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# Embryo Germination Development of *Coffea arabica* L. at Various Media Composition, Subcultures Stages and Embryo Size

Rina Arimarsetiowati[1*)]

[1)]Indonesian Coffee and Cocoa Research Institute, Jl. P.B. Sudirman 90, Jember, Indonesia
[*)]Corresponding author: arimarsetiowati@gmail.com
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**Abstract**

Most reliable and efficient protocol for *Coffea arabica* L. of Sigararutang variety plant regeneration was established using embryoid as an early explant from the induction of embryogenic callus phase. A completely randomized designs with 5 replications was designed to accomplish 20 protocols of embryo germination methods with different steps of subculture, size of embryo and germination medium. The embryogenic calluses from the flush leave explant were induced embryoid on a half-strength MS medium fortified with a half-strength combination vitamin of 1.8 mg/L nicotinic acid, 10.1 mg/L thiamin HCl and 3.1 mg/L pyridoxine, 50 mg/L myo inositol, 33 mg/L L.cistein, 1 mg/L Kinetin, 0.1 mg/L NAA, 20 gr/L sucrose, 2.4 gr/L gelrite and pH 5.5. The result showed that Protocol 17 was the most effective, with 59,2% of rooted cotyledons, 4.04 cm of length of roots, 1.68 cm of length of hypocotyl, 20.8% of opened cotyledons and 100% of cotyledonary embryo at the end of 8 weeks which used the B medium, large embryos and twice phase of subculture from liquid medium to solid medium. The Protocol 17 is stable protocol from low to high value. Protocol 8 is the steady protocol from high to low value. Protocols 17 and 8 are the highest and lowest ranking, respectively, for each parameter. Protocol 17 is the most suitable for the germination embryo somatic.

**Keywords:** Arabica coffee, embryo somatic, embryoid, cotyledon, hypocotyl, germination

## INTRODUCTION

As one of major important commodities in the world, coffee cultivation provides livelihood to more than 80 million people and more than 11 million hectares are cultivated around the world annually (Layola-Vargas *et al*., 2016). Coffee (*Coffea arabica* L.) is one of the most commercial powerful crops for more than 60 countries in the world, mainly in Latin America, Africa, and Asia (USDA, 2011) and it is highly valuable for beverage consumption universally. In addition, coffee is the most traded commodity, next to oil, on world markets (Etienne, 2005). Arabica coffee is the most extensively commercially cultivated species in the world (Gatica *et al*., 2007), famous for producing high-quality coffee with a low caffeine content and a fine aroma (Lashermes *et al*., 2009), and tolerant to the major fungal diseases such as leaf rust and coffee berry disease (Silva *et al*., 2006).

Sigararutang is a superior variety of Arabica coffee which has been legally distributed as stated in Decree by the Agriculture Ministry number 205/Kpts/SR.120/4/2005 regarding the variety releasing of Sigararutang as superior coffee variety.

It grows in the highlands more than 1,000 m asl. Based on the coffee cupping, Sigararutang coffee is a world-class specialty coffee that has the most favored attributes of coffee winners in all over the world.

Conventional breeding of coffee is quite difficult because of the long duration of processes involved in selection, hybridization, progeny evaluation, backcrossing and interspecific crossing before seeds are set (Oliveira *et al*., 2007). Coffee production is affected by planting material, multiplication method rates and susceptibility to diseases and pests. Technique such as plant tissue culture and plant transformation can contribute to coffee improvement and accelerate the release of varieties with new traits (Santos-Briones & Hernandez-Sotomayor, 2006).

