Journal of Tropical Crop Science Vol. 2 No. 3, October 2015
www.j-tropical-crops.com

The Induction of Primary and Secondary Somatic Embryogenesis for Arabica Coffee Propagation

Meynarti Sari Dewi Ibrahim[AB], Raden Roro Sri Hartati[A], Rubiyo[A], Agus Purwito[B], and Sudarsono*[B]

[A]Indonesian Agency for Agricultural Research and Development (IAARD),
Jl Ragunan 29 Pasar Minggu Jakarta Selatan 12540, Indonesia
[B]PMB Lab., Department of Agronomy and Horticulture, Faculty of Agriculture, Bogor Agricultural University, Jl. Meranti, Kampus IPB Darmaga, Bogor 16680, Indonesia

*Corresponding author; email : s_sudarsono@ymail.com

Abstract

The primary and secondary somatic embryogenesis (SE) can be used for clonal propagation of *Coffea arabica* L. However, the success of SE induction depends on plant growth regulators (PGR) in the propagation media and varieties. This study was conducted to investigate (1) the effects of 2,4-D and thidiazuron to induce primary SE, (2) the use of thidiazuron in solid and semi-solid media to induce secondary SE to support Arabica coffee clonal propagation. The use of PGR promoted fresh weight, number of torpedo and germinated embryos, but has no effects on callus formation. The best medium to induce primary SE varied with variety; media supplemented with 4.52 µM 2,4 -D +18.16 µM thidiazuron was the best for "AS2K" and "Sigarar Utang"; 4.52 µM 2,4-D + 9.08 µM thidiazuron for "S 795"; whereas 4.52 µM 2,4-D + 13.62 µM thidiazuron was for "Kartika". All treatments resulted in morphologically normal somatic embryos. Primary somatic embryos were developed indirectly, through callus, whereas the secondary embryos were directly from somatic embryos. The use of 9.08 µM thidiazuron increased the percentage and number of secondary somatic embryos and the number of regenerated Arabica coffee plantlets.

Keywords**:** *Coffea arabica* L., 2,4-D, thidiazuron, semi-solid media, indirect somatic embryogenesis

Introduction

Somatic embryogenesis is one of tissue culture techniques to propagate plant clonally. Somatic embryogenesis of Arabica coffee has been successfully developed to obtain plant rapidly, uniformly, and true-to-type. Coffee embryogenesis can be done through primary and secondary somatic embryogenesis (Bertrand-Desbrunais et al., 1988; Menendez-Yuffá and García, 1997; Fernández-Da Silva et al., 2005). Secondary somatic embryos are somatic embryo formed from primary somatic embryos. Coffee somatic embryos can be regenerated either directly without callus formation or indirectly through callus formation (Molina et al., 2002; Santana-Buzzy et al., 2007; Ibrahim et al., 2013a).

Propagation using indirect somatic embryogenesis was more promising than direct embryogenesis to produce large quantities of seeds. Embryo maturation process, which was more uniform in indirect embryogenesis, is considered more profitable in mass propagation. Induction of somatic embryogenesis of Arabica coffee using plant growth regulator (PGR) have been reported by Gatica-Arias et al. (2008), Samson et al. (2006), Etienne (2005), Berthouly and Etienne (2000), and Ibrahim et al. (2013a; 2013b) supporting the success use of indirect somatic embryogenesis for propagation.

PGR has essential roles in inducing somatic embryogenesis. The success of using synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D) for the induction of somatic embryos (embryoids) on cultured explants have been reported (Raghavan, 2004). Thidiazuron is effective to induce adventitious shoot formation and to promote axillary shoot proliferation *in vitro* (Lu, 1993). Studies by Santos-Briones and Hernández-Sotomayor (2006) and Samson et al. (2006) reported that in addition to PGR, plant genotypes also affected leaf explant's ability for somatic embryogenesis. The optimum PGR concentrations to induce somatic embryogenesis in coffee genotypes requires further studies.

Study by Ibrahim et al. (2013) on the use of 2,4-D and thidiazuron on *C. arabica* "Kartika" reported that the

increase in number of somatic embryo was in accordance to with the increase of PGR concentration. Therefore, more studies on types and concentration of PGR are required to optimize coffee propagation *in vitro*.

