

## Evaluation of Somatic Embryogenesis Ability in Robusta Coffee (*Coffea canephora* Pierre)

### *Evaluasi Kemampuan Embriogenesis Somatik pada Kopi Robusta (Coffea canephora Pierre)*

Priyono<sup>1\*)</sup>

#### Summary

Somatic embryogenesis (SE) should be very effective for propagation of coffee species. Evaluation of indirect somatic embryogenesis (ISE) and direct somatic embryogenesis (DSE) are useful for describing cell proliferation precedes embryogenesis. The research aimed to evaluate the ISE and DSE ability of *Coffea canephora* Pierre which performed on a great genetic diversity, was carried out at the Nestlé R&D Centre Tours France including the accessions derived from a coffee core collection of Nestlé and three clones of ICCRI. Three aspects, namely: SE process, SE diversity and SE stability were evaluated. The results showed that both DSE and ISE process were observed in present study. These results indicated that both SE processes in *C. canephora* appeared to be independent mechanism. Under given experimental conditions, SE ability depended on the genotype. Diversity for SE ability was observed not only among three genetic groups of *C. canephora* (Congolese, Guinean, and Conillon), but also within the genetic groups. Moreover, SE was reproducible and stable for explants from cuttings of the given genotype. Interestingly, among three clones of ICCRI, namely BP409, BP961 and Q121 which were used as parents for controlled crosses, showed significant differences suggesting which were possible segregation within their progenies for ISE ability.

#### Ringkasan

*Embriogenesis somatik diharapkan sebagai metode perbanyakan tanaman yang sangat efektif pada kopi. Evaluasi dua jenis proses embriogenesis somatik, yaitu proses langsung dan tidak langsung akan bermanfaat untuk menggambarkan kemampuan proliferasi sel. Penelitian untuk mengevaluasi embriogenesis somatik kopi Robusta (Coffea canephora) yang mempunyai tingkat keragaman genetik tinggi telah dilakukan di Nestlé R&D Centre Tours, Perancis. Bahan tanam menggunakan kopi Robusta koleksi Nestlé Perancis dan tiga klon koleksi Pusat Penelitian Kopi dan Kakao Indonesia (Puslitkoka). Tiga aspek, yaitu proses embriogenesis, keragaman embriogenesis dan kemantapan embriogenesis dievaluasi dalam penelitian ini. Hasil penelitian menunjukkan bahwa baik embriogenesis somatik langsung maupun tidak langsung dapat diamati. Penelitian ini menunjukkan bahwa kedua proses embriogenesis somatik tersebut merupakan dua mekanisme yang berbeda. Dalam penelitian ini ditunjukkan bahwa kemampuan embriogenesis somatik tergantung pada genotipe, baik antar maupun di dalam kelompok genetik kopi Robusta, yaitu Congolese, Guinean dan Conillon. Lebih lanjut diketahui bahwa kedua proses embriogenesis*

---

Naskah diterima (*received*) 15 Juni 2010, disetujui (*accepted*) 2 Juli 2010.

1) Pusat Penelitian Kopi dan Kakao Indonesia, Jl. PB. Sudirman No. 90, Jember, Indonesia.

\*) Alamat penulis (*Corresponding Author*) : priyono\_iccri@yahoo.com.

somatik tersebut stabil terhadap indukan sebagai sumber eksplan. Kemampuan embriogenesis somatik tidak langsung ketiga klon Puslittkoka (BP409, BP961 dan Q121) sangat beragam, sehingga memberikan harapan adanya pola segregasi yang baik berdasarkan kemampuan embriogenesis somatik tidak langsung pada populasi yang dibuat dari silangan klon tersebut.

**Key words:** *Coffea canephora*, somatic embryogenesis, variability, stability, genotype.

## INTRODUCTION

Robusta coffee (*Coffea canephora* Pierre) is genetically highly diverse (Lashermes *et al.*, 1996). Due to its strict allogamous nature, each plant derived from crossing can be considered as a unique genotype. Although, the superior individual progeny could be selected, but only vegetative propagation will produce uniform plants.

One of the major problems of coffee growing in Indonesia is low productivity due to the minimum use of high yielding planting materials derived from vegetative propagation. Small farmers commonly do not grow clonal planting material due to unavailability and more sophisticated production than seedlings. To minimize these constraints, the use of planting material obtained from mass vegetative propagation is expected to be an effective and efficient approach.

Regeneration efficiency via somatic embryogenesis (SE) is now possible to produce somatic embryos and plantlets for a large number of crops. The SE can be classified in two different types, namely direct somatic embryogenesis (DSE) and indirect somatic embryogenesis (ISE). In the DSE, explants cultivated on initiation medium will form embryogenic units which develop directly into bipolar somatic embryos. It has been suggested that in DSE, embryogenic cells are already present and only favourable condition and a minimal re-programming are required for embryo development (Quiroz-Figueroa *et al.*, 2005).

In the ISE, the initiated embryogenic units do not develop beyond the pre-globular stage but break up into new embryogenic units. Thus in the ISE the re-programming of differentiated cells to acquire the embryogenic status is required (Quiroz-Figueroa *et al.*, 2006). Consequently, the time required for ISE commonly is longer than DSE. In the ISE process i.e., callus initiation followed by embryogenesis is the major reason explaining long of time required for the embryogenesis (Kumar *et al.*, 2007; Pinto-Sintra, 2007).