Somatic embryogenesis (SE) has been effectively conquered on an industrial level for the *C. arabica* species (Bobadilla Landey *et al.*, 2013). There have been several reports on somatic embryogenesis in *C. arabica* (Da Silva *et al*., 2005; Etienne, 2005; Samson *et al*., 2006; Ducos *et al*., 2007; Ebrahim *et al*., 2007; Gatica *et al*., 2008a,b; Lopez *et al*., 2010; Rezende *et al*., 2008). In coffee, this process has been achieved via direct somatic embryogenesis from pro-embryogenic cells of leaf tissue in the absence of visible callus proliferation or by indirect somatic embryogenesis (ISE) via friable embryogenic callus formation (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 conversion into plants (van Boxtel & Berthouly, 1996). Embryogenic callus cultures are obtained after culturing of explants on an auxin containing medium. Thereafter, subculture on auxin free medium induces embryo regeneration (van Boxtel & Berthouly, 1996). The use of liquid medium has enabled the proliferation and mass production of somatic embryos in erlernmeyer flasks in *C. arabica* and *C. canephora* (Zamarripa *et al*., 1991; van Boxtel & Berthouly, 1996; Barry-Etienne *et al*., 2002a; Quiroz-Figueroa *et al*., 2002; Santana *et al*., 2004).

Previous investigations have been conducted to investigate the efficiency of liquid system using erlenmeyer flasks (Berthouly *et al*., 1995), the composition of culture media (Neuenschwander & Baumann, 1992; van Boxtel & Berthouly, 1996; Quiroz-Figueroa *et al*., 2001), the culture density (van Boxtel & Berthouly, 1996; Santana *et al*., 2004), the callus age (Santana *et al*., 2004), and the subculture intervals (van Boxtel & Berthouly, 1996). However, the Arabica coffee SE process is still hindered by two practical bottlenecks: an embryo-to-planlet conversion rate that is too low, and exaggerated plant losses in the nursery at each step of the acclimatization and hardening process, which causes to be this propagation system insufficiently cost-effective and productive (Etienne *et al*., 2013). Currently, these extreme production costs are a serious disadvantage to the mass distribution of Arabica coffee in Indonesia. The *C. canephora* and many other woody species face a similar case resulting in too expensive production costs of somatic seedling, thereby discontinuing the large-scale utilization of somatic seedlings as planting material (Lelu-Walter *et al*., 2013).

Embryo germination is the critical step for the planlet conversion. Successful germination of somatic embryo is influenced by the medium composition, stage of the subculture and embryo size. The composition of the media relates to the growth regulator utilization. The subculture stage is related to the process of transfer of solid media and liquid media. Embryo size associated with embryo formation to be germinated to produce buds. Presumably, the greater and more perfect of the embryo size, the higher of

the successful rate of shoot formation. There are few reports on the events occurring during pre-germination stages leading to young planlet of coffee. Moreover, there are no reports on an efficient and reproducible germination protocol for the most preferred, Sigararutang variety of Arabica coffee.

Therefore, the aim of this research was to establish the most reliable, efficient and reproducible protocol for embryo development and germination of somatic embryos from embryoid explant of coffee (*Coffea arabica* L. variety Sigararutang) by studying the influence of *in vitro* culture system at various of media composition, subcultures stages and embryo size, to be subsequently used for enhancement the plant conversion of SE during acclimatization.

## MATERIALS AND METHODS

The research was conducted in Tissue Culture Laboratory of Indonesian Coffee and Cocoa Research Institute, Jember, Indonesia. A completely randomized design with five replications was designed to accomplish 20 protocols of embryo germination with different step of subculture, size of embryo and germination medium. A brief description of the mentioned protocols is represented in Table 1.

Embryoids as a starting materials were collected from embryogenic callus induction from the flush of Sigararutang variety of Arabica coffee using 5 µM 2,4-D and 5 µM 2-ip as specified by Arimarsetiowati (2011) protocol. The embryogenic calluses were then induced to embryoid on a half-strength Murashige-Skoog (MS) (1962) medium supplemented with a half-strength combination vitamin of 1.8 mg/L nicotinic acid, 10.1 mg/L thiamin HCl, 3.1 mg/L pyridoxine as according to Sondhal *et al*., (1994), 50 mg/L myo inositol, 33 mg/L L.cistein, 1 mg/L Kinetin, 0.1 mg/L naphthalene acetic acid (NAA), 20 g/L sucrose, 2.4 gr/L gelrite and pH 5.5. The culture was set at the temperature of 25$^{O}$C in dark condition. The embryoid culture will be used for stock material as a starting explant for the further of 20 protocols.

