

Callogenesis and Embryogenic Potential of New Superior Cocoa (*Theobroma cacao* L.) Genotypes Treated with Ascorbic Acid

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Abstract

Clonal propagation is an alternative method of maintaining the genetic purity of superior cocoa plants (*Theobroma cacao* L.). The somatic embryogenesis technique is likely the most effective and efficient method for plant propagation. This study aimed at investigating the callogenesis and embryogenic potential of new superior cocoa clones on culture media added with various concentrations of antioxidants and variation in the concentration of auxin hormone combined with ascorbic acid. The superior clones used in this study were MCC 01, MCC 02, Sulawesi 03, ICCRI 07, and ICCRI 09. The parameters observed in this study were percentage of embryogenic callus formation, number of secondary somatic embryos, and percentage of embryo somatic cells that develop into planlets. The addition of 2,4,5-T combined with ascorbic acid affected the initiation of cocoa secondary somatic embryogenesis. Treatment combination of ascorbic acid 100 mg.L⁻¹ and 2,4,5-T 1 mg.L⁻¹ had a significant effect on the initiation of secondary somatic embryogenesis of cocoa in the embryogenic callus phase (4.73%), globular embryos (2.53%), torpedo embryos (4.67%) and sprouts (0.47%). Three clones i.e ICCRI 07, Sulawesi 03 and ICCRI 09 clones showed a higher percentage (3.0%, 2.10%, and 1.80%, respectively) of embryogenic callus growth than the other clones. Meanwhile, on MCC 01 clone, all treatments did not affect the regeneration of embryogenic callus. The combination treatment of organic acid and adenine showed a low embryogenic callus response in MCC 01 and MCC 02 clones. However, MCC 02 clone also did not show a response in form of globular, torpedo, and germination phase somatic embryo regenerations. This indicates that different plant cultivars show different responses to the addition of organic acids. Treatment combinations of adenine 0.0125 mg.L⁻¹ + ascorbic acid 100 mg.L⁻¹ gave the best response to the regeneration of somatic embryos for the globular, torpedo, and germination stages, started 3 weeks after being transferred to regeneration medium.

Keywords: somatic embryogenesis, cocoa, *in vitro*, somatic embryo, ascorbic acid, browning

INTRODUCTION

Theobroma cacao L. is one of the important plantation commodities that has significant economic value for the chocolate industry in the world (Squicciarini & Swinnen, 2016). ICCO (2020) shows that world cocoa production in 2019-2020 decreased by 4.750 million

tons compared to 2018-2019 (4.780 million tons). The reason behind this decline in production is caused by the reduced number of cocoa plantations and the decrease in crop productivity.

Along with its development, in addition to the aging of cocoa trees and high input costs, there are several obstacles in cocoa

cultivation related to abiotic stress problems such as drought (Towaha & Wardiana, 2015) and biotic stress such as pests and diseases attack that affect cocoa productivity. Development of superior cocoa clones that have resistance to biotic stress in form of pests and diseases can be obtained through a breeding program carried out periodically and continuously from genetic materials, which may provide opportunities for genetic conservation of cocoa plants. Environmental stress might be caused by both biotic and abiotic factor.

Procurement of superior planting materials conventionally often facing problems because it is difficult to get high quality seedlings in large quantities and in a short time. According to Pancaningtyas (2013), cocoa somatic embryogenesis is a tissue culture technique that produces primary embryos from cocoa plant organs, i.e. the flower parts (staminodes and petals). This technique has several advantages over conventional propagation techniques for large-scale plant propagation while maintaining genetic stability and integrity, i.e., obtaining plants with a uniform genotype in large numbers and in a short time (Maximova *et al.*, 2002; Quainoo & Dwomo, 2012).

The development of this technology to date has produced plantlets with a conversion rate of 65% and relatively low percentage of somaclonal variation (Lopez *et al.*, 2010). Modifications related to the growth medium for somatic embryogenesis (SE) have been reported recently by Kouassi *et al.* (2017) and Modeste *et al.* (2017). Further development of SE protocol modified by various commercial companies has been outlined in various international patent documents (Wickramasuriya & Dunwell, 2018). Despite continuous progress, the low reproducibility and regeneration efficiency of the developed methods and the differences in responses between genotypes in the SE process are still challenges for mass propagation of elite cocoa plants on a commercial

scale (da Silva *et al.*, 2008). The diversity of responses to somatic embryogenesis is mainly due to differences in genotypes (Issali *et al.*, 2008; Kouassi *et al.*, 2017).

However, variation of somatic embryos for each cocoa genotype is one of the obstacles that must be overcome. The application of somatic embryogenesis in cocoa plants is considered to be quite complicated due to the recalcitrant character of cocoa seed and plant genetics that affect the ability of gene expression to regenerate. Recalcitrant is defined as the inability of plant cells, tissues, and organs to thrive in tissue culture (Benson, 2000). One of the components that affect the disability in vitro is the high phenol content and phenol oxidase compounds. Oxidation of this compound is an inhibiting factor for multiplication and tissue regeneration. The ability of embryogenesis is also influenced by the concentration and distribution of polyphenolic compounds (Gallego *et al.*, 2016). High concentrations of phenolic compounds are associated with non-regenerating responses of somatic embryos (Alemanno *et al.*, 2003). These compounds can further inhibit the development of plant tissue in vitro. The addition of polyvinylpyrrolidone (PVP) and antioxidants such as ascorbic acid and citric acid is a way to inhibit the production of phenolic compounds or reduce their accumulation in tissue culture media (Krishna *et al.*, 2008). Overcoming this bottleneck requires optimization of existing protocols.