Somatic embryogenesis should be very effective for propagation of coffee species. Staritsky (1970) has been the first to report the somatic embryogenesis in *C. canephora* by describing the induction of embryoids from callus tissues (ISE). On the contrary, Hatanaka *et al.* (1991) obtained somatic embryos directly from explant sections (DSE). The advantages of DSE are the rapid embryo formation and high embryo germination rate (Priyono, 1992). The main benefit of ISE is the high frequency of somatic embryogenesis which has clear implications with regards to large scale production (Ducos, 2000). Unlike plants from cuttings, those derived from SE have a shape and development comparable to that of seed derived trees, even when using leaf explants from plagiotropic branches (Priyono *et al.*, 2000).

Early studies on somatic embryogenesis have demonstrated that an auxin is critical for embryo initiation. The decrease of auxin concentration or its absence pro-

motes embryo expression. Thus, in general terms, the classic SE protocol involves successively the culture on a first medium containing auxin and then a subculture on a second medium without growth regulator, both media containing a substantial supply of reduced nitrogen. According to this current protocol, the majority of culture media developed for induction of coffee SE contain a mixture of auxin and cytokinin (Giridhar *et al.*, 2004). However, Priyono (2000) and Ducos *et al.* (2003) showed that coffee SE can also be induced from leaf explants using a cytokinin as a sole growth regulator. Whatever plant growth regulators, the somatic embryogenesis ability in *Coffea* sp. is extremely variable depending on genotype (Michaux-Ferrière *et al.*, 1989) and species (Priyono, 2004). The present research aimed to evaluate the DSE and ISE relationship, SE ability among genotype as well as the SE stability of Robusta coffee genotype having wide genetic distance.

## MATERIAL AND METHODS

Plant materials were from three different origins i) one set of seven accessions from Nestlé Coffee Core Collection, which represent the three groups of *C. canephora*, i.e. Congolese group (FRT 23 and FRT 56), Guinean group (FRT 09 and FRT 16) and Conilon group (FRT 31 and FRT 80), ii) a set of two elite parental clones of Indonesian Coffee and Cocoa Research Institute (ICCRI), i.e. BP 409 and BP 961 (Congolese group), and iii) Q121 (an hybrid between Guinean and Congolese).

The DSE and ISE methods developed by Priyono (1992) and Ducos (2000), respectively, were used in this study. Leaves were harvested from the 3<sup>rd</sup> to 4<sup>th</sup> leaf pair position from the tip. The leaves were

washed and then sterilized with 70% ethanol for 30 seconds. Sterilization was continued in 4% NaOCl solution with a few drops of Tween 20 for 30 minutes, and then rinsed three times with sterile water. Sterilized leaves were cut into pieces (10 mm x 10 mm) and placed in a Petri dish containing 40 ml of solid medium.

Medium for DSE was half strength concentration of MS basal medium added with half strength concentration of Gamborg's vitamins and supplemented with 1 mg/l L-cystein, 10 mg/l Ca-Pantotenate, 100 mg/l casein hydrolysate, 50 mg/l adenine sulphate, 5 mg/l kinetin, and 40 g/l sucrose while 3g/l of Gelrite were added as gelling agent.

Two different media were used for ISE, namely callus induction and somatic embryo production media. Medium for callus induction comprised MS basal medium and Gamborg's vitamins added with 1.125 mg/l BAP and 30 mg/l sucrose. 8 g/l of Bacto agar were used as gelling agent. Medium used for somatic embryo production was MS basal medium and Gamborg's vitamin added with 30 mg/l sucrose, and 3 g/l gelrite as gelling agent. The pH of all media was adjusted to 5.7 before autoclaving under saturated steam at 121°C for 20 min.

For DSE, all cultures were incubated at 25°C under cool-white fluorescent lamps, providing approximately of 30  $\mu$ mol photon/m<sup>2</sup> over a 16 h photoperiod. For ISE technique, the culture conditions, as well as for the media, were different according to the stage of the protocol for callus induction. the Petri dishes were placed at 25°C in darkness, then for somatic embryo production, they were transferred in the same culture conditions than those described above for DSE.

### Evaluation of Somatic Embryogenesis Abilities

In DSE technique, the number of explants that formed micro callus and subsequently somatic embryos were recorded. The number of somatic embryo was counted after

3 and 6 months of culture.

In ISE, the number of primary explants that formed calli or embryogenic calli were recorded after six months at the end of the first culture stage. Then, three months later at the end of the second cul-

Table 1. Description of criteria for evaluation of somatic embryogenesis ability in Robusta coffee (*Coffea canephora*)

Tabel 1. Deskripsi kriteria untuk evaluasi kemampuan embriogenesis somatik pada kopi Robusta (*Coffea canephora*)