Embryo germination method consisted of 20 kinds of protocols (Table 1). Embryoids were layed on solid medium directly in the first and second protocols. Solid medium was for inducing shoot and root that consisted of two kinds medium. Medium A was composed of a half-strength of MS medium supplemented with vitamin (1 mg/L pyridoxine, 10 mg/L thiamine, 1 mg/L nicotinic acid), 30 g/L sucrose, 0.7 µM indole acetic acid (IAA), 0.5 mg/L benzyl amino purine (BAP), 2.3 mg/L Adenin, 0,1 mg/L NAA, 10% coconut water dan 50 mg/L AgNO3. On the other hand, medium B contained a half-strength of macro MS medium, a half-strength of micro driver and Kuniyuki walnut (DKW) supplemented with vitamin DKW, 2 mg/L glycine, 10 g/L glucose, 5 g/L sucrose. Moreover, embryoid was placed by immersing in 50 mL liquid medium at the third until twentieth protocols. The liquid medium consisted of full MS medium (without plant growth regulators). These cultures were put on the shaker at 100 rpm during three 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 (0.01-0.25 cm), medium (0.26-0.50 cm), big (0.51-0.70 cm), and mixed embryo explants which had been selected in each phase of sub-cultures. While, at the first, second, nineteenth and twentieth protocols, the embryoid was not selected.

Table 1. The 20 protocols of embryo germination at the various phase of subculture, the size of the embryo and germination medium

| Protocols | Phase of Sub-Culture | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | | | | 4 | | | | 5 | | | |
| | Embryoid Medium | Solid Medium | Liquid Medium | | | | A Germination Medium | | | | B Germination Medium | | | |
| | | | S | M | L | Mix | S | M | L | Mix | S | M | L | Mix |
| 1 | ✓ | ✓ | | | | | | | | ✓ | | | | |
| 2 | ✓ | ✓ | | | | | | | | | | | | ✓ |
| 3 | ✓ | ✓ | ✓ | | | | | | | ✓ | | | | |
| 4 | ✓ | ✓ | | ✓ | | | | | | ✓ | | | | |
| 5 | ✓ | ✓ | | | ✓ | | | | | ✓ | | | | |
| 6 | ✓ | ✓ | | | | ✓ | | | | ✓ | | | | |
| 7 | ✓ | ✓ | ✓ | | | | | | | | | | | ✓ |
| 8 | ✓ | ✓ | | ✓ | | | | | | | | | | ✓ |
| 9 | ✓ | ✓ | | | ✓ | | | | | | | | | ✓ |
| 10 | ✓ | ✓ | | | | ✓ | | | | | | | | ✓ |
| 11 | ✓ | ✓ | | | | ✓ | ✓ | | | | | | | |
| 12 | ✓ | ✓ | | | | ✓ | | ✓ | | | | | | |
| 13 | ✓ | ✓ | | | | ✓ | | | ✓ | | | | | |
| 14 | ✓ | ✓ | | | | ✓ | | | | ✓ | | | | |
| 15 | ✓ | ✓ | | | | ✓ | | | | | ✓ | | | |
| 16 | ✓ | ✓ | | | | ✓ | | | | | | ✓ | | |
| 17 | ✓ | ✓ | | | | ✓ | | | | | | | ✓ | |
| 18 | ✓ | ✓ | | | | ✓ | | | | | | | | ✓ |
| 19 | ✓ | ✓ | | | | ✓ | | | | ✓ | | | | |
| 20 | ✓ | ✓ | | | | ✓ | | | | | | | | ✓ |

Notes: S, M, L, and Mix are the size of embryos (S = Small, M = Medium, L = Large, Mix = Mixed); A and B are germination medium; In the Protocols 19 and 20 liquid medium phase was repeated twice in the same medium prior germination medium.

The alteration of embryoid growth into mature embryo and germination embryo was observed at 2, 4, 6, and 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 Duncan's multiple range test at P = 0.05.