Somatic embryogenesis can also be optimized by utilizing the formation of secondary somatic embryos. Some advantages of using the secondary somatic embryos are the availability of sterile explants that can be directly used for propagation and explant proliferation is faster because requiring no external somatic embryo induction. Secondary somatic embryos can be used to propagate mutans or transgenic plants.

The success of inducing primary and secondary somatic embryos is influenced by various factors, such as the combination and concentration of PGR and plant genotypes. This study aimed to examine the effects of 2,4-D and thidiazuron in the culture media on primary and secondary somatic embryo formation of several Arabica coffee genotypes, i.e. "AS2K", "S 795", "Kartika", and "Sigarar Utang", to support their clonal propagation.

Materials and Methods

The Induction of Primary Somatic Embryogenesis of C. arabica Using 2,4-D and Thidiazuron

Young leaves of "AS2K", "Kartika", "S 795", and "Sigarar Utang" were harvested from greenhouse-grown plants. The leaves were cleaned under running water, sterilized with 0.2% Dithane M-45 and 0.1% Agrimicin for 30 minutes, rinsed with water, re-sterilized for ten minutes in 50% alcohol, followed by 10 to 15 minutes in 10% calcium hypochlorite, then rinsed three times with sterilized water. Leaf blades were cut into 10 mm x 10 mm slices and planted on callus induction media. The cultures were incubated in the dark at ± 25°C and relative humidity of 60%.

Basic medium for callus induction was half-strength of Murashige and Skoog (MS) medium supplemented with B5 vitamins, sucrose (30 g L^{-1}), Polyvinylpolypyrrolidone (250 mg L^{-1}), and Phytagel (2.5 g L^{-1}). The pH of media prior to autoclaving was 5.6 ± 0.1. PGR combinations tested were 4.52 µM 2,4-D + 4.54 µM thidiazuron; 4.52 µM 2,4-D + 9.08 µM thidiazuron; 4.52 µM 2,4-D + 13.62 µM thidiazuron; 4.52 µM 2,4-D + 18.16 µM thidiazuron; 4.52 µM 2,4-D + 22.70 µM thidiazuron; 9.04 µM 2,4-D + 4.54 µM thidiazuron; 9.04 µM 2,4-D + 9.08 µM thidiazuron; 9.04 µM 2,4-D + 13.62 µM thidiazuron; 9.04 µM 2,4-D + 18.16 µM thidiazuron; 9.04 µM 2,4-D + 22.70 µM thidiazuron.

Explants were subcultured into advanced callus induction media consisting of ½ MS, supplemented with sucrose (30 g.L^{-1}), Phytagel (2.5 g.L^{-1}), 2,4-D (4.50 µM) and BAP (17.75 µM) (van Boxtel and Berthouly, 1996). The embryogenic calli of about 200 mg were weighed and subcultured onto ½ MS medium supplemented with kinetin (9.30 µM), sucrose (35 g.L^{-1}), and phytagel (2.5 g.L^{-1}) to solidify the medium (Van Boxtel and Berthouly, 1996). Cotyledonary-stage embryos were then sub cultured into MS medium containing sucrose (40 g.L^{-1}), Phytagel (2.5 g.L^{-1}), and BAP (1.33 µM) (Etienne, 2005). The cultures were incubated at ± 25°C room with irradiation intensity of 1000-1500 lux and relative humidity of 60%.

The Morphological Development of C. arabica Somatic Embryo

The development of Arabica coffee somatic embryo from callus to globular embryos, and to plantlets were recorded using Zeiss AxioVision microscope connected to computer using AxioVision software version 4.82.

The Induction of Secondary Somatic Embryogenesis Using Thidiazuron in Solid and Semi-Solid Media

The explants used to induce secondary somatic embryos were torpedo-stage of primary somatic embryos from four coffee varieties. Thidiazuron at 4.54 µM or 9.08 µM were added into modified MS solid and semi-solid media (Etienne, 2005). Germination medium added with 1.33 µM BAP was used as control. Explants forming secondary somatic embryos were subcultured into germination media. The cultures were then incubated in dark room at ± 25°C until secondary somatic embryos developed.