Application of the somatic embryogenesis protocol to new superior cocoa clones requires optimization at the secondary embryogenic callus regeneration stage, which is the most important stage in somatic embryo production. Therefore, this study aimed to evaluate the callogenesis and embryogenic potential of new superior cocoa clones on culture media equipped with various concentrations of antioxidants and variations in the concentration of auxin hormone.

MATERIALS AND METHODS

The genotypes used in this study were Sulawesi 03, MCC 01, MCC 02, ICCRI 07, and ICCRI 09. Each clone used 100 budding flowers. The used as explants material were cocoa flower parts.

This research was carried out at the Biotechnology Laboratory of the Indonesian Coffee and Cocoa Research Institute, taking place from February 2019 to December 2020. Research on cocoa propagation through the somatic embryogenesis technique was carried out in integrated procedures, starting from mother tree selection, explant collection, flower induction, primary embryo production, embryogenic callus initiation, embryogenic callus multiplication, secondary embryo production, germination, and planlet maturation. The initial stage of laboratory works began with the orientation of the medium used for each stage of development. Different stages require different culture mediums, as well as different genotype, require different medium.

The growing medium used in the cocoa genotypes protocol included modified Murashige and Skoog, and Driver and Kuniyuki basal medium, PGR (2,4-D, 2,4,5-T, kinetin, adenine sulfate, NAA, IBA), ascorbic acid, glucose, sucrose, gelrite, aquadest, sodium hypochlorite 10%, aluminum foil, NaOH, HCl, alcohol.

Embryogenic Callus Initiation

Primary embryos obtained from the previous stage were re-initiated to produce secondary embryogenic callus, incubated for eight weeks in the dark at 25°C. Secondary embryogenesis was used to increase the number and quality of somatic embryos produced (Maximova *et al.*, 2002). The culture medium

used consisted of a combination of basic media Murashige and Skoog (1962) and Driver & Kuniyuki (1984), adenine sulfate 0.25 mg.L⁻¹, glycine 2 mg.L⁻¹, L-lysine 0.40 mg.L⁻¹, L-leucine 0.40 mg.L⁻¹, L-arginine 0.40 mg.L⁻¹, L-tryptophan 0.20 mg.L⁻¹. Embryogenic callus from each cocoa clone had a different regeneration rate. Therefore, the addition of ascorbic acid antioxidant treatment and variations in the concentration of auxin (2,4,5-T) in the growing media were as following:

M0 (Control/embryogenic callus initiation medium - SEC).

M1 (ascorbic acid 100 mg.L⁻¹ + 2,4,5-T 0.5 mg. L⁻¹).

M2 (ascorbic acid 100 mg.L⁻¹ + 2,4,5-T 1 mg.L⁻¹).

M3 (ascorbic acid 200 mg.L⁻¹ + 2,4,5-T 0.5 mg.L⁻¹).

M4 (ascorbic acid 200 mg.L⁻¹ + 2,4,5-T 1 mg.L⁻¹).

The study was arranged according to a factorial completely randomized design, where the first factor was five cocoa clones (Sulawesi 03, MCC 01, MCC 02, ICCRI 07, and ICCRI 09) and the second factor was five types of media with three replications. The total treatment combinations were 75. Furthermore, analysis of variance was carried out using Duncan multiple range test at 5% significant level. The characteristics of embryogenic callus are having a friable structure and forming crystals like dew. The formation of the embryogenic callus is an important key in the somatic embryogenesis process of propagation. The optimal time for secondary embryogenic maintenance is 21 to 26 weeks after initiation of the primary embryo (Maximova *et al.*, 2002).

Somatic Embryo Regeneration

This stage is a process for the formation of somatic embryos derived from secondary embryogenic callus. Multiplicated embryogenic callus was sub-cultured on expression media with balanced hormone composition. This process lasted for four weeks in the dark. Embryos that have been formed were maintained until they were ready to be harvested. The characteristics of normal embryos that are ready to be harvested are embryos in the torpedo-cotyledon phase. At this stage, there is still a diversity of various stages of embryonic growth produced, including the globular phase to the cotyledon phase. Embryos before the torpedo phase and the cotyledon phase were sub-cultured on the same medium.

Phenolic oxidation is one of the obstacles in the propagation of cocoa embryogenesis. To overcome this problem, organic acid was added to increase the potential of somatic embryos from the resulting callus biomass. The formulation of the media for enhancing the regeneration of embryos from biomass with the addition of organic acids, as follows:

Media C0 (Control /expression medium)

Media C1 (adenin 0.025 mg.L⁻¹ + ascorbic acid 50 mg.L⁻¹)

Media C2 (adenin 0.0125 mg.L