## RESULTS AND DISCUSSION

Beforehand, the development and germination of Sigararutang Arabica coffee somatic embryos using 20 different protocols were comparable in the present study. 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. Five morphological types were described on the basis of percentages of rooted cotyledons, length of roots, length of hypocotyl, percentages of opened cotyledons and percentages of cotyledonary embryo after 2, 4, 6, and 8 weeks of growth.

### Rooted Cotyledons

The highest percentage of rooted was achieved from Protocol 16, while the lowest occurred in Protocol 7 in week 2. This was due to the utilization of large-sized embryos (torpedo) in Protocol 16 which had complete and perfect performance embryo phase. Large and perfect embryos have high opportunity to develop further phase faster than smaller embryo. This embryo was derived from liquid

phase that had passed through maturation phase and then subcultured using medium B. Otherwise in Protocol 7, although using large-sized embryos, but the embryo was selected early from solid media then subcultured in liquid media containing MS without hormone then subcultured to medium A. Avivi *et al*. (2010) reported that the ability of smaller embryozigote to induce callus was higher compared to larger size embryo in cocoa.

In week 4, the highest percentages of rooted cotyledons occur in Protocol 2. This is because of all embryoids were subcultured in media B without going through the selection process. Similarly in week 2, the lowest percentages of rooted cotyledon was occurred in Protocol 7 after 4 weeks. In week 6 and 8, percentages of rooted cotyledons occur in Protocols 16 and 17. This was because of the utilization of embryos derived from liquid media so that ready to germinated in solid media. In addition, in this phase the embryo was large-sized and perfect performance so that it had enough nutrition to germinate. The advantages of liquid media for enhancing shoot propagation (Hammerschlag, 1982; Harris & Mason, 1983), growth (Snir & Erez, 1980; Skidmore *et al*., 1988) or somatic embryogenesis (Jones & Petolino, 1988; Gawel & Robacker, 1990) have been reported for several species. Mechanisms involved in this improved performance are not known with precisely. The absence of gelling agent may increase the availability of water and dissolved substances to explants (Debergh, 1983).

The use of A and B media (with and without auxin hormone) and embryo size in the later stage of subculture had no effect on the percentages of rooted cotyledons. The most important thing is the utilization of complete performance embryo after passing through the liquid phase. On the other hand, the lowest percentages of rooted cotyledons was achieved in Protocol 8 because the embryo had a root death. According to Yang *et al*. (2013) to obtain intact plant derived from somatic embryo, it was absolutely necessary competent development embryo as a source material for conversion. Table 2 shows the response of the percentages of rooted cotyledons Arabica of Sigararutang variety.

Table 2. Percentage of rooted cotyledons in twenty protocols in Sigararutang variety after 2, 4, 6, and 8 weeks of growth.

| Protocols | Culture periods (weeks) | | | |
|---|---|---|---|---|
| | 2 | 4 | 6 | 8 |
| 1 | 8 cd | 10 efg | 16.4 bcd | 16.4 bcd |
| 2 | 42.8 a | 48 a | 49.6 a | 49.6 a |
| 3 | 1.6 d | 2.4 fg | 2.8 bcd | 2.8 bcd |
| 4 | 1.2 d | 3.6 fg | 5.6 bcd | 5.6 bcd |
| 5 | 3.2 d | 3.6 fg | 3.2 bcd | 3.2 bcd |
| 6 | 0.8 d | 2.8 fg | 3.6 bcd | 3.6 bcd |
| 7 | 0.4 d | 0 g | 1.2 cd | 1.2 cd |
| 8 | 2.8 d | 1.6 g | 0 d | 0 d |
| 9 | 6 cd | 8.8 efg | 4.8 bcd | 4.8 bcd |
| 10 | 3.6 d | 9.6 efg | 9.2 bcd | 9.2 bcd |
| 11 | 9.2 bcd | 11.2 efg | 21.2 b | 21.2 b |
| 12 | 38 a | 30.8 bc | 20.4 b | 20.4 b |
| 13 | 44.4 a | 45.6 a | 48.8 a | 48.8 a |
| 14 | 18 bc | 20.4 cde | 19.2 bc | 19.2 bc |
| 15 | 14.8 bcd | 15.6 def | 12 bcd | 12 bcd |
| 16 | 45.2 a | 45.6 a | 59.2 a | 59.2 a |
| 17 | 39.2 a | 40 ab | 59.2 a | 59.2 a |
| 18 | 22 b | 28.4 bcd | 43.6 a | 43.6 a |
| 19 | 12.8 bcd | 25.2 cd | 8.4 bcd | 8.4 bcd |
| 20 | 22.8 b | 28.4 bcd | 12 bcd | 12 bcd |

Note: Means within the same column having the same letter were not significantly different by the Duncan test 5%.