Data analysis

The experiment was arranged in a completely randomized design with two factors, i.e. coffee varieties and culture media as described above. The data were subjected to analysis of variance (ANOVA) and significant treatment effects at $P < 0.05$. Means comparison were conducted using Duncan Multiple Range Test (DMRT) with SAS software version 9.1. Factors tested at induction of primary somatic embryos were media combination (2,4-D + thidiazuron) and varieties, whereas at induction of secondary somatic embryos were PGR type and concentrations, and media density. The

experiments had ten replications with five leaf slices for each replication. For primary somatic embryos induction each replication consists of one jar containing ± 200 mg of callus. For secondary somatic embryo induction one replication consists of 10 torpedo-stage of primary somatic embryos. Parameters measured were percentage of callus formation, fresh weight of callus, number of torpedoes, number of germinated embryos, percentage of secondary somatic embryo formation, and number of secondary somatic embryos.

Results and Discussion

The Induction of Primary Somatic Embryogenesis of C. arabica Using 2,4-D and Thidiazuron

When cultured on media, all of the coffee varieties in this study showed high percentage (above 80%) of callus formation (Table 1). Calli were developed from edges of the leaf sections after about three weeks in the callus induction media. Considering the high phenolic content of perennial plants, including Arabica coffee, these results were promising as indirect embryogenesis is highly dependent on the quantity of callus formation. Coffee varieties did not significantly affect the percentage of callus formation, but they substantially affected callus fresh weight (Table 1). "AS2K" had the heaviest callus fresh weight than the others. These results demonstrated that each coffee variety requires a specific media to induce somatic embryogenesis. Similarly, Molina (2002), Santos-Briones and Hernández-Sotomayor (2006) and Samson et al. (2006) reported that different coffee genotypes had different rates of somatic embryo formation in different media.

Increasing the concentrations of 2,4-D and thidiazuron increased the percentage of callus formation and callus fresh weight (Table 2). Media containing 9.04 µM of 2,4-D and 13.62 µM thidiazuron had the highest percentage of explant formation, whereas media containing 9.04 µM 2,4-D and 22.70 µM thidiazuron had the greatest culture fresh weight (Table 2). Addition of 2,4-D significantly increased callus formation. Thidiazuron in the culture media did not affect the percentage of callus formation and the fresh weight of culture.

The combination of 2,4-D and thidiazuron in the media induced a better callus formation than addition of thidiazuron alone (Gatica-Arias et al., 2008). These results suggested that high concentrations of thidiazuron, which belong to

Table 1. Callus formation and callus fresh weight of four *C. arabica* varieties at three months after subculture.

Varieties	Callus formation (%)	Callus fresh weight (mg)
"AS 2K"	86.00	410 a
"S 795"	85.80	370 b
"Kartika"	87.20	360 bc
"Sigarar Utang"	87.20	330 c

Note : Numbers in the same column followed by the same letter were not significantly different at ∝= 5%.

Table 2. Effects of 2,4-D and thidiazuron in the callus induction media on *C. arabica* callus formation and callus fresh weight at three months after subculture.

PGR in Callus induction media		Callus formation (%)	Callus fresh weight (mg)
2,4-D (µM)	Thidiazuron (µM)		
4.52	4.54	76.00 e	310 c
4.52	9.08	78.50 de	340 bc
4.52	13.62	83.00 cd	350 bc
4.52	18.16	85.00 bc	360 abc
4.52	22.70	88.50 abc	370 abc
9.04	4.54	90.50 ab	370 abc
9.04	9.08	90.00 ab	390 ab
9.04	13.62	92.50 a	390 ab
9.04	18.16	91.00 ab	400 ab
9.04	22.70	90.50 ab	410 a

Note : Numbers in the same column followed by the same letter were not significantly different at ∝= 5%.

cytokines group with strong activity (Sakakibara, 2004), were not able to induce callus without 2,4-D. The concentration of 2,4-D and thidiazuron in the callus induction media significantly affected somatic embryogenesis. However, the increase of 2,4-D and thidiazuron concentration were not always followed by increased in torpedo stage number (Table 3). “AS2K” had the best response in generating torpedo stage at 4.52 µM 2,4-D + 18.16 µM thidiazuron, “S 795” at 4.52 µM 2,4-D + 22.70 µM thidiazuron, “Kartika” at 9.04 µM 2,4-D + 18.16 µM thidiazuron and “Sigarar Utang” at 4.52 µM 2,4-D + 18.16 µM thidiazuron. Similarly, number of germinated embryo was also affected significantly by 2,4-D and thidiazuron concentrations (Table 4).

