsequently, leaf explants were cultured on a half-strength MS medium supplemented with various composiston medium (Table 1). The pH of all media was adjusted to 5.6, which was consistent throughout all experiments. The culture was incubated in a dark growth room at 25 ± 2 ˚C. The percentage of callus formation was recorded after eight weeks of culture. All of the experiments were carried out in a completely randomized design (CRD) and each set of callus induction experiment comprised of ten explants in triplicate.

2.3 Callus differentiation into somatic embryo

The embryogenic callus thus induced were then sub-cultured onto fresh embryo regeneration medium (ERM) and maintained under the same conditions for further 90 days. The ERM composed a half-strength MS containing different concentration (0.5, 1.0, 2.0 mg/l) BAP alone and in combination with 2.0 g/L activated charcoal for differentiation of somatic embryo and their maturation (Table 2). The cultures were maintained in the dark room at 27˚C with 75% relative humidity, for 90 days. Number of each phase embryo development which consist of globular, heart-shape,

torpedo and cotyledonary embryos was recorded. All of the experiments were carried out in a completely randomized design (CRD) and each set of differentiation somatic embryo experiments comprised seven callus fragments in triplicate.

Table 1: Composition of callus embryogenic induction medium of *C. arabica* L.

Medium components	Culture medium variants				
	M1	M2	M3	M4	M5
MS macro and micro salt	½	½	½	½	½
Gamborg's B5 vitamins	+	+	+	+	+
BAP. mg/L	3	2.25	2.25	–	1
2.4-D. mg/L	–	–	–	–	1
GA3. mg/L	0.05	–	–	–	–
IBA. mg/L	1	–	–	–	–
2-ip. mg/L	–	1	–	–	4
Kinetin. mg/L	–	–	5	1	–
L-cysteine. mg/L	–	30	30	33	80
NAA. mg/L	–	–	–	0.1	–
Casein hydrolysate. g/L	–	–	–	–	0.1
Malt extract. g/L	–	–	–	–	0.4
Sucrose. g/L	30	30	30	30	30
Gellan gum. g/L	4	4	4	4	4
pH	5.6	5.6	5.6	5.6	5.6

2.4 Germination of somatic embryos into plantlets

A completely randomized designs with 5 replications was designed to accomplish the 20 protocols of regeneration of somatic embryo into plantlets through the different step of subculture, size of embryo and germination medium. A brief description of the mentioned protocols is represented in Table 3. The embryoid as a starting material was collected from embryogenic callus induction. Embryoids were laid on solid medium directly in the first and second protocols. Solid medium is for inducing shoot and root that consists of two kinds medium. Medium A is composed of a half-strength of Murashige and Skoog (MS, 1962) medium supplemented with vitamin (1 mg/L piridoxine, 10 mg/L thiamine, 1 mg/L nicotinic acid), 30 gr/L sucrose, 0.7 µM IAA, 0.5 mg/L BAP, 2.3 mg/L Adenin, 0,1 mg/L NAA, 10% coconut water and 50 mg/L AgNO3. In the other hand, medium B contained a half-strength of Macronutrient of MS medium, a half-strength of Micro DKW supplemented with vitamin DKW, 2 mg/L glycine, 10 gr/L glucose, 5 gr/L sucrose. Moreover, embryoid was placed by immersing in 50 ml liquid medium at the third until twentieth protocols. The liquid medium consists of full hormone-free MS medium. These cultures were put on the shaker with speed of 100 rpm during 3 weeks for each phase of subculture to break and divide the embryoid cells and also to develop the embryoid into a perfect embryo. After three weeks, the mature embryo was placed on A dan B solid medium. Especially for nineteenth and twentieth protocols, transferring in liquid medium were conducted twice. In the third until eighteenth protocols, the liquid phase applied small, medium, large and mixed embryo explants which have been selected in each phase of sub-cultures. While, at the first, second, nineteenth and twentieth protocols, the embryoid was not selected.

The alteration of embryoid growth into mature embryo and germination embryo was observed at 8 weeks. The observations were carried out by measuring the percentage of the number of root formation, root length, hypocotyl length, percentage of open cotyledons and percentage of cotyledonary embryo. The observed data were interpreted by analysis of variance. If there is a significant difference, the differences between treatments are determined according to Tukey test at P=0.05.

Table 2: Composition of embryo regeneration medium (ERM) for differentiation and maturation somatic embryo of *C. arabica* L.

Medium components	Embryo regeneration medium (ERM)					
	ERM1	ERM2	ERM3	ERM4	ERM5	ERM6
MS macro and micro salt	½	½	½	½	½	½
Gamborg's B5 vitamins	+	+	+	+	+	+
BAP. mg/L	0.5	1	2	0.5	1	2
L-cysteine. g/L	0.04	0.04	0.04	0.04	0.04	0.04
Malt extract. g/L	0.8	0.8	0.8	0.8	0.8	0.8
Casein hydrolysate. g/L	0.2	0.2	0.2	0.2	0.2	0.2
Activated charcoal. g/L	2.0	2.0	2.0	–	–	–
Sucrose. g/L	30	30	30	30	30	30
Gellan gum. g/L	2.8	2.8	2.8	2.8	2.8	2.8
pH	5.6	5.6	5.6	5.6	5.6	5.6

Table 3: The 20 protocols of embryo germination at the various phase of subculture. the size of the embryo and germination medium.

Protocols	Embrioid	Phase I		Phase II		Phase III	
P1			MA				
P2			MB				
P3			SE		MA		
P4			ME		MA		
P5			LE		MA		
P6			MixE		MA		
P7			SE		MB		
P8			ME		MB		
P9			LE		MB		
P10			MixE		MB		
P11			MixE		MA. SE		
P12			MixE		MA. ME		
P13			MixE		MA, LE		
P14			MixE		MA. MixE		
P15			MixE		MB. SE		
P16			MixE		MB. ME		
P17			MixE		MB, LE		
P18			MixE		MB, MixE		
P19			MixE		MixE		MA
P20			MixE		MixE		MB

Note: SE: Small Embryos (globular and heart stage). ME: Medium Embryos (torpedo stage). LE: Large Embryo (long torpedo before entering the cotyledonary stage). Mix: Mix Embryos consist of small. medium and large embryos. MA (Medium A) and MB (Medium B) are germination medium. During the 19th and 20th protocols. liquid medium phase was repeated twice in the same medium prior to germination medium.