Criteria <i>Kriteria</i>	Code <i>Kode</i>	Remark <i>Keterangan</i>
Frequency of callusing <i>Frekuensi terbentuknya kalus</i>	FC	Number of explants showing calli in callus induction stage of indirect somatic embryogenesis  <i>Jumlah eksplan yang membentuk kalus pada tahap induksi kalus pada embriogenesis somatik tidak langsung</i>
Frequency of embryogenic calli <i>Frekuensi kalus embriogenik</i>	FEC	Number of explants showing embryogenic calli in induction stage of indirect somatic embryogenesis  <i>Jumlah eksplan yang membentuk kalus embriogenik pada tahap induksi kalus pada embriogenesis somatik tidak langsung</i>
Score of embryogenic calli <i>Skor kalus embriogenik</i>	SEC	Number of embryogenic clusters of indirect somatic embryogenesis
<i>Jumlah gerombol kalus embriogenik pada tahap induksi kalus pada embriogenesis somatik tidak langsung</i>		
Frequency of micro calli without somatic embryogenesis showing micro calli projection <i>Frekuensi terbentuknya kalus mikro tanpa embriogenesis somatik langsung</i>	FMC	Number of explants without  <i>Jumlah eksplan yang</i>
somatic embryo expression of direct embryogenesis <i>Frekuensi terbentuknya kalus mikro tanpa embriogenesis somatik langsung</i>		
Frequency of embryogenic explant which produces at least one embryo inoculated explants <i>Frekuensi eksplan embriogenik</i>	FSE *	Number of explants per 15  <i>Jumlah eksplan yang membentuk minimal satu embrio per 15 eksplan</i>
Intensity of embryogeni <i>Intensitas embriogenik</i>		ANERE * Number of embryo(s) <i>Jumlah</i>
Global embryogenic potential <i>Potensi embriogenik global</i>		ANEP * Total embryo(s) per <i>Jumlah</i>
Petri dish = FSE x ANERE <i>embrio per cawan petri = FSE x ANERE</i>		

Notes (*Catatan*): \* this trait is observed for indirect somatic embryogenesis and direct somatic embryogenesis. (\* *kriteria ini diamati baik pada tipe embriogenesis somatik tidak langsung maupun langsung*).

ture stage the number of primary explants forming embryo (from calli) and the number of somatic embryos were counted.

The observation and counting of SE were carried out under the binocular microscope. The recorded data at the end of the somatic embryogenesis observation of both protocols are presented in Table 1.

Several experiments were conducted as described below:

- 1) SE ability according to the *C. canephora* genetic group. The experiments were set up in randomized complete block design of 10 genotypes representing all genetic groups of *C. Canephora* in 10 replications of 15 primary explants. The tested genotypes included the parental clones of the segregating populations.
- 2) Stability of SE ability according to the origin of the explant origin (mother plant effect). The experiment was set up in factorial completely randomized design, with 10 replications of 15 primary explants. The first factor was the mother plant consisting of two different cuttings of the same clone, and the second factor was the genotype consisting of 6 different genotypes.

The statistical analysis was done according to Pearson for the correlation study. The clustering analysis was performed by hierarchy dendrogram method with complete linkage. The dissimilarity distance was used to evaluate genotypes relationship. The ANOVA followed by the Tukey-Kramer comparison test was used for the evaluation of SE ability. All statistical analysis was performed by Statbox.6 software.

## RESULT AND DISCUSSIONS

### Effect of somatic embryogenesis protocol

In the DSE, within 30 days of culture, a part of leaf piece directly developed a number of small, smooth and translucent globules that rapidly evolved into typical bipolar embryo structures as reported by Priyono *et al.* (2010). In the ISE protocol the calli first appeared from the cut of leaf explants. Three types of response were generally observed for induction of calli. The first type was some leaf explants characterized by a slight swelling of explants without subsequent surface modification. Maintaining the culture for more than 6 months did not result in any further visible modification. The explants finally turned brown and died. The second and third types were characterized by a swollen at the cut end or on surface of the leaf explant. Primary calli were observed already after three months of culture. The calli were slightly compact and white to yellowish. At the end of the callus induction stage (7 months), primary calli can be separated in two categories, namely aqueous and friable embryogenic calli. No embryo was observed during the callus induction. Following 7 months on callus induction medium, all leaf explants were transferred onto a medium for induction/production of embryos. Three types of response were observed for this stage. The first type was a slight swelling of explants with no embryo formation. The second type was leaf explants with embryogenic calli derived from callus induction medium. After 3 months, the embryogenic cells derived from friable calli developed into globules which evolved in somatic embryos by a large basal connection to the callus. The third type was leaf explants with aqueous calli derived from callus induction medium. After 3 months, the embryogenic cells derived from aqueous calli developed into globules which evolved similarly to those from embryogenic calli.

In both protocol, some embryos were able to germinate on the same medium or to proliferate into larger embryo masses producing secondary embryogenesis. Pro-embryo globules appeared on the surface of primary embryos and then differentiated into embryos easily separated from their origin. Most of them were individual bipolar embryos. The successive stages of development from globular to mature bipolar and germinated embryos are totally similar with the two techniques. The embryos were characterized by the presence of apical meristem and root axis. Shoots always developed before the roots. It clearly shows that with the Priyono (1992) protocol the appearance of structures were capable to develop into bipolar somatic embryos which has been observed for most of the genotypes directly at cut edges or on the surface of leaf explants. All stages from globular shape through cotyledonary embryos were observed. On the contrary, with the Ducos (2000) protocol the embryogenic tissue did not develop beyond the pre-globular stage but broke up into new embryogenic tissues. This result shows that culture conditions strictly determine the type of SE: 1) direct somatic embryogenesis with the protocol of Priyono (1992) and 2) indirect somatic embryogenesis with the protocol of Ducos (2000). With DSE, the explants had a minimum of proliferation before forming somatic embryos, whereas with ISE, the explants had an extensive proliferation before initiating somatic embryos. These two processes led to the production of the same cotyledonary somatic embryos showing no apparent influence of the tissue origin.