The combination of 2,4-D at 4.52 µM or 9.04 µM and thidiazuron at the range of 4.54 to 22.70 µM in the culture media resulted in significant effect of somatic embryo germination (Table 4). The best media for each variety was determined by number of embryogenic callus in callus induction phase, number of torpedos and number of germinated embryos. The best medium to generate the highest number of torpedo and germinated embryos was variety specific. Media containing 4.52 µM 2,4-D + 18.16 µM thidiazuron was best for AS2K“ and “Sigarar Utang”; 4.52 µM 2,4-D + 22.70 µM thidiazuron for “S 795" and 9.04 µM 2,4-D + 18.16 µM thidiazuron for "Kartika". (Tables 3 and 4). However, selection of the best media was not only determined by number of torpedo and germinated embryos, but also the costs of PGR. When the costs of PGR were taken into account, media with 4.52 µM 2,4-D and 9.08 µM of thidiazuron was the best medium for “S 795”; media containing 4.52 µM 2,4-D + 13.62 µM thidiazuron was for “Kartika”, whereas “AS2K” and “Sigarar Utang” were best cultured in

Table 3. Interaction effects between coffee variety and callus induction media on torpedo stage embryo development at eight months after subculture.

Callus induction media		Coffee varieties			
2,4-D (µM)	Thidiazuron (µM)	“AS2K”	“S 795”	“Kartika”	“Sigarar Utang”
4.52	4.54	56.60 dA	45.10 bA	43.40 dA	48.90 dA
4.52	9.08	53.60 dA	48.30 abA	48.80 cdA	44.80 dA
4.52	13.62	66.10 bcdAB	53.80 abB	87.90 abA	56.60 cdB
4.52	18.16	99.10 aA	58.90 abB	62.40 bcdB	84.00 aA
4.52	22.70	94.20 aA	61.20 aB	63.90 bcdB	78.40 abAB
9.04	4.54	59.00 cdA	44.80 bA	56.10 cdA	47.80 dA
9.04	9.08	71.40 bcdA	46.50 abA	64.00 bcdA	72.40 abcA
9.04	13.62	80.40 abcA	45.70 abB	71.50 bcA	64.70 abcdAB
9.04	18.16	95.10 aA	56.60 abB	99.40 aA	58.30 cdB
9.04	22.70	82.20 abA	59.20 abB	85.20 abA	62.90 bcdB

Note : Numbers followed by the same lower case letters on the same column and the same uppercase letters on the same row were not significantly different at DMRT 5%.

Table 4. Interaction effects between coffee variety and callus induction media on somatic embryo germination at two months after subculture.

Callus induction media		Coffee varieties			
2,4-D (µM)	Thidiazuron (µM)	“AS2K”	“S 795”	“Kartika”	“Sigarar Utang”
4.52	4.54	40.80 deA	33.10 bA	31.40 dA	38.60 cdA
4.52	9.08	37.60 eA	36.30 abA	36.40 cdA	35.10 dA
4.52	13.62	51.00 cdeAB	41.80 abB	75.90 abA	47.10 cdB
4.52	18.16	84.40 aA	46.90 abB	50.40 bcdB	80.10 aA
4.52	22.70	78.20 abA	49.20 aB	51.90 bcdB	75.20 abA
9.04	4.54	43.00 cdeA	32.80 bA	44.10 cdA	40.00 cdA
9.04	9.08	55.40 cdeAB	34.50 abB	62.00 bcdAB	76.10 abA
9.04	13.62	64.40 abcA	33.70 abB	59.50 bcA	61.40 abcA
9.04	18.16	79.10 abA	44.60 abB	87.40 a A	52.40 cdB
9.04	22.70	62.00 bcdAB	47.20 abC	73.20 abA	56.90 bcdBC

Note : Numbers in the same column followed by the same lower case letters, or in the same row followed by the same upper case letters were not not significantly different at $\propto$= 5%.

media containing 4.52 µm 2,4-D + 18.16 µm thidiazuron was best for "AS2K" and "Sigarar Utang".