2.5 Statistical analyses

For all the experiments completely randomized design (CRD) was followed. The results were analyzed as mean ± standard errors (SE) and the significant difference ($p \leq 0.05$) among the mean was assesses by analysis of variance followed by Tukey test using Rstudio software (Version 1.4.1106). Principal component analysis (PCA), a multivariate statistical technique has been carried out with the findings from Table 4 and Table 5, pertained to the responses of callus induction by different part of leaf explants and the responses of 20 different protocols of *in vitro* cultures, respectively. The combination of different medium and different protocol were considered to be various treatments present at the bi-plots, which shows the overall distribution of the condired parameters. The analysis was performed at 95% confidence interval with Rstudio software package (Version 1.4.1106) to delineate the cumulative distribution of ten different explants in relation to considered five various medium, as well as the performance embryo in the 20 different protocol of plantlet conversion.

2.6 Morphological and histological analysis

The morphological characteristics of embryogenic callus through indirect somatic embryogenesis in AS2K clones Arabica coffee and embryos development were observed under a stereo-zoom binocular microscope (SZ 4045 TR Olympus® Tokyo, Japan) coupled to computer running image capture software (DP2-BSW). Histological analysis on the embryogenic callus and the differentiation of

somatic embryogeensis was observed for each developmental stage of somatic embryogenesis in the surface of explants by anatomic preparation using paraffin method according to Ruzin (1999). The anatomic samples in the glass slides were observed using light microscope (Olympus, Japan).

3 RESULTS

3.1 Induction of embryogenic callus

The efficiency of M1, M2, M3, M4, M5 media supplemented with different PGRs on the induction of embryogenic callus from different part of leaf explants was compared and the results are presented in Table 4. The characteristics of embryogenic callus were friable and yellowish with the presence of somatic embryos. There was a significant ($P \leq 0.05$) effect of all media on the percentages of embryogenic callus formation. It was noted that M5 media supplemented with 1 mg/L 2,4-D, 1 mg/L BAP and 4 mg/L 2-ip induced maximum 50% of third leaf tip explant to form embryogenic callus. In case of shoot explants, the same medium gave 33,11% of embryogenic callus, followed by second leaf tip (30,36%), third leaf base (22,24%), first leaf base (21,22%), third leaf middle (20%), second leaf middle (15,76%), first leaf middle (14,09%) and second leaf base (9,17%).

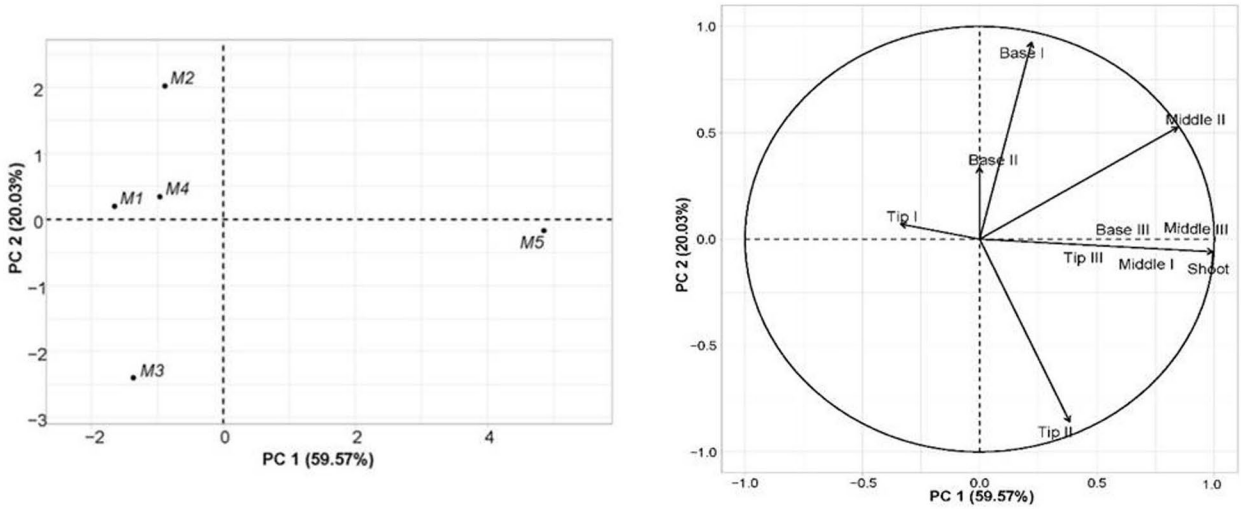
On M1 media supplemented with 3 mg/L BAP, 0.05 mg/L GA3, and 1 mg/L IBA, the highest percentage of embryogenic callus formation was 20% from the first leaf tip explant. On the other hand, the maximum percentage of embryogenic callus on M2, M3, M4 media was from first leaf base (32,17%), second leaf tip (42,28%), and first leaf base (21,22%), respectively. Result clearly indicated that M5 media is most suitable medium for calus embryogenic callus in third leaf tip explant.

Now to delineate the cumulative distribution of ten different types of explant for the induction of embryogenic callus on five culture medium a concerted effort has also been made by the use of principle component analysis (PCA). The reliability of this statistical tool has now been proved in different plant tissue culture experiments to conclude their findings (Żur et al., 2015; Navarro et al., 2017). In PCA, the data based on the percentage of the part of leaf explants forming embryogenic callus (Table 4), were projected over first two principal components. The eigenvalues of two PCs (PC1: 5.957, PC2: 2.003) and tehir percentage of variance (PC1: 59.57%, PC2: 20.03%) are presented in Figure 2. In this bi-plot, PC1 correlated positivily with shoot, first leaf base, third leaf base, first leaf middle, second leaf middle, third leaf middle, second leaf tip, and third leaf tip, whereas PC2 positively correlated with first leaf base, second leaf base, second leaf middle, and first leaf tip (Figure 2). The first leaf base and second leaf middle explants exhibiting positive correlation both in PC1 and PC2 indicated the promising responses accounting to the closer proximity with different media when compared to that other part of the leaf explants with same treatment. The M5 medium supplemented 1 mg/L 2,4-D, 1 mg/L BAP and 4 mg/L 2-ip was closely associated with third leaf tip explants, when compared to the other medium (M1, M2, M3, M4). It suggests that M5 medium might be considered as a better option than that of other medium to get the desired responses from the explants of *C. arabica* clone AS2K. Our data from Table 4 and PCA analysis clearly indicated that the third leaf tip explant is more suitable explant than the others, and M5 is the best culture medium.

Table 4: Effect of five different combinations medium on the induction callus from ten different part of leaf explants of *Coffea arabica* L. AS2K clone.