Some reports referred the SE process has been published. Some species such as *Paulownia elongate* (Ipekci & Gozukirmizi, 2003), *Azadirachta* (Gairi & Rashid, 2006),

*Linum usitatissimum* (Salaj *et al.*, 2005), *Epipremnum aureum* (Zhang *et al.*, 2005) only DSE was observed. In other crops, such as *Cyclamen persicum* (Winkelmann *et al.*, 2004), *Scaevola aemula* (Wang & Bhalla, 2004), several *Gossypium* species (Sun *et al.*, 2006), and *Theobroma cacao* (Niemenak *et al.*, 2008) only ISE was obtained. However, in certain crops such as *Allium sativum* (Sata *et al.*, 2000), and *Manihot esculenta* (Raemakers *et al.*, 2005) direct and indirect types of somatic embryogenesis have been described. Both types of SE occur simultaneously by selecting the proper explants and growth regulators.

The significant differences among genotypes, SE protocols and their interaction (Table 2), and the absence of correlation between the two protocols (Table 3) for all observed criteria were noted. Present results suggest that these protocols are performed by independent mechanism. It clearly indicates that the type of SE protocol plays a major role for production of embryogenic cells like in DSE but not in the ISE protocol. The occurrence of one or the other depends on culture conditions including media and lighting (Gatica-Arias *et al.*, 2008b). The use of cytokinin as sole plant growth regulator to induce SE are carried out in present study. However, different types and concentration of plant growth regulators are recognized for both protocols. These different conditions namely a combination of an agar solidified medium with darkness used in ISE protocol and the combination of gelrite solidified medium with a 16-hour photoperiod used in DSE protocol. It is likely that other experimental conditions would lead to a different observed diversity and then to conclude to a genotype x protocol interaction. This phenomenon is giving the advantage for the application

Table 2. Variance analysis of somatic embryogenesis ability in Robusta coffee (*Coffea canephora*)Tabel 2. Analisis varian kemampuan embriogenesis somatik kopi Robusta (*Coffea canephora*)

Source Sumber		Somatic embryogenesis ability Kemampuan embriogenesis somatik					
		3 months of culture for direct somatic embryogenesis and 10 months of culture for indirect somatic embryogenesis 3 bulan setelah kultur pada embriogenesis langsung dan 10 bulan setelah kultur pada embriogenesis somatik tidak langsung			6 months of culture for direct somatic embryogenesis and 10 months of culture for indirect somatic embryogenesis 6 bulan setelah kultur pada embriogenesis langsung dan 10 bulan setelah kultur pada embriogenesis somatik tidak langsung		
		FSE	ANERE	ANEPE	FSE	ANERE	ANEPE
F-Ratio F-Ratio	A: Genotype <sup>1)</sup> A: Genotipe	25.56	11.80	16.87	24.78	11.11	13.14
	B: Protocol <sup>2)</sup> B: Protokol <sup>2)</sup>	338.49	238.69	269.04	119.60	64.07	80.65
	AB: interaction AB: interaksi	15.96	14.25	19.71	11.98	11.22	14.16
Prob level Aras peluang	A: Genotype A: Genotipe	< 0.00001	< 0.00001	< 0.00001	< 0.00001	< 0.00001	< 0.00001
	B: protocol B: Protokol	< 0.00001	< 0.00001	< 0.00001	< 0.00001	< 0.00001	< 0.00001
	AB: interaction AB: interaksi	< 0.00001	< 0.00001	< 0.00001	< 0.00001	< 0.00001	< 0.00001

Note (Catatan): <sup>1)</sup> The 16 genotypes tested were 10 genotypes of Nestle R&D Centre Tours collection and each of 3 progenies selected randomly from BP 961 x Q 121 and BP 409 x Q 121, respectively. <sup>2)</sup> The two protocols were direct somatic embryogenesis and indirect somatic embryogenesis protocol. See Table 1 for the abbreviation of criteria of observation. (<sup>1)</sup> 16 genotipe yang diuji adalah 10 genotipe koleksi Nestle R&D Centre Tours dan masing-masing 3 individu dari silangan BP 961 x Q 121 dan BP 409 x Q 121. <sup>2)</sup> Dua protokol yang diuji adalah tipe embriogenesis langsung dan tipe embriogenesis tidak langsung. Lihat Tabel 1 untuk singkatan kriteria pengamatan).

Table 3. Correlation between criteria of somatic embryogenesis ability in Robusta coffee (*Coffea canephora*)Tabel 3. Korelasi antara kriteria kemampuan embriogenesis somatik kopi Robusta (*Coffea canephora*)

Criteria Kriteria		Indirect somatic embryogenesis Embriogenesis somatik tidak langsung				Direct somatic embryogenesis Embriogenesis somatik langsung			
		FC	FEC	FSE	ANEP	ANERE	FMC	FSE	ANEP
Indirect somatic embryogenesis Embriogenesis somatic tidak langsung	FEC	0.48							
	FSE	0.50	<b>0.93</b>						
	ANEP	0.40	<b>0.70</b>	<b>0.83</b>					
	ANERE	0.09	<b>0.57</b>	<b>0.68</b>	<b>0.93</b>				
Direct somatic embryogenesis Embriogenesis somatic langsung	FMC	0.36	-0.41	-0.22	0.05	-0.12			
	FSE	0.37	0.34	0.39	0.14	0.00	-0.02		
	ANEP	0.33	0.36	0.28	-0.03	-0.14	-0.15	<b>0.82</b>	
	ANERE	0.36	0.48	0.38	0.07	-0.04	-0.11	<b>0.65</b>	<b>0.84</b>

Note (Catatan): <sup>1)</sup> The analysis based on 16 genotypes; Bold letters term significant correlation for  $\alpha = 0.001$  as analysis by Pearson method performed by statbox 6 software (<sup>1)</sup> Analisis berdasarkan pada 16 genotipe; huruf tebal menunjukkan korelasi secara nyata pada  $\alpha = 0,001$  menurut metode Perason dengan menggunakan program statbox 6).

of these techniques of vegetative propagation in *C. canephora* improvement. SE ability is therefore genotype dependent but probably not for the absence or presence of embryogenesis whatever the culture conditions. It is likely that this phenomenon is involving some genes expressed for zygotic embryogenesis but with a regulation of their expression determined by culture conditions. It means that, even if it may vary a lot in intensity according to the genotype, the SE phenomena would be mainly determined by the culture conditions, especially the composition of culture medium and lighting.