The Morphological Development of Arabica Coffee Somatic Embryo

Somatic embryos were formed from embryogenic callus at callus induction phase (Figure 1 A and B) after about four weeks of culture in embryo induction medium. Embryogenic callus was population of meristematic cells forming aggregates known as pro-embryogenic mass (PEM). PEM have no strong bond among cells, so they could easily be separated from each other. Pro-embryo developed into a globular-stage embryo at three months after subculture on regeneration medium (Figure 1C).

The size of Arabica coffee globular embryo was only about ± 200 µm in diameter (Figure 1D). Globular embryos were then developed into oblong-stage embryo of about the similar size, but the upper parts had stretched so the shape of oblong-stage embryo was ovate (Figure 1D). Figure 1E represented heart-stage embryo (400 to 500 µm in diameter), with distinct curvature at the top, indicating the beginning of cotyledon formation. Cotyledon was observed as a small protrusion at the tip of the apical embryo and its development was indicated by heart-shaped stage (Yeung, 1995; Quiroz-Figueroa et al., 2006). Cotyledon of dicotyledonous plants emerged as two meristematic protrusions located at the apical tip of the embryo. These protrusions occurred due to the expansion of the apical tip of the embryo laterally. Both protrusions caused the embryos split symmetrically (Nugroho et al., 2008).

Embryos elongated before developing into torpedo-stage (Figure 1F). The stages of development in this study were consistent with those reported by Quiroz-Figueroa et al. (2002). The average measurement of embryos and torpedo-stage embryo were 500 µm by 100 µm, and 600 µm by 2000 µm, respectively (Figure 1G).

The early phase of cotyledonary stage started with the opening of upper part of embryo, which was then developed into leaf (Figure 1H). The apex of both cotyledones formed apical meristem (Nugroho et al., 2008). Cotyledonary-stage can be observed when the cotyledon was ± 600 µm in diameter and 2000-3000 µm in height, with radicle and leaves of the embryo. Development of plantlets was indicated by the growth of green leaves and tap roots (Figure 1I).