Medium	Percentage of explants forming embryogenic callus (%)									
	Shoot	Base leaf I	Base leaf II	Base leaf III	Middle leaf I	Middle leaf II	Middle leaf III	Tip leaf I	Tip leaf II	Tip leaf III
M1	0 c	12.5 ± 0.29 bc	6.42 ± 0.30 c	0 c	0 c	5.22 ± 0.12 c	0 c	20 ± 0.58 abc	0 c	0 c
M2	0 c	32.17 ± 10.87 abc	21.47 ± 0.29 abc	0 c	0 c	9.03 ± 0.58 bc	0 c	0 c	0 c	0 c
M3	0 c	0 c	11.04 ± 0.58 bc	0 c	0 c	0 c	0 c	0 c	42.28 ± 0.64 ab	0 c
M4	0 c	25 ± 0.58 abc	0 c	0 c	0 c	7.23 ± 0.62 c	0 c	0 c	11.04 ± 0.58 bc	0 c
M5	33.11 ± 0.59 abc	21.22 ± 2.44 abc	9.17 ± 0.60 bc	22.24 ± 0.62 abc	14.09 ± 0.58 bc	15.76 ± 5.66 bc	20 ± 0.58 abc	0 c	30.36 ± 1.84 abc	50 ± 0.58 a

Values are expressed as mean ± standard error (SE) of three replicated experiments. each with ten explants per treatments. Means followed by the same letters within columns are not significantly different at $P \leq 0.05$ level according to Tukey test. All data were taken after eight weeks of culture.



	Eigenvalue	Percentage of variance
PC1	5.957	59.575
PC2	2.003	20.031

	Coefficients of PC 1	Coefficients of PC 2
Shoots	0.994	-0.060
Leaf base I	0.221	0.925
Leaf base II	-0.001	0.336
Leaf base III	0.994	-0.060
Leaf middle I	0.994	-0.060
Leaf middle II	0.844	0.526
Leaf middle III	0.994	-0.060
Leaf tip I	-0.337	0.071
Leaf tip II	0.383	-0.858
Leaf tip III	0.994	-0.060

Figure 2: Bi-plot with first and second components of PCA based on the frequency of callus induction from ten different part of leaf explants (shoot. first leaf base. second leaf base. third leaf base. first leaf middle. second leaf middle. third leaf middle. first leaf tip. second leaf tip. and third leaf tip) of *Coffea arabica* L. AS2K clone. cultured on five different combinations medium (M1. M2. M3. M4. M5). Distributional bi-plot with 5 different composistion medium (M1-M5) shown in this figure. The eigenvalues of two PCs and their percentage of variance have been shown in this figure. In this bi-plot. PC1 correlated positively with shoot. first leaf base. third leaf base. first leaf middle. second leaf middle. third leaf middle. second leaf tip. and third leaf tip. whereas PC2 positively correlated with first leaf base. second leaf base. second leaf middle. and first leaf tip.

3.2 Callus differentiation into somatic embryo

In ERM medium, somatic embryos were successfully regenerated from embryogenic callus. Embryogenic callus were noticed that friable and yellowish (Figure 3B). The somatic embryo begins with a protrusion on the surface of the embryogenic callus which then produces several stages of somatic embryo development starting from the globular, heart-shape, torpedo and cotyledonary phases (Figures 3D). Differentiation and various stages of somatic embryo development were clearly seen when embryogenic callus was transferred to ERM medium containing various concentrations of BAP (0.5, 1, 2, mg/L) and supplemnted with the addition of 2 g/L activated charcoal.

The maximum number of globular, heart-shape, torpedo and cotyledonary (9, 3, 9, 1) were achieved on ERM6 medium containing 2 mg/L BAP without activated charcoal on 60th day

(Figure 3). While on 90th days, the number of globular, heart-shape, torpedo and cotyledonary increased by 18, 4, 12, 4, respectively (Figure 4). Somatic embryos are formed normally, completely and separate from one another. The number of somatic embryos formation was strongly influenced by the concentration of BAP added. Cotyledonary stage embryos was observed after 60 days of culture on ERM 4. Significants variations in mean number of globular, heart-shape, torpedo and cotyledonary embryos were observed among the various ERM medium (Figure 4) and the morphological analysis of development somatic embryogenesis in *C. arabica* were described in Figure 5.

3.3 Germination of somatic embryos into plantlets

The plantlet formation of AS2K clone Arabica coffee somatic embryos using 20 different protocols were comparable in the present study (Table 5). The 20 protocols devide based on two germination media (with and without plant growth regulator), three subcultures stages and four embryo size (small, medium, large and mixed), were chosen to assess their effect on embryos development and germination.

There was a significant ($P \leq 0.05$) effect of all protocols on the their percentages of rooted cotyledons, the length of roots, the length of hypocotyl, the percentages of opened cotyledons and the percentages of live cotyledons after 8 weeks of culture. These cotyledonary embryo will be transferred for plantlet regeneration in MS medium with free plant growth regulator. Among the protocols tested, the 17th protocol was the most effective, with 65,6% of rooted cotyledons, 5.12 cm of length of roots, 1.68 cm of length of hypocotyl, 23.2% of opened cotyledons and 100% of embryos germination at the end of 8 weeks which used the B medium, large embryos and twice phase of subculture from liquid medium to solid medium.

Now, the cumulative distribution of twenty different protocols for plantlet regeneration must be interpreted on their their percentages of rooted cotyledons, the length of roots, the length of hypocotyl, the percentages of opened cotyledons and the percentages of live cotyledons after 8 weeks of culture, a coordinated effort has also been contributed by the use of principle component analysis (PCA). In PCA, the data based on the effects of 20 different protocols *in vitro* cultures on the embryo germination in the performance embryo such as