Many studies have suggested that callus formation and regeneration are regulated by independent genes (Gatica-Arias *et al.*, 2008a; Mano & Komatsuda, 2002). In this study, there is no significant correlation between FMC and FSE with DSE or FC and FSE with ISE. These results are in agreement with the idea that these traits are regulated by different genes. On the contrary, significant correlations were observed between FSE and the number of produced embryos per explant (ANERE) with both protocols. The high correlation between these two different traits might suggest that these traits are under the same control and that the ability of explants to form somatic embryos is linked to the intensity of the phenomena. Finally, a positive correlation was observed between these two characteristics in the present study, which suggests that the SE ability response could be assessed by any of these two characteristics.

A significant correlation ( $r = 0.93$ ) was observed between frequency of embryogenic calli and frequency of SE with ISE protocol. This logical correlation validates the classification between aqueous calli and embryogenic calli made at the end of the first culture stage. If aqueous calli were sig-

nificantly capable to produce embryos this correlation would not be as much significant. Identifying and selecting embryogenic calli are therefore a crucial steps for establishing cell strains regenerating embryos. For other species, it is not so obvious to identify and separate embryogenic calli from non embryogenic ones (Sun *et al.*, 2006). Wan *et al.* (1992) have run some RFLP analysis on regenerating calli formed from embryo-like structures in maize. They hypothesized that some regions might be related to both induction embryogenic calli and development of the embryos. It has also been suggested that, in DSE, some cells have already been embryogenically determined and simply require favourable conditions for embryo development (William & Maheswaran, 1986). On the contrary, ISE would require the re-determination of differentiated cells (Quiroz-Figueroa *et al.*, 2006).

With DSE (Priyono *et al.*, 2010) the number of embryos observed after 6 months was significantly higher than after 3 months. Interestingly, in the present study, the secondary SE was also observed with ISE protocol which is hormone free during the embryo formation stage. Similarly to these observations, somatic embryos of walnut, pecan, and cassava underwent repetitive somatic embryogenesis on basal medium devoided of growth regulators after a single exposure to an induction medium containing some hormone. The suitability of hormone free medium to sustain secondary embryogenesis has also been reported on different oak embryogenic systems (Fernandez-Guijarro *et al.*, 1995).

### **Diversity and stability of somatic embryogenesis ability**

The SE ability of the two protocols has



Table 4. Indirect somatic embryogenesis ability of the three groups of Robusta coffee (*Coffea canephora*)Tabel 4. Kemampuan somatik embriogenesis tidak langsung pada tiga grup kopi Robusta (*Coffea canephora*)

Genotype <sup>1)</sup> Genotipe <sup>1)</sup>	Somatic embryogenesis ability Kemampuan embriogenesis somatik %					
	Callus induction stage Tahap induksi kalus			Somatic embryo production stage Tahap produksi embrio somatik		
	FC	FEC	ECS	FSE	ANERE	ANEP
FRT 80 (Cn)	100 c	100 d	11.5 e	63 b	12 ab	155 a
FRT 31(Cn)	98 c	96 cd	8.3 cd	99 c	23 bcd	343 bc
FRT 16 (G)	99 c	3 a	0.5 ab	2 a	0 a	1 a
FRT 09 (G)	97 c	96 cd	9.8 ed	85 bc	27 cd	386 c
FRT 23 (Cg)	92 c	11 ab	0.5 ab	12 a	6 a	13 a
FRT 51 (Cg)	23 a	21 b	2.5 ab	14 a	11 ab	47 a
FRT 56 (Cg)	98 c	98 d	10.9 e	93 c	30 d	400 c
BP 409 (Cg)	98 c	94 cd	7.3 c	91 c	13 abc	180 ab
BP 961 (Cg)	85 bc	79 c	3.1 b	64 b	13 abc	140 a
Q 121 (Cg x G)	69 b	79 c	0.4 a	7 a	5 a	10 a

Note (Catatan): <sup>1)</sup> Cn (Conillon), G (Guinean), Cg (Congolese). Results in the same column followed by the same letters are not significantly different according to Tukey-Kramer Multiple-Comparison Test for  $\alpha = 0.05$ . (<sup>1)</sup> Cn (Conillon), G (Guinean), Cg (Congolese). Angka-angka dalam kolom yang sama jika diikuti oleh huruf yang sama menunjukkan tidak berbeda nyata uji Tukey-Kramer pada  $\alpha = 0.05$ .