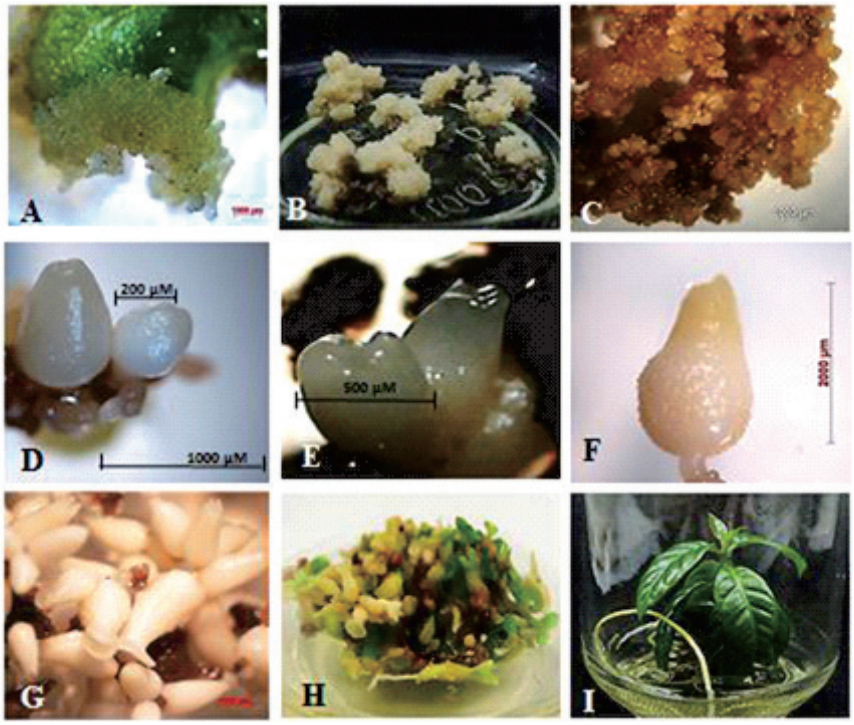

Figure 1. Developmental stages of indirect somatic embryogenesis of *C. arabica* "AS2K" on media containing 2,4-D and thidiazuron: (A) callus on initial induction medium; (B) embryogenic callus on advanced callus induction medium; (C) pro-embryonic mass (PEM); (D) globular stage; (E) heart-stage; (F) elongated embryo; (G) torpedo stage; (H) cotyledonary- stage; (I) a fully grown planlet.

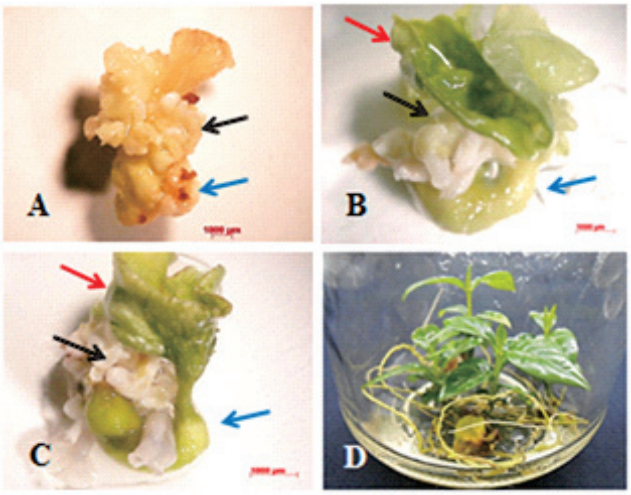

Figure 2. Secondary somatic embryogenesis of *C. arabica* "AS2K" : (A) globular stage; (B) torpedo stage; (C) cotyledonary stage; (D) fully grown plantlet. Blue arrow indicates primary somatic embryo, black arrow indicates secondary somatic embryogenesis, and red arrow indicates cotyledonary stage of primary somatic embryo.

The developmental stages of Arabica coffee somatic embryo in this study, i.e. pro-embryo, globular, oblong, heart, elongated embryos, torpedo, cotyledonary, germinated embryos, until plantlets were fully developed, were considered normal and followed the pattern of somatic embryogenesis reported by Zimmerman (1993). This study had succesfully described and documented somatic embryo developmental stages of Arabica coffee.

The Induction of Secondary Somatic Embryo Using Thidiazuron in Solid and Semi-Solid Media

The addition of 4.54 and 9.08 µM thidiazuron into the culture media resulted in higher percentages of secondary somatic embryogenesis than that of 1.33 µM BAP media. Medium solidity did not affect the percentages of somatic embryo formation (Table 5).

Previous studies reported the success of secondary somatic embryogenesis induction in Arabica coffee "Catimor" that were previously frozen with liquid nitrogen and stored (Menendez-Yuffá and García, 1997; Bertrand-Desbrunais et al., 1988). Furthermore, Oktavia et al., 2003) regenerated secondary somatic embryos using IAA and BAP-containing media. However, no studies reported the use of semi-solid media to regenerate secondary somatic embryos in Arabica coffee. This study has demonstrated an alternative protocol to induce coffee somatic embryogenesis which could potentially be used for other crops.

Table 5. Percentage of secondary somatic embryo formation of *C. arabica* "AS2K" on solid and semi-solid media at two months after culture.

Treatment	Percentage of secondary somatic embryo formation		
	Solid media	Semi-solid media	
Thidiazuron 4.54 µM	52.00	49.00	50.50 a
Thidiazuron 9.08 µM	64.00	61.00	57.50 a
BAP 1.33 µM (control)	2.00	1.00	1.50 c
Average	39.33	33.67	

Note : Numbers in the same column followed by the same letters were not significantly different at ∝= 5%.