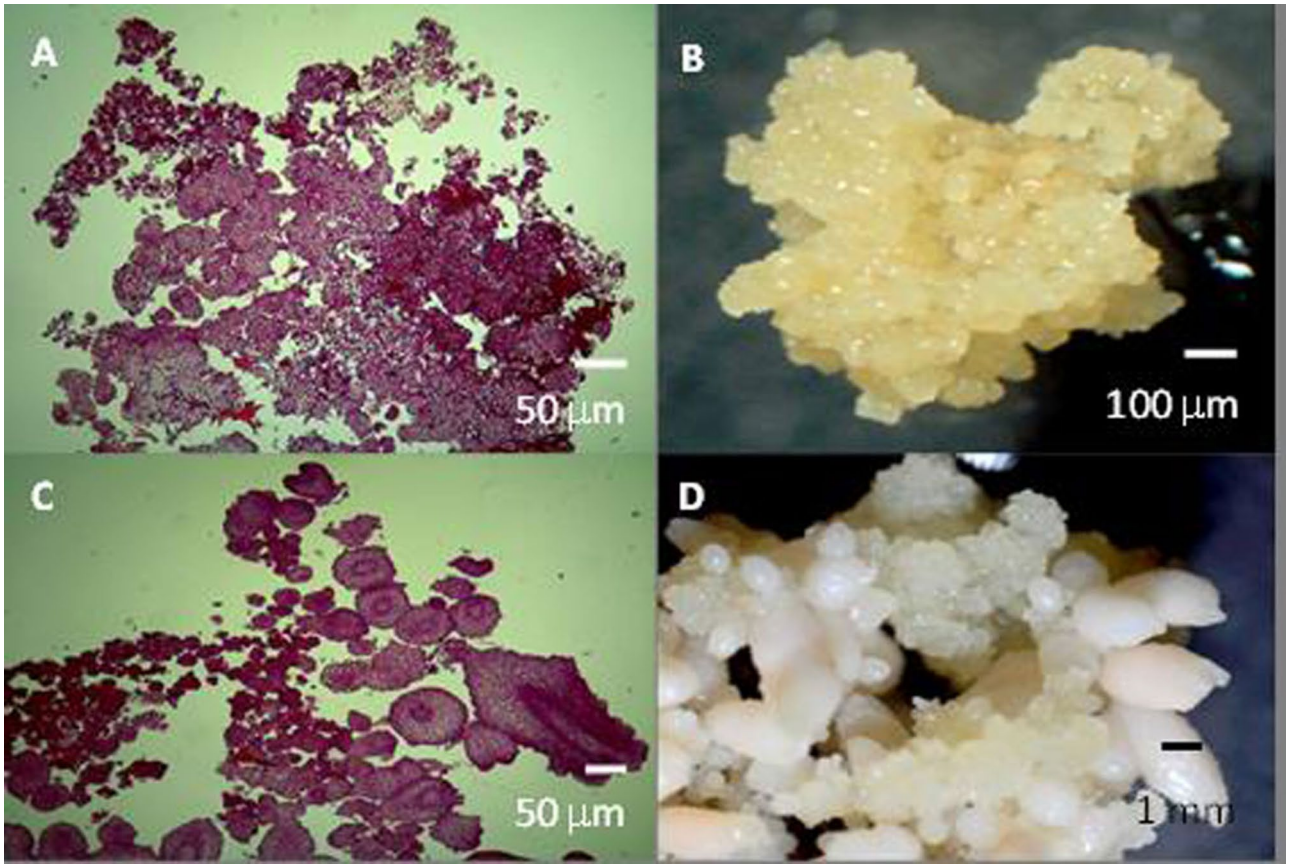


Figure 3: Histological and morphological characteristics of indirect somatic embryogenesis in AS2K clones Arabica coffee. (A and B) Embryogenic calli at 60 days after culture on CIM medium; (C and D) Somatic embryo clusters in differentiation phase of globular. heart. torpedo at 60 days after culture on ERM medium.

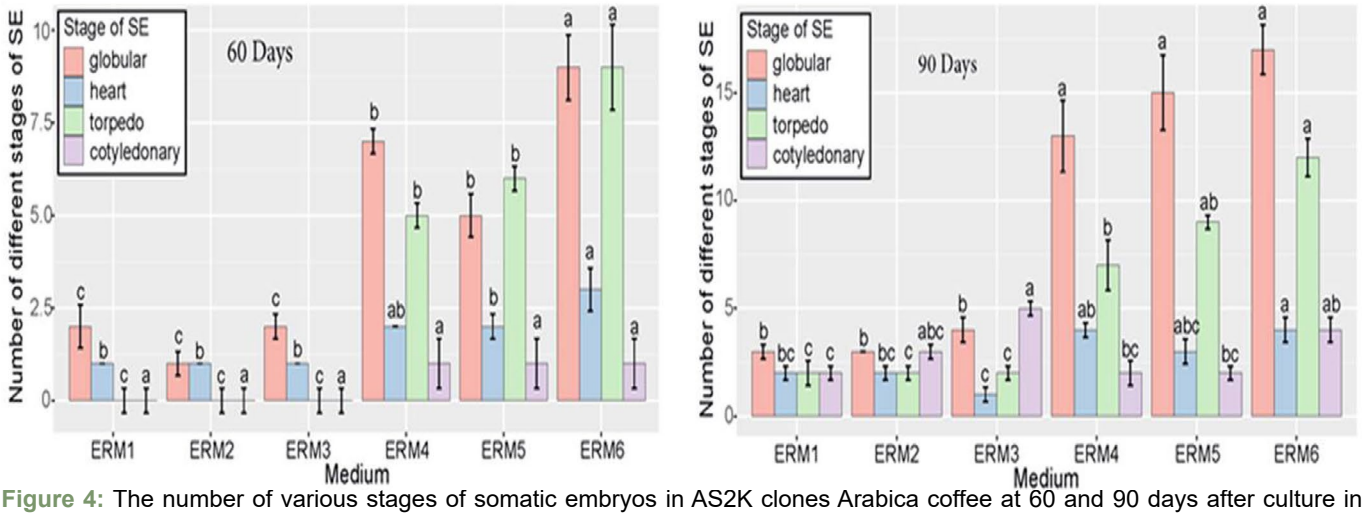


Figure 4: The number of various stages of somatic embryos in AS2K clones Arabica coffee at 60 and 90 days after culture in ERM (Embryo Regeneration Medium). ERM1: 0.5 mg/L BAP + 2 mg/L activated charcoal; ERM2: 1 mg/L BAP + 2 mg/L activated charcoal; ERM3: 2 mg/L BAP + 2 mg/L activated charcoal; ERM4 medium: 0.5 mg/L BAP; ERM5: 1 mg/L BAP +; ERM6: 2 mg/L BAP. Mean globular. heart. torpedo. cotyledonary number followed by the same letter do not differ by Tukey's test at 5% probability level ($P \leq 0.05$).