Table 5. Variance analysis of indirect somatic embryogenesis ability of 6 genotypes in Robusta coffee (*Coffea canephora*)Tabel 5. Analisis varian kemampuan embriogenesis somatik tidak langsung pada kopi Robusta (*Coffea canephora*)

Source Sumber	Somatic embryogenesis ability Kemampuan embriogenesis somatik						
	Callus induction stage Tahap induksi kalus			Somatic embryo production stage Tahap produksi embrio somatik			
	FC	FEC	SEC	FSE	ANERE	ANEP	
A: Genotype <sup>1)</sup>							
F-Ratio F-Ratio	A: Genotipe <sup>1)</sup>	1.84	17.54	39.05	87.32	48.36	46.99
	B: explant origin <sup>2)</sup>						
	B: asal eksplan <sup>2)</sup>	0.07	0.24	0.02	0.07	0.01	0.12
	AB: interaction						
	AB: interaksi	0.23	0.79	0.22	0.05	0.07	0.02
	A: Genotype <sup>1)</sup>	0.11523	< 0.00001	< 0.00001	< 0.00001	< 0.00001	< 0.00001
Prob level Aras peluang	A: Genotipe <sup>1)</sup>						
	B: explant origin <sup>2)</sup>	0.79500	0.62276	0.89451	0.79736	0.92435	0.73075
	B: asal eksplan <sup>2)</sup>						
	AB: interaction	0.94716	0.55729	0.95167	0.99873	0.99652	0.99986
	AB: interaksi						

Note (Catatan): <sup>1)</sup> The 6 genotypes tested were each of 3 genotypes selected randomly from BP 961 x Q 121 and BP 409 x Q 121, respectively. <sup>2)</sup> The explant origin was explant derived from cutting of mother plant. See Table 1 for the abbreviation of criteria of observation (<sup>1)</sup> enam genotipe yang diuji adalah masing-masing 3 individu yang dipilih secara acak dari silangan BP 961 x Q 121 dan BP 409 x Q 121. <sup>2)</sup> Sumber eksplan yang diuji adalah eksplan yang berasal dari setek setiap individu silangan. Lihat Tabel 1 untuk singkatan kriteria pengamatan).

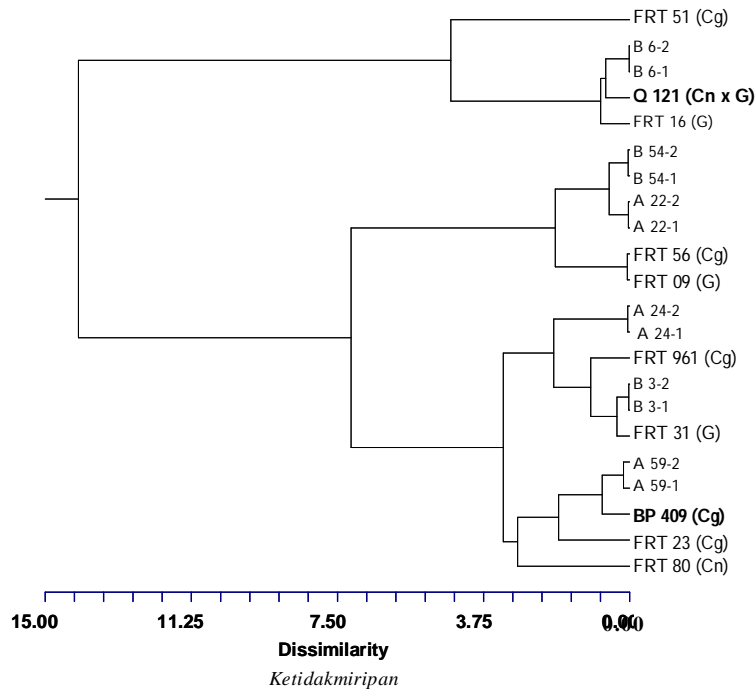


Figure 1. Dendrogram of the somatic embryogenesis (SE) ability in Robusta coffee. The SE ability data are collected from two SE protocols, namely ISE and DSE.

Gambar 1. Dendrogram kemampuan embriogenesis somatik kopi Robusta. Data kemampuan embriogenesis somatik diperoleh dari dua protokol embriogenesis somatik, yaitu embriogenesis somatik langsung dan tidak langsung.

\*) Notes (Catatan): Cg (Congolese), G (Guinean), Cn (Conillon). Cg (Congolese), G (Guinean). Cn (Conillon).

been compared for 7 genotypes representative of *C. canephora* of genetic diversity other than the three parents of the hybrid populations. In our previous results, significant differences were observed between these genotypes for DSE (Priyono *et al.*, 2010).

Present study, with ISE (Table 4), the FSE observed after 10 months (7 months for callogenesis and 3 months for embryo formation) were 2% for the Guinean clone (FRT 16) and 12% for the Congolese one (FRT 23). In the same experiment SE frequency reached 85% for another Guinean clone (FRT 09), 93% for another Congolese clone (FRT 56), and 99% for a Conillon clone (FRT 31).

Deeply, the highest values of FSE were recorded for BP 409 (Congolese) with DSE and FRT 31 (Conillon) with ISE. Two Guinean clones (FRT16 and FRT 09) showed 0% of FSE with DSE, but with ISE this value became 2% for FRT 16 and 85% for FRT 09. Present results again confirm our previous report (Priyono *et al.*, 2010), which showed an important diversity for SE ability not only between the three groups of *C. canephora* (Congolese, Guinean and Conillon) but also within a given group (Figure 1). It means that SE is influenced by a strong genotype x protocol interaction. Although totipotency is an important characteristic of plant cells, under specific conditions all

cells do not express it. Even assuming that all cells are equally totipotent, the number of formed embryos is minimal compared to the total number of cells from a cellular aggregate (Quiroz-Figueroa *et al.*, 2006). Furthermore, the endogenous hormone level might be of great importance, since it regulates the explant differentiation. It has been postulated to be the main difference between genotypes exhibiting various grades of competence (Grieb *et al.*, 1997). Interestingly, in the agreement with DSE (Priyono *et al.*, 2010) the ISE frequency of the three ICCRI clones (BP 409, BP 961 and Q 121) used as parents for controlled crosses, showed significant differences for SE suggesting possible segregation within their progenies for this trait.