## Root Length

The lowest of the length of roots occurs in Protocols 6 and 7. This is because the embryo was selected from solid medium early then subcultured in liquid medium containing MS without hormones in week 2. Liquid cultures enable relatively easier manipulation of successive embryo development stages (Lilien-Kipnis *et al*. 1994), and as the mechanization of production requires, among others, the synchronization of embryo development. *Narcissus* L. micropropagation in liquid media showed that liquid culture system was more efficient compared to cultivation on solid media. While the highest roots length was obtained at Protocol 16 similar with on the observation from the percentages of rooted cotyledons. Similar results were obtained in week 4 where Protocols 16 and 7 were the highest and the lowest, respectively. In week 6 and 8, the highest root length was obtained at Protocols 16 and 17 similar with on the observation from the percentages of rooted cotyledons. While, the lowest root length was achieved at Protocol 8. The percentages of rooted cotyledons and the length of root are interconnected. The length of root is shown in Table 3.

## Hypocotyl Length

The highest hypocotyl length was obtained in Protocol 2, because embryoids were directly subcultured into media B without going through a process of embryo size selection in week 2. While, the lowest results were achieved at Protocol 7. In week 4, the highest hypocotyl length was obtained at Protocol 19 because this stage undergoing a long subculture process of 3 times, using mixed embryo (without going through the selection process) and using media A at the end of the subculture stage. While the lowest results were obtained at Protocol 7. In week 6 and 8, the highest hypocotyl length was obtained in Protocol 17 and 19. This is because

Table 3. Length of roots in twenty protocols in Sigararutang variety after 2, 4, 6 and 8 weeks of growth (cm)

| Protocols | Culture periods (weeks) | | | |
|---|---|---|---|---|
| | 2 | 4 | 6 | 8 |
| 1 | 0.52 defg | 0.58 defg | 0.8 def | 0.8 def |
| 2 | 1.56 b | 1.88 bc | 1.92 cde | 1.92 cde |
| 3 | 0.1 g | 0.22 efg | 0.6 ef | 0.6 ef |
| 4 | 0.1 g | 0.6 defg | 1.02 def | 1.02 def |
| 5 | 0.20 fg | 0.58 defg | 0.86 def | 0.86 def |
| 6 | 0.06 g | 1.02 cdefg | 2 cde | 2 cde |
| 7 | 0.06 g | 0 g | 0.16 f | 0.16 f |
| 8 | 0.18 fg | 0.14 fg | 0 f | 0 f |
| 9 | 0.38 efg | 0.94 cdefg | 0.48 ef | 0.48 ef |
| 10 | 0.30 efg | 1.24 bcdef | 1.34 def | 1.34 def |
| 11 | 0.38 efg | 0.52 defg | 0.64 def | 0.64 def |
| 12 | 0.6 cdefg | 0.86 cdefg | 0.74 def | 0.74 def |
| 13 | 1.12 bcd | 1.36 bcde | 2.9 abc | 2.9 abc |
| 14 | 0.86 bcdef | 1.1 cdefg | 0.8 def | 0.8 def |
| 15 | 1.2 bcd | 1.24 bcdef | 2.18 bcd | 2.18 bcd |
| 16 | 2.56 a | 3.08 a | 4.02 a | 4.02 a |
| 17 | 1.14 bcd | 1.42 bcde | 4.04 a | 4.04 a |
| 18 | 1.44 b | 2.36 ab | 3.44 ab | 3.44 ab |
| 19 | 0.94 bcde | 1.56 bcd | 1.06 def | 1.06 def |
| 20 | 1.28 bc | 1.42 bcde | 0.92 def | 0.92 def |

Note: Means within the same column having the same letter were not significantly different by the Duncan test 5%.