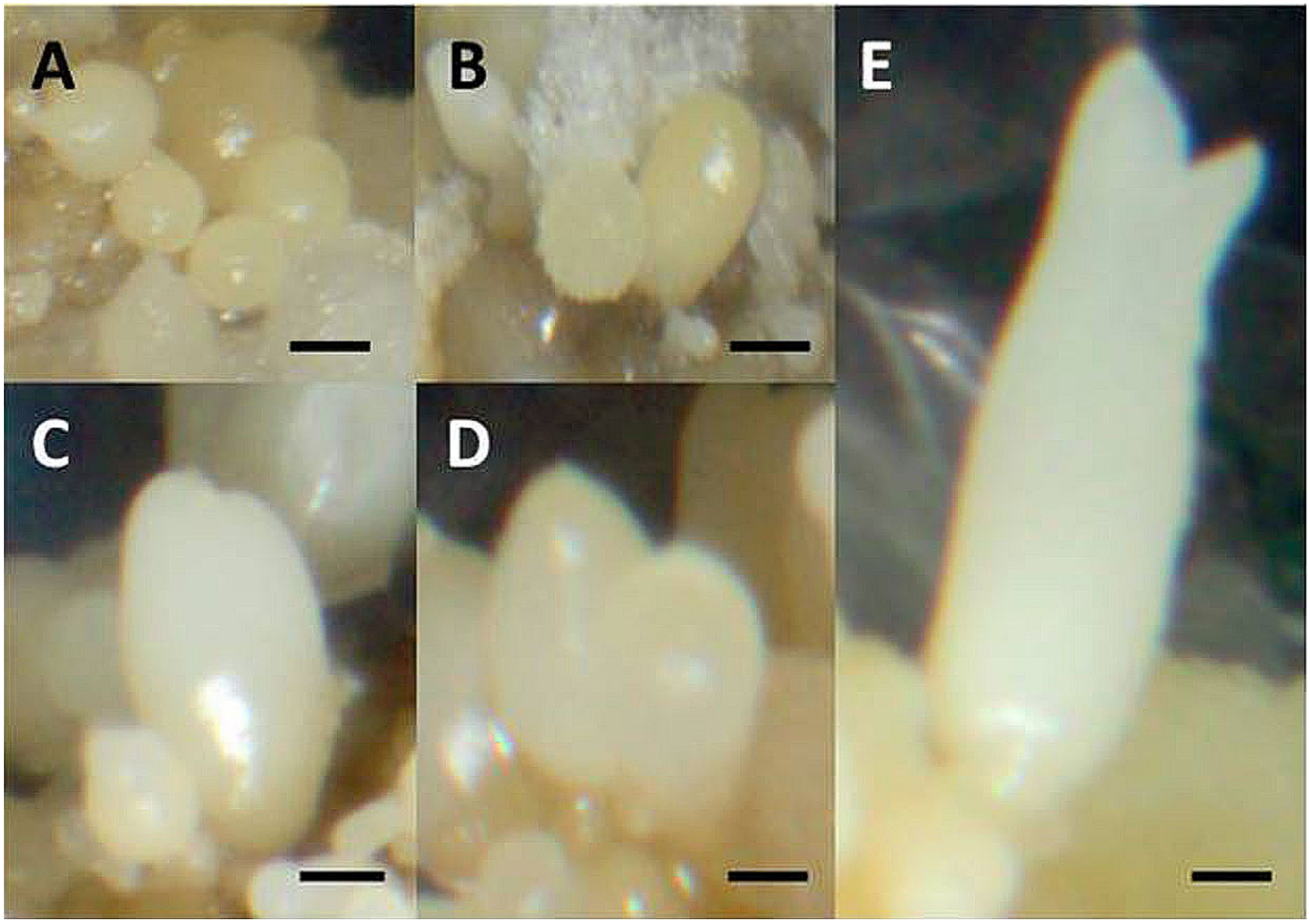


Figure 5: Morphological analysis of development somatic embryogenesis in *C. arabica*. A. Globular embryoid. B. Group of embryogenic cells forming preoembryogenic masses and proembryos. C. Elongated somatic embryoids with globular embryoid. D. Heart shape embryoid. E. Torpedo shaped embryoids. All the scale bar is set to 100 μm.

roots percentage, length of roots, length of hypocotyl, open cotyledonary and germination percentages of *C. arabica* L clone AS2K (Table 5), were projected over first two principal components. The eigenvalues of two PCs (PC1: 3.844, PC2: 0.605) and their percentage of variance (PC1: 76.884%, PC2: 12.097%) are presented in Figure 6. In this bi-plot, PC1 correlated positivily with all the parameter, susc as: roots percentage, length of roots, length of hypocotyl, open cotyledonary and germination percentages, whereas PC2 positively correlated with length of roots, length of hypocotyl and open cotyledonary (Figure 6). The length of roots, length of hypocotyl and open cotyledonary exhibiting positive correlation both in PC1 and PC2 indicated the promising responses accounting to the closer proximity with different protocols when compared to that other parameter with same treatment. The 17th protocols which used the B medium, large embryos and twice phase of subculture from liquid medium to solid medium was closely associated with the length of roots, length of hypocotyl and open cotyledonary, when compared to the other protocols. It suggests that the 17th protocol might be considered as a better option than that of other protocols to get the desired responses from the germination resposnses of *C. arabica* clone AS2K. Our data from Table 5 and PCA analysis clearly indicated that the length roots is most influence paramater on plantlet regeneration, and the 17th protocol is the best protocol for plantlet regeneration.

3.4 Histology of regenerating structure

Because the primary goal of this research was to verify the embryogenic origin of regenerating structures in the mentioned *in vitro* propagation system, embryogenic callus and somatic embryos at the globular, heart-shaped, torpedo, and cotyledonary stages were histologically examined. The leaf-derived callus culture demonstrated the formation of meristamatic zones of embryogenic callus (Figure 3A) as well as active cell division. After 6 weeks of culture, globular and heart-shaped somatic embryos were observed in the transverse section of embryogenic callus (Figure 3C). The investigation