Since, for a given protocol, the *in vitro* conditions are the same, it might have been speculated that the embryogenic response depends on physiological status of the tissues from the primary explants (Molina *et al.*, 2002; Quiroz-Figueroa *et al.*, 2002). In present study, the stability of somatic embryogenesis ability according to the mother plant had therefore to be controlled. This factor is the so called "mother plant" effect. It was studied on 6 genotypes, for two cuttings of a given clone. All observed parameters were significantly ( $\alpha = 0.01$ ) affected by the genotype. More importantly, no significant difference between mother plants from a given clone and their interaction has been recorded meaning that differences observed between successive experiments could not be due to the specific "mother plant" used as a source of primary explants (Table 5). It indicated that ISE ability was stable to mother plant derived from cutting. This result was in agreement with previous result (Priyono *et al.*, 2010) with DSE observed in the three parameters possibly influencing SE ability, mother

plant cutting, period of harvest of the explant, and location of the laboratory, has also shown that the DSE process was stable and reproducible for these three factors.

The already achieved, high efficient, coffee somatic embryogenesis (Ducos *et al.*, 2007) offers the possibility to massively propagate elite genotypes of both *C. arabica* (selected F<sub>1</sub> hybrids) and *C. canephora* (clonal varieties). Pilot productions already permitted some commercial applications. Due to its considerable multiplication potential, somatic embryogenesis constitutes the greatest opportunity for large-scale clonal propagation of coffee worldwide, including Indonesia. Using superior clones selected for yield, yield-related traits, resistance to most important pests and diseases, drought tolerance, and easy propagation by somatic embryogenesis or cuttings, coffee production could be more sustainable even if reducing the coffee cultivation area.

## CONCLUSIONS

Direct and indirect somatic embryogenesis in *C. canephora* are suggested as an independent mechanisms. It is stable to mother plant of a given genotype. Moreover, SE of *C. canephora* is strictly influenced by genotypes. The diversity of SE ability is not only between the three groups of *C. canephora* (Congolese, Guinean, and Conillon) but also within a given group. The SE ability of three parental clones had significant differences suggesting possible segregation within their progenies for SE ability.

## ACKNOWLEDGEMENTS

The author thank to Dr. Teguh Wahyudi (Director of Indonesian Coffee and Cocoa Research Institute), Dr. Pierre Broun,

Dr. Bruno Florin, and Dr. Dominique Crouzillat (Nestle R&D Centre Tours), Dr. Vincent Petiard (Nature Source Genetics) and Mr Wismandjaja (PT Nestle Indonesia) for their supporting this project.

## REFERENCES

- Docos, J.P. (2000). *Large Scale of Robusta Clones by Somatic Embryogenesis. Handbook*. Nestle R & D Centre Tours. 46p.
- Ducos, J.P.; R. Alenton; J.F. Reano; C. Kanchanomai; A. Deshayes & V. Pétiard (2003). Agronomic performance of *Coffea canephora* P. Tress derived from large-scale somatic embryo production in liquid medium. *Euphytica*, 215, 223–223.
- Ducos, J.P.; C. Lambot & V. Pétiard (2007). Bioreactor for coffee mass propagation by somatic embryogenesis. *Int. J. of Plant Dev. Biol.*, 1, 1–12.
- Fernandez-Guijarro, B.; C. Celestino & M. Toribio (1995). Influence of internal factors on secondary embryogenesis and germination in somatic embryo of leaves of *Quercus suber*. *Plant Cell Tissue and Organ. Culture*, 41, 99–106.
- Gairi, A. & A. Rashid (2006). Direct differentiation of somatic embryos on different regions of intact seedling of *Azadirachta* in response to thidiazuron. *J. Plant Physiol.*, 161, 1073–1077.
- Gatica-Arias, A.M.; G. Arrieta-Espinoza & A.M. Espinosa (2008b). Direct somatic embryogenesis in *Coffea arabica* L cvs catura and catuai: effect of triacontanol, light condition, and medium consistence. *Agronomia Costarricense*, 32, 139–147.
- Gatica-Arias, A.M.; G. Arrieta-Espinoza & A.M.S. Esquirel (2008a). Plant regeneration via indirect somatic embryogenesis and optimisation of genetic transformation in *Coffea Arabica* L cvs catura and catuai. *Electronic Journal on Biotechnology*, 11, 1–15.
- Giridhar, P.; V. Kumar; E.P. Indu; G.A. Ravishankar & A. Chandrasekar (2004). Thidiazuron induced somatic embryogenesis in *Coffea arabica* and *C. canephora*. *Acta. Bot. Croat.*, 63, 25–33.
- Grieb, B.; F. Schaffer; J. Imani; K.N. Masyhayekki; B. Arnholdt-Smith & K.H. Neumann (1997). Change in soluble protein and phytohormone concentrations of cultured carrot petiole explant during induction of somatic embryogenesis in *Daucus carota* L. *Journal of Applied Botany*, 71, 94–103.
- Hatanaka, T.; O. Arakawa; T. Yasuda; N. Uchida & T. Yamaguchi (1991). Effect of plant growth regulators on somatic embryogenesis in leaf cultures of *Coffea canephora*. *Plant Cell. Reports*, 10, 179–182.
- Ipekci, Z. & N. Gozukirmizi (2003). Direct somatic embryogenesis and synthetic seed production from *Paulownia elongate*. *Plant Cell Reports*, 22, 16–24.
- Kumar, V.; M. Madhava-Naidu & G.A. Ravishankar (2007). Development of coffee biotechnology in vitro plant propagation and crop improvement. *Plant Cell Tissue and Organ Culture*, 87, 49–65.
- Lashermes, P.; J. Cros; M.C. Combes; P. Trouslot; F. Anthony; S. Hamon & A. Charrier (1996). Inheritance and restriction fragment length polymorphism of chloroplast DNA in the genus *Coffea*. *Theor. Appl. Genet.*, 93, 626–632.
- Mano, Y. & T. Komatsuda (2002). Identification of QTLs controlling tissue-culture traits barley (*Hordeum vulgare* L.). *Theor. Appl. Genet.*, 105, 708–715.
- Michaux-Ferrière, N.; D. Biyesse; D. Alvard & D. Dublin (1989). Etude histologique de l'embryogenèse somatique chez *Coffea arabica*, induite par culture sur milieux uniques de fragments foliaires de génotypes déférents. *Café Cacao*