Table 4. Length of hypocotyl in twenty protocols in Sigararutang variety after 2, 4, 6 and 8 weeks of growth (cm)

| Protocols | Culture periods (weeks) | | | |
|---|---|---|---|---|
| | 2 | 4 | 6 | 8 |
| 1 | 0.86 b | 0.8 abcd | 0.94 cd | 0.94 cd |
| 2 | 3.82 a | 1.4 ab | 0.98 cd | 0.98 cd |
| 3 | 0.32 b | 0.48 cd | 0.46 def | 0.46 def |
| 4 | 0.38 b | 1.2 abcd | 0.96 cd | 0.96 cd |
| 5 | 0.38 b | 0.98 abcd | 0.66 de | 0.66 de |
| 6 | 0.42 b | 0.98 abcd | 1.02 bcd | 1.02 bcd |
| 7 | 0.16 b | 0.42 d | 0.22 ef | 0.22 ef |
| 8 | 0.4 b | 0.76 abcd | 0 f | 0 f |
| 9 | 0.54 b | 1.42 a | 0.68 cde | 0.68 cde |
| 10 | 0.5 b | 1.16 abcd | 0.78 cde | 0.78 cde |
| 11 | 0.6 b | 0.82 abcd | 0.98 cd | 0.98 cd |
| 12 | 1.26 b | 1.18 abcd | 0.86 cde | 0.86 cde |
| 13 | 1.26 b | 1.36 abc | 1.38 abc | 1.38 abc |
| 14 | 0.92 b | 1.1 abcd | 0.78 cde | 0.78 cde |
| 15 | 0.52 b | 0.52 bcd | 0.68 cde | 0.68 cde |
| 16 | 1.36 b | 1.36 abc | 1.64 ab | 1.64 ab |
| 17 | 1.46 b | 1.58 a | 1.68 a | 1.68 a |
| 18 | 0.98 b | 1.06 abcd | 1.38 abc | 1.38 abc |
| 19 | 1.4 b | 1.62 a | 1.68 a | 1.68 a |
| 20 | 1.12 b | 1.3 abcd | 0.76 cde | 0.76 cde |

Note: Means within the same column having the same letter were not significantly different by the Duncan test 5%.

of the utilization medium A at the end of the subculture stage. Media A contains more complete composition hormone than media B. The lowest results is obtained in Protocol 8 due to the death of embryos. The response of hypocotyl length of Sigararutang variety is found in Table 4.

## Opened Cotyledons

Most of the protocols have not shown yet opened cotyledons in week 2. Only in Protocol 14 showed the highest of the percentage of opened cotyledons because medium-size embryos were germinated in media B was derived from liquid medium. In 4, 6, 8 weeks, the percentages of opened cotyledon were achieved at Protocol 17. The percentages of opened cotyledon are found in Table 5. The embryo development starts from globular, heart and torpedo. Torpedo formation is characterized by the cell differentiation and growth polarization, especially characterized by the formation of cotyledon. Opened cotyledon can trigger shoot apical dominance result in shoot formation.

## Cotyledonary Embryo

The percentages of cotyledonary embryo determine the success of plant conversion during acclimatization. In week 2, the highest percentages of cotyledonary embryos was obtained in Protocols 11, 13, and 17, while the lowest percentages was in Protocol 3. In week 4, the highest percentages were obtained in Protocols 15 and 16, while the lowest percentages was in Protocol 7. In week 6 and 8, the highest percentage was obtained in Protocols 13 and 17 but the percentages is decreased compared the previous weeks because of the death of embryos. While the lowest percentages is in the Protocol 8. Embryos with the mature physiology have better germination capacity, even though the plant conversion from somatic embryos still low. The percentages of cotyledonary embryo is found in Table 6.