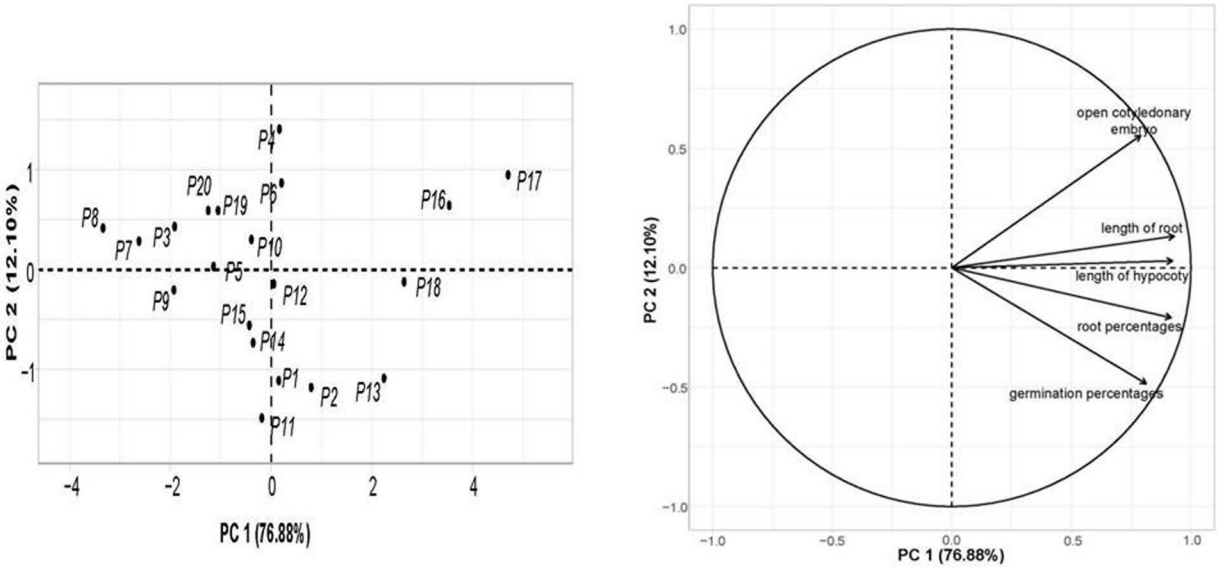
Table 5: Effects of 20 different protocols *in vitro* cultures on the embryo germination in the performance embryo of *C. arabica* L clone AS2K.

Protocol	Root percentages (%) (Mean ± SE)	Length of root (cm) (Mean ± SE)	Length of hypocotyl (cm) (Mean ± SE)	Open cotyledonary (%) (Mean ± SE)	Germination percentages (%) (Mean ± SE)
P1	17.6 ± 3.54 cd	0.88 ± 0.09 cde	0.98 ± 0.06 abc	0.8 ± 0.49 d	93.6 ± 2.79 ab
P2	51.2 ± 14.39 ab	2.1 ± 0.63 bcde	1 ± 0.28 abc	2 ± 1.10 bcd	72.4 ± 18.6 abcde
P3	3.2 ± 2.73 d	0.64 ± 0.52 de	0.46 ± 0.12 bc	4 ± 2.19 bcd	25.2 ± 12.45 bcde
P4	8.4 ± 3.49 d	1.1 ± 0.29 cde	0.9 ± 0.26 abc	17.6 ± 5.98 ab	55.2 ± 18.34 abcde
P5	3.6 ± 1.83 d	0.86 ± 0.43 cde	0.66 ± 0.28 abc	5.2 ± 2.65 bcd	52.8 ± 21.79 abcde
P6	8.4 ± 4.12 d	2.56 ± 0.69 abcde	1.02 ± 0.26 abc	10 ± 2.97 abcd	48 ±16.52 abcde
P7	1.6 ± 1.17 d	0.22 ± 0.13 e	0.24 ± 0.15 bc	1.6 ± 1.17 bcd	16.4 ± 10.17 de
P8	0 d	0 e	0 c	0 d	0 e
P9	6.4 ± 4.12 d	0.34 ± 0.21 de	0.5 ± 0.31 abc	1.2 ± 0.80 cd	36.4 ± 22.29 abcde
P10	12 ± 5.81 d	1.58 ± 0.87 bcde	0.8 ± 0.35 abc	7.6 ± 3.92 abcd	52.8 ± 21.75 abcde
P11	24 ± 10.60 bcd	0.76 ± 0.22 de	0.96 ± 0.14 abc	4.8 ± 0.49 bcd	93.2 ± 3.72 abc
P12	24.8 ± 7.84 bcd	0.82 ± 0.24 de	0.86 ± 0.24 abc	9.2 ± 3.26 abcd	70.8 ± 18.65 abcde
P13	52.8 ± 6.34 ab	2.92 ± 0.75 abcd	1.38 ± 0.10 ab	6.8 ± 1.36 bcd	100 ± 0 a
P14	19.2 ± 5.31 cd	0.8 ± 0.20 de	0.8 ± 0.21 abc	5.2 ± 1.62 bcd	74.4 ± 18.69 abcd
P15	12.8 ± 2.73 d	2.18 ± 0.17 bcde	0.68 ± 0.08 abc	2.4 ± 0.98 bcd	67.6 ± 9.07 abcde
P16	60 ± 8.09 a	4.1 ± 0.68 ab	1.68 ± 0.19 a	17.2 ± 4.59 abc	78.8 ± 19.22 abcd
P17	65.6 ± 2.04 a	5.12 ± 1.04 a	1.68 ± 0.13 a	23.2 ± 7.89 a	100 ± 0 a
P18	47.6 ± 6.43 abc	3.46 ± 0.62 abc	1.4 ± 0.07 ab	12.8 ± 2.87 abcd	93.6 ± 1.6 ab
P19	2.8 ± 1.86 d	0.54 ± 0.26 de	1.18 ± 0.32 abc	5.2 ± 1.50 bcd	26.8 ± 10.03 abcde
P20	8.8 ± 4.32 d	0.86 ± 0.42 cde	1.04 ± 0.43 abc	4.4 ± 3.12 bcd	20 ± 9.82 cde

Values are expressed as mean ± standard error (SE) of five replicated experiments. each with ten explants per treatments. Means followed by the same letters within columns are not significantly different at $P \leq 0.05$ level according to Tukey test. All data were taken after eight weeks of culture.

of somatic embryos confirmed the germination of cotyledonary staged embryos (Figure 7). As a center point for active cell division, the meristematic regions demonstrated the origin of root and shoot differentiation. Histological examination of friable and non-friable callus identified the presence of actively dividing cells only in friable callus. As a result, during the tissue culture practical, the selection of the best friable calli is mandatory because only the friable callus has the potential for regenerating plantlet. Embryogenic cell clusters produced globular and heart-shaped embryos (Figure 7C). The presence of two meristems in the globular structures, namely the root and shoot axes, proved that they were true somatic embryos (Kumari; Baskaran; Van Staden, 2017). The germination of embryos into plantlets in the current study was consistent with the above report.