- Thé*, 33, 207–217.
- Molina, D.M.; M.E. Aponte; H. Cortina & G. Moreno (2002). The effect of genotype and explant age on somatic embryogenesis of coffee. *Plant, Tissue and Organ Culture*, 71, 117–123.
- Niemenak, N.; S. Surminski; C. Rohsius; D.O. Ndoumou & R. Lieberei (2008). Regeneration of somatic embryos in *Theobroma cacao* L. in temporary immersion bioreactor and analyses of free amino acid in different tissue. *Plant Cell Reports*, 27, 667–676.
- Pinto-Sintra, A.L. (2007). Establishment of embryogenic cultures and plant regeneration in the Portuguese cultivar 'Touriga Nacional' of *Vitis vinifera* L. *Plant Cell Tissue and Organ Culture*, 88, 253–265.
- Priyono; B. Florin; M. Rigoreau; J.P. Ducos; U. Sumirat; S. Mawardi; C. Lambot; P. Broun; V. Pétiard; T. Wahyudi & D. Crouzillat (2010). Somatic embryogenesis and vegetative cutting capacity are under distinct genetic control in *Coffea canephora* Pierre. *Plant Cell Reports*, 29, 343–357.
- Priyono; Matsaleh & D. Suhendi (2000). Regeneration and plant morphism of orthotropic and plagiotropic leaf culture of *Coffea canephora* through direct somatic embryogenesis. *Pelita Perkebunan*, 16, 65–74.
- Priyono (1992). Direct somatic embryogenesis in Arabica coffee derived from hypocotyls skin explant. *Indonesia Journal of Tropical Agriculture*, 3, 64–66.
- Priyono (2004). *In vitro* culture of coffee leaves for evaluating the capability of somatic embryogenesis of several coffee species. *Pelita Perkebunan*, 20, 110–122.
- Quiroz-Figueroa, F.R.; C.F.J. Fuentes-Cerda; R. Rojas-Herrera & V.M. Loyola-Vargas (2002). Histological studies on the developmental stages and differentiation of two different somatic embryogenesis system of *Coffea arabica*. *Plant Cell Tissue and Organ Culture*, 20, 1141–1149.
- Quiroz-Figueroa, F.R.; M. Gonzalez; R.M. Galaz-Avalos & V.M. Loyola-Vargas (2005). Direct somatic embryogenesis in *Coffea canephora*. p.111–117. **In:** Loyola-Vargas, Vazquez-Flota (Eds). *Methods in Molecular Biology vol. 318: Plant Cell Culture Protocol*. Humana Press Inc., Totowa, New Cork.
- Quiroz-Figueroa, F.R.; R. Rojas-Herrera; R.M. Galaz-Avalos & V.M. Loyola-Vargas (2006). Embryo production through somatic embryogenesis can be used to study cell differentiation in plants. *Plant Cell Tissue Organ Culture*, 86, 285–301.
- Raemakers, K.; I. Pereira; H.K. Van Putten & R. Visser (2005). Indirect somatic embryogenesis in cassava for genetic modification purposes. p. 101–109. **In:** Loyola-Vargas & Vazquez-Flota (Eds). *Methods in Molecular Biology vol. 318: Plant Cell Culture Protocol*. Humana Press Inc., Totowa, New Cork.
- Salaj, J.; B. Petrovska; B. Obert & A. Pret'ova (2005). Histological study of embryolike structures initiated from hypocotyl segments of flax (*Linum usitatissimum* L.). *Plant Cell Reports*, 24, 590–595.
- Sata, S.J.; S.B. Bagatharia & V.S. Thaker (2000). Induction of direct somatic embryogenesis in garlic (*Allium sativum*). *Methods in Cell Science*, 22, 299–304.
- Staritsky, G. (1970). Embryoid formation in callus culture of coffee. *Acta Botanica Neerlandica*, 19, 509–514
- Sun, Y.; X. Zhang; C. Huang; X. Guo & Y. Nie (2006). Somatic embryogenesis and plant regeneration from different wild diploid cotton (*Gossypium*) species. *Plant Cell Reports*, 25, 289–296.
- Wan, Y; T.R. Rocheford & J.M. Widhrom (1992). RFLP análisis to identify putative chromosomal regions involved in the anther culture response and callus formation of maize. *Theor. Appl. Genet.*, 85, 360–365.
- Wang, Y.H. & P.L. Bhalla (2004). Somatic embryogenesis from leaf explants of Australian fan flower (*Scaevola aemula* R.Br.). *Plant Cell Reports*, 22, 408–414.