Tabel 6. Number of secondary somatic embryo of *C. arabica* "AS2K" on solid and semi-solid media at four months after culture.

Treatment	Average number of secondary somatic embryos per explant		
	Solid media	Semi solid media	
Thidiazuron 4.54 µM	3.94	4.04	3.99 b
Thidiazuron 9.08 µM	5.56	4.94	5.24 a
BAP 1.33 µM (control)	0.20	0.10	0.15 c
Average	3.23	3.02	

Note : Numbers in the same column followed by the same lower case letters were not significantly different at ∝= 5%.

Media containing 9.08 µM thidiazuron resulted in the highest somatic embryogenesis (Table 6). The number of secondary somatic embryos was affected by thidiazuron concentrations in the induction media. These results suggested increasing number of somatic embryos can bedone by increasing thidiazuron concentration in the media. Results of this study also demonstrated that media density did not significantly affect the number of somatic embryos. The induction of secondary somatic embryos using thidiazuron at 4.54 dan 9.08 µM could use either solid or semi-solid media. The secondary somatic embryos in this study developed directly without callus induction. However, the development of secondary somatic embryos resembled the development of primary somatic embryo. The development of secondary somatic embryo from primary somatic embryo started with pro-embryo, globular, heart, torpedo, cotiledonary stages, and planlets (Figure 2).

Conclusion

The effects of 2,4-D and thidiazuron on primary somatic embryo induction was variety specific. "AS2K" and "Sigarar Utang" were best grown on media containing 4.52 µM 2,4-D + 18.16 µM thidiazuron; "S 795" on media containing 4.52 µM 2,4-D + 9.08 µM thidiazuron, and "Kartika" on media containing 4.52 µM 2,4-D + 13.62 µM thidiazuron. The morphology of Arabica coffee somatic embryo was similar to somatic embryo in other dicotyledonous plants. The development of secondary somatic embryos from torpedo-phase in this study occurred directly without callus induction. Either solid or semi-solid media can be used to induce secondary somatic embryos of Arabica coffee. The highest percentage and number of somatic embryos were obtained from media with 9.08 µM thidiazuron. Thidiazuron at 9.08 µM increased the percentage and number of secondary somatic embryos, hence was suitable for propagating Arabica coffee.

Acknowledgment

This research was funded by the Indonesian Agency for Agricultural Research and Development (IAARD), Ministry of Agriculture through the Indonesian Center for Estate Crops Research and Development.

References

Berthouly, M., and Etienne, H. (2000). Somatic embryogenesis of Coffee *In* "Coffee Biotechnology and Quality, Proceedings of the 3rd International Seminar on Biotechnology in the Coffee Agro-Industry" (T. Sera, C.R. Soccol, A. Pandey, S, Roussos, eds), pp 71-90. Kluwer Academic Publishers.

Bertrand-Desbrunais, A., Fabre, J., Engelmann, F., Dereuddre, J., and Charrier, A. (1988). Reprise de l'embryogenèse adventive à partir d'embryons somatiques de caféier (*Coffea arabica* L.) après leur congélation dans l'azote liquide. *C. R. Academie Science Paris* **307**, 795-801.

Etienne, H. (2005). Somatic embryogenesis protocol: *Coffea arabica* L. and *C. canephora* P. *In* "Protocol for Somatic Embryogenesis in Woody Plants" (S.M.Jain, and P.K.Gupta, eds). pp.167-179. Springer.

Fernández-Da Silva, R., Hermoso-Gallardo, L., and Menéndez-Yuffá, A. (2005). Primary and secondary somatic embryogenesis in leaf sections and cell suspensions of *Coffea arabica* Cv. Catimor. *Intercia* **30**, 694-698.

Gatica-Arias, A.M., Arrieta-Espinoza, G., and Esquivel, A.M.E. (2008). Plant regeneration via indirect somatic embryogenesis and optimisation of genetic transformation in *Coffea arabica* L. cvs. Caturra and Catuaí.

Electronic Journal of Biotechnology **11**, 1-12.