On the surface of the calli, single, densely stained, non-vacuolated, starch-containing cells with thick walls were observed 6 to 7 days after culture initiation. Karlsson and Vasil (1986) reported similar characteristics of embryogenic cells. After two weeks, these single cells developed embryogenic characteristics, with internal sectioning divisions leading to the formation of discrete two and four cells proembryos. This explains why somatic embryos are unicellular. Somatic embryos can be formed from a single cell in other species as well (Xie; Hong, 2001). The existence of clusters of rapidly dividing embryogenic cells in the periphery of proliferating



	Eigenvalue	Percentage of variance
PC1	3.844	76.884
PC2	0.605	12.097

	Coefficients of PC 1	Coefficients of PC 2
Root percentages	0.917	-0.209
Length of root	0.930	0.132
Length of hypocotyl	0.923	0.029
Open cotyledonary embryo	0.790	0.554
Germination percentages	0.813	-0.486

Figure 6: Bi-plot with first and second components of PCA based on the performance embryo such as roots percentage. length of roots. length of hypocotyl. open cotyledonary and germination percentages of *C. arabica* L. clone AS2K . cultures on 20 different protocol combined subculture. medium and size of embryo. Distributional bi-plot with 20 different protocols (P1-P20) shown in this figure. The eigenvalues of two PCs and their percentage of variance have been shown in this figure. In this bi-plot. PC1 correlated positively with open cotyledonary embryo. length of root. length of hypocotyl. root percentages and germination percentages. whereas PC2 positively correlated with open cotyledonary embryo. length of root and length of hypocotyl only.

calli tissues contributed to proembryogenic masses. These proembryogenic masses were divided by clusters of loose and vacuolated parenchyma cells (Figure 7A). Following the acquisition of embryogenic determination, further cell division and differentiation in the proembryos resulted in the development of globular somatic embryos within 10-12 days. Globular embryos had a protoderm that encircled a density of tightly packed parenchymatous-like vacuolated cells with meristematic locations and a procambial regions (Figure 7B). The structure of a well-defined boundary called protoderm around globular embryoids and the availability of independent vascular system are general characteristic that distinguishes somatic embryoids from shoot buds (Quiroz-Figueroaet al., 2002). Suspensor or suspensor-like cells were not observed in the globular embryoids. Whereas, somatic embryos were discovered to be linked to the parent tissue via the embryonary axis. In overall, embryos with a narrow suspensor-like organ are more likely to have a unicellular origin, whereas somatic embryos with a multicellular origin appear to be widely linked to the explant via the embryonary axis (Williams; Maheswaran, 1986). The cytoplasm of newly formed cells was dense, with an abundance of starch grain. The globular structures grew longer and more conical, and a notch formed centrally on the developing somatic embryoids, giving it a heart shape (Figure 7C-D). Heart-shaped embryoids differentiated further into torpedo stage with the development of cotyledonary initials and the establishment of polarity with distinct plumule and root pole (Figure 7E). The absence of vascular continuity between the two confirmed the complete independence of somatic embryoids from the subadjacent callus tissue.

According to the results of the above analysis, we developed a protocol for micro propagation process through indirect somatic embryogenesis (ISE) techniques using leaf explants. A summary of the procedure is shown in Figure 8. Briefly, leaf explants (Figure 8A) were cultured in M5 medium for callus induction (Figure 8B). Subsequently, the callus were transferred to the new medium to performed embryogenic calli (Figure 8C). The embryogeneic calli will become the different stage of development embryo (Figure 8D) in ERM6 medium, such as the globular stage, the heart stage, the torpedo stage and the cotyledonary stage. Finally, the mature somatic embryos were transferred to the 17th protocol for further germination and plantlet regeneration (Figure 8E and 8F).

4 DISCUSSION

The leaf tip explant have more nutrients than other part of the leaf. As a result, it have higher embryogenic callus formation. According to Prado and Maurel (2013), in the schematic of the water transport mechanism from root to leaf, nutrients are delivered to all leaf lamina via the leaf bone. The water transport flow from the midrib in the stalk and ends at

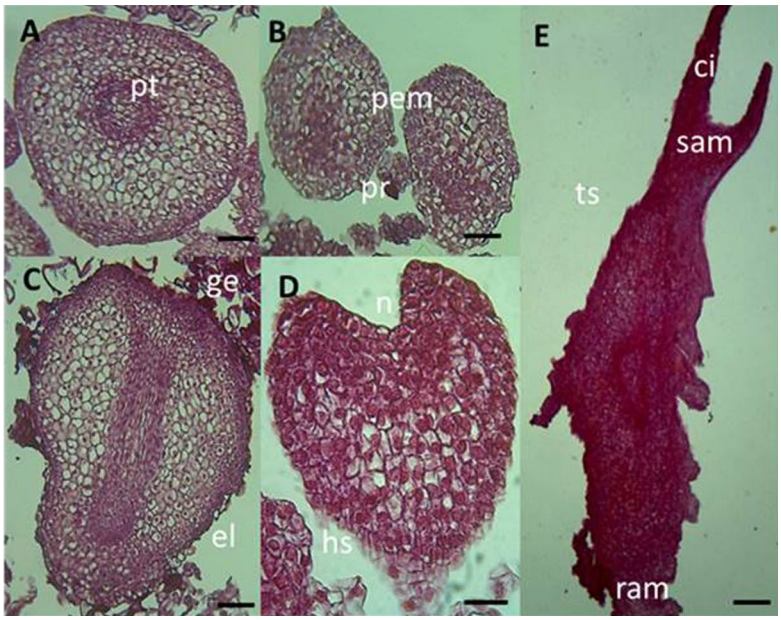


Figure 7: Histological analysis of development somatic embryogenesis in *C. arabica*. A. Globular embryoid developed within the callus tissue with well differentiated protoderm (pt). B. Group of embryogenic cells forming preoembryogenic masses (pem) and proembryos (pr) on the surface as well as within callus tissue. C. Elongated somatic embryoids (el) with globular embryoid (ge) formed on the surface of callus tissue. D. Development of notch (n) in the centre of globular embryoid leading to theformation of heart shape embryoid. E. Torpedo shaped embryoids showing polarity with differentiation of cotyledonary initials (ci) and apical pole forming shoot apical meristem (sam) and root apical meristem (ram). All the scale bar is set to 50 µm.