Table 5. Percentage of opened cotyledon in twenty protocols in Sigararutang variety after 2, 4, 6 and 8 weeks of growth

| Protocols | Culture periods (weeks) | | | |
|---|---|---|---|---|
| | 2 | 4 | 6 | 8 |
| 1 | 0.4 b | 0 e | 0.8 d | 0.8 d |
| 2 | 0 b | 0 e | 2 cd | 2 cd |
| 3 | 0 b | 1.2 de | 3.6 cd | 3.6 cd |
| 4 | 0.4 b | 4 bcde | 17.6 ab | 17.6 ab |
| 5 | 0 b | 0.8 de | 4.8 cd | 4.8 cd |
| 6 | 0 b | 2 cde | 8.8 bcd | 8.8 bcd |
| 7 | 0 b | 0.8 de | 1.6 d | 1.6 d |
| 8 | 0 b | 0.8 de | 0 d | 0 d |
| 9 | 0.4 b | 0.4 e | 4.4 cd | 4.4 cd |
| 10 | 0 b | 0.8 de | 6.4 cd | 6.4 cd |
| 11 | 0.4 b | 3.6 bcde | 0.8 d | 0.8 d |
| 12 | 4.8 a | 9.2 ab | 8 bcd | 8 bcd |
| 13 | 1.6 b | 6.4 abcd | 5.6 cd | 5.6 cd |
| 14 | 4 a. | 7.6 abc | 5.2 cd | 5.2 cd |
| 15 | 0 b | 1.2 de | 2.4 cd | 2.4 cd |
| 16 | 1.6 b | 5.2 abcde | 12.4 abc | 12.4 abc |
| 17 | 0 b | 10.4 a | 20.8 a | 20.8 a |
| 18 | 0 b | 5.2 abcde | 10 bcd | 10 bcd |
| 19 | 0.4 b | 3.6 bcde | 4 cd | 4 cd |
| 20 | 0.4 b | 3.2 cde | 4.4 cd | 4.4 cd |

Note: Means within the same column having the same letter were not significantly different by the Duncan test 5%.

Table 6. Percentage of cotyledonary embryo in twenty protocols in Sigararutang variety after 2, 4, 6 and 8 weeks of growth

| Protocols | Culture periods (weeks) | | | |
|---|---|---|---|---|
| | 2 | 4 | 6 | 8 |
| 1 | 99.2 a | 96.8 ab | 97.2 a | 97.2 a |
| 2 | 75.6 ab | 90 abc | 77.2 abc | 77.2 abc |
| 3 | 41.2 b | 40 de | 28.4 def | 28.4 def |
| 4 | 54.4 ab | 54.4 abcde | 55.6 abcde | 55.6 abcde |
| 5 | 51.6 ab | 51.6 bcde | 41.2 cdef | 41.2 cdef |
| 6 | 49.6 ab | 49.2 cde | 49.2 bcde | 49.2 b cde |
| 7 | 47.6 ab | 27.6 e | 16.4 ef | 16.4 ef |
| 8 | 78.4 ab | 40 de | 0 f | 0 f |
| 9 | 79.2 ab | 80 abcd | 58.8 abcde | 58.8 abcde |
| 10 | 60 ab | 60 abcde | 56.4 abcde | 56.4 abcde |
| 11 | 100 a | 100 a | 95.2 ab | 95.2 ab |
| 12 | 84.8 ab | 83.6 abcd | 70.8 abcd | 70.8 abcd |
| 13 | 100 a | 100 a | 100 a | 100 a |
| 14 | 86.8 ab | 80 abcd | 75.2 abcd | 75.2 abcd |
| 15 | 99.2 a | 100 a | 69.6 abcd | 69.6 abcd |
| 16 | 90.4 ab | 100 a | 99.2 a | 99.2 a |
| 17 | 100 a | 100 a 100 | a 100 | a |
| 18 | 68.8 ab | 96.8 ab | 95.2 ab | 95.2 ab |
| 19 | 80.4 ab | 74.4 abcd | 40.4 cdef | 40.4 cdef |
| 20 | 72.8 ab | 70.4 abcde | 30.8 cdef | 30.8 cdef |

Note: Means within the same column having the same letter were not significantly different by the Duncan test 5%.