Ibrahim, M.S.D., Hartati, R.S., Rubiyo, Purwito, A., and Sudarsono. (2013a). Embriogenic calli induction and regeneration potential of Arabica coffee by 2,4-dichlorophenoxyacetic acid and 6-benzyladenine. *Buletin Ristri* **4**, 91-96.

Ibrahim, M.S.D., Hartati, R.S., Rubiyo, Purwito, A., and Sudarsono. (2013b). Direct and indirect somatic embryogenesis on Arabica coffee (*Coffea arabica*). *Indonesian Journal of Agricultural Science* **14**, 79-86.

Lu, Chin-Yi (1993). The use of thidiazuron in tissue culture. *In Vitro Cellular and Developmental Biology* **29**, 92- 96.

Menéndez-Yuffá, A. and de Garcia, E.G. (1997). Morphogenic events during indirect somatic embryogenesis in coffee "Catimor". *Protoplasma* **199**, 208–214.

Molina, D.M., Aponte M.E., Cortina, H., and Moreno, G. (2002). The effect of genotype and explant age on somatic embryogenesis of coffee. *Plant Cell Tissue and Organ Culture* **71**,117-123.

Nugroho, L.H, Purnomo, and Sumardi, I. (2008). "Struktur dan Perkembangan Tumbuhan". 177 pp. Penebar Swadaya.

Oktavia, F., Siswanto, Budiani A, and Sudarsono. (2003). Direct somatic embryogenesis and regeneration of arabica coffee plantlets (*Coffea arabica*) from different explant. *Menara Perkebunan* **71**, 44-55.

Quiroz-Figueroa, F.R., Fuentes-Cerda, C.F.J., Rojas-Herrera R., and Loyola-Vargas, V.M. 2002. Histological studies on the developmental stages and differentiation of two different somatic embryogenesis systems of *Coffea arabica*. *Plant Cell Report* **20**, 141–1149.

Quiroz-Figueroa, F.R., Rojas-Herrera, R., Galaz-Avalos, R.M., and Loyola-Vargas, V.M. 2006. Embryo production through somatic embryogenesis can be used to study cell differentiation in plants. *Plant Cell Tissue and Organ Culture* **86**, 285-301.

Raghavan, V. 2004. Role of 2,4-dichlorophenoxyacetic acid (2,4-D) in somatic embryogenesis on cultured zygotic embryos of *Arabidopsis*: cell expansion, cell cycling, and morphogenesis during continuous exposure of embryos to 2,4-D. *American Journal of Botany* **91**, 1743-1756.

Sakakibara, H. (2004). Cytokinin biosynthesis and metabolism In "Plant Hormones: Biosynthesis, Signal, Action" (P.J. Davies, eds.), third Edition. pp 95-114. Kluwer Academic Publisher.

Samson, N.P., Campa, C., Le Gal L., Noirot M., Thomas G., Lokeswari T.S., and Kochko A.de. (2006). Effect of primary culture medium composition on high frequency somatic embryogenesis in different Coffea species. *Plant Cell Tissue and Organ Culture* **86**, 37-45.

Santana-Buzzy, N., Rojas-Herrera R., Galaz-Ávalos R.M., Ku-Cauic J.R., Mijangos-Cortés J., Gutierrez-Pacheco L.C, Canto A., Quiroz-Figueroa F., and Loyala-Vatgas V.M. (2007). Advances in coffee tissue culture and its practical applications. *In Vitro Cellular and Developmental Biology* **43**, 507-520.

Santos-Briones, C.D.L, and Hernández-Sotomayor, S.M.T. (2006). Coffee biotechnology. *Brazilian Journal of Plant Physiology* **18**, 217-227.

van Boxtel, J., and Berthouly, M. (1996). High frequency of somatic embryogenesis from coffee leaves. *Plant Cell Tissue and Organ Culture* **44**, 7-17.

Yeung, E.C. (l995). Structural and development patterns in somatic embryogenesis *In* "*In vitro* Embryogenesis in Plants" (T.A.Thorpe, ed). pp 205-247. Kluwer.

Zimmerman, J.L. (1993). Somatic embryogenesis: A model for early development in higher plants. *The Plant Cell* **5**, 1411-1423.