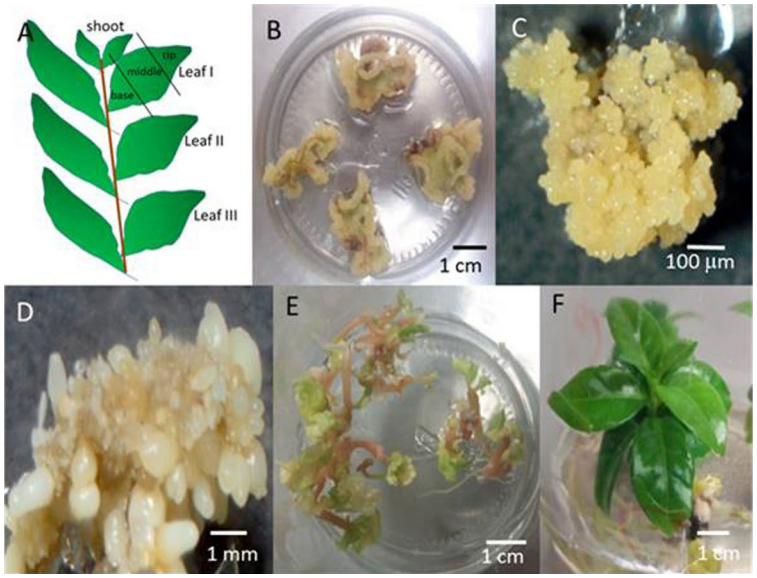


Figure 8: Schematic representation of the *Coffea arabica* micro propagation process through indirect somatic embryogenesis (ISE) techniques using leaf explants. (A) Leave explants; (B) Callus induction; (C) Embriogenic callus regeneration; (D) Differentiation somatic embryo consist of globular. heart-shape. torpedo phase; (E) Embryo germination; (F) Plantlet regeneration.

the tip of the leaf. After in the midrib, nutrients flow into the branch bone and the leaf veins, which are the final branch on the leaves. As stated in the explanation, the circulation of nutrient content stops at the tip of the leaf, where the nutrients that are completed accumulate. As a result, even when the leaves are cut, they possess the nutrients contained in midrib. Kalve, De Vos and Beemster (2014) divides the leaf into two parts to separate the two growth processes. These two parts are a part of cell division at the base of the leaf and cell elongation at the tip of the leaf. The absorption of nutrients and water increased with increased wet weight as a result of expressing the cells that occur at the tip. Wijayati and Solichatun (2005) explained that elongation cell will be followed by cell expansion and greater wet weight. The rise in wet weight is primarily due to excessive water capture by the cell. Furthermore, within the mother tree, the leaf tip is utilized as nutrient storage facility. Fitriana et al. (2019) describe that the plant part utilised is one of the factors influencing callus initiation in somatic embryogenesis. The plant parts used in this study are the base, middle, and tip of the leaf explants. The meristematic properties of the leaf's base and tip differ. Because it is near to the SAM (Shoot Apical Meristem), the base of the leaf is assumed to have a more meristematic connectivity, whereas the tip is considered to have more mature cells.

Somatic embryogenesis is dependent on plant cell totipotency as well as their ability to dedifferentiate and differentiate into a new cell type (Su et al., 2020; Salaün; Lepiniec; Dubreucq, 2021). Cytokinins are essential for controlling numerous stages of plant growth and development (Zürcher; Müller 2016; Kieber; Schaller, 2014). The absense of exogenous auxin from the growth medium enhances embryo development in Arabica coffee, eliminates the major chemical ingredient that maintains plants cell a totipoten (Nic-Can; Loyola-Vargas, 2016). Active charcoal has been used in plant propagation because of its adsorption capability exogenous 2,4-D compound, as proved by its inhibition activity on direct somatic embryogenesis in *C. canephora* (Hatanaka et al., 1991). Activated charcoal is frequently used to improve cell growth and development by neutralizing advantageous plant growth enhancing compounds from the media (Buckseth et al., 2018). Unlike the previous findings for indirect somatic embryogenesis of Arabica coffee, in which the 2,4-D accumulation was adsorbed by active charcoal, we accomplished embryo development in the current study by adding BAP without using actived charcoal. The addition of active charcoal did not expect for an accelerated determination and differentiation phase. It was most likely of a different genotype that was used, resulting in different response of embryo development. Seasonality, concentration of plant growth regulators and genotype all influence the duration and frequency of the explant embryogenic response (Berthouly; Etienne 1999, Almeida et al., 2008).

The growth and development of somatic embryos into plantlet is a crucial step in somatic embryogenesis process. The 17th protocol was the most effective. This is because of the utilization of large-sized embryos (torpedo) in protocol 17 which have complete and perfect performance embryos phase. Large and perfect embryo have high opportunity to develope further phase faster than smaller embryo. This embryo are derived from the liquid phase that has passed through the

maturation phase and then subcultured using medium B. In the other hand, the eighth protocol shows the minimum value for each parameter in eight week. All the parameter such as the percentages of rooted cotyledons, the length of roots, the length of hypocotyl, the percentages of opened cotyledons and the percentages of live cotyledons did not responses. Embryos with the mature physiology have better germination capacity, even though the plant conversion from somatic embryos is still low. The higher number of embryos do not germinate due to several factors. Firstly, there is no meristem shoot formation or embryo development is very limited. This occurs because of the embryo develops under sub-optimal environmental conditions. Secondly, physiologically somatic embryos are insufficiently developed. The seventeenth protocol is the most suitable for the development and germination embryo somatic.

5 CONCLUSIONS

Because of conventional coffee breeding programs are frequently hindered by the long duration of processes such as selection, hybridization, progeny evaluation, backcrossing, and inter-specific crossing before seeds are set, interesting alternative and novel breeding strategies such as transgenics are being attempted for genetic improvement. SE are an effective tool for regeneration and such breeding methods. Recognizing the factors that lead to a higher percentage of somatic embryogenesis can benefit in rapid multiplication and novel breeding programs. In this study, somatic embryos of AS2K clone Arabica coffee were successfully induced from the third position of the leaf tip explants using 1 mg/L 2,4-D, 1 mg/L BAP, and 4 mg/L 2-ip and subjecting the regeneration of embryo somatic to 90 days of culture period using ERM6 medium containing 2 mg/L BAP without activated charcoal. Histological studies show that there are several stages of somatic embryo development, beginning with the globular, heart, torpedo, and cotyledonary phases. The length of the roots has the greatest influence on plantlet regeneration, and the 17th protocol, which used B medium, large embryos, and a two-phase subculture from liquid medium to solid medium, is the best protocol for plantlet regeneration. Plants derived from somatic embryogenesis can be useful for producing a large number of plantlets from a single elite mother plant. This influence on the effectiveness may also support future attempts to improve through genetic transformation. The protocols developed during the research might be advantageous for other coffee cultivars that haven't been evaluated for their somatic embryogenic potential through certain modifications.

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7 AUTHORS' CONTRIBUTIONS

RA wrote the manuscript and performed the experiment, BSD supervised the experiment and co-worked the manuscript, YTMA supervised the experiment and co-worked the manuscript, EP conducted all statistical analyses, ES reviewed and approved the final version of the work.

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