## Protocols Consistency

The maximum percentages of rooted cotyledons, the length of roots, the length of hypocotyl, the percentages of opened cotyledons and the percentages of cotyledonary embryo were obtained when embryoid coffee explants were cultured on Protocol 17, in week 8.

The top two of the high consistency protocols starting from the two weeks until eight weeks for percentages of rooted cotyledons, the length of roots, the length of hypocotyl, the percentages of opened cotyledons and the percentages of cotyledonary embryo were obtained by using Protocols 16 and 17 (Figure 1). To sum up, Protocols 17, 16, 18, and 13 are the best consistent protocols during eight weeks observation in each parameter.

Among the protocols tested, Protocol 17 was the most effective, with 59.2% of rooted cotyledons, 4.04 cm of length of roots, 1.68 cm of length of hypocotyl, 20.8% of opened cotyledons and 100% of cotyledonary embryo at the end of 8 weeks which used the B medium, large embryos and twice phase of subculture from liquid medium to solid medium. There is consistency parameter in their responsiveness between number of roots, length of root, length of hypocotyl, percentages of open cotyledon and percentages of cotyledonary embryo during 2 until 8 weeks. The Protocol 17 is stable protocol from low to high value. Concluding, the Protocol 17 is the most suitable for the development and germination embryo somatic.

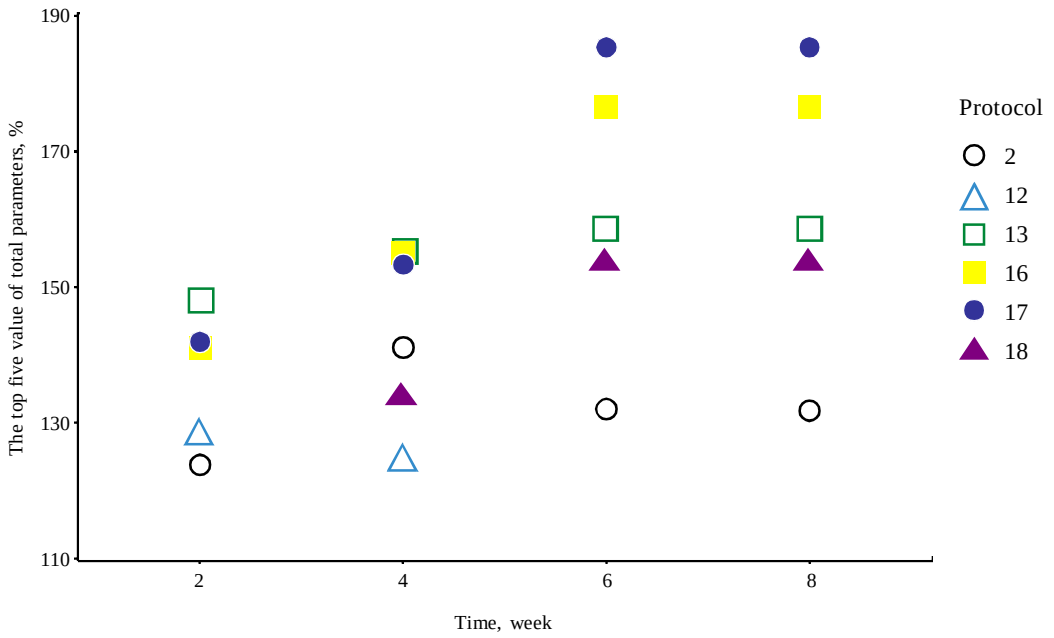


Figure 1. Top five of the high consistency protocol on the rooted cotyledons, length of roots, length of hypocotyl, opened cotyledon and cotyledonary embryo of Sigararutang variety after 2, 4, 6, and 8 weeks of growth

## CONCLUSION

The successful germination of somatic embryo is influenced by the composition medium, stage of the sub-culture and embryo size. The *in vitro* embryos germination of *Coffea arabica* can be achieved at a higher rate by choosing the appropriate protocols. The Protocol 17 is the most suitable for the development and germination embryo somatic. The Protocol 17 is stable protocol from low to high value were the protocol use embryoid medium followed by solid medium than mixed size of embryos followed by germination using medium B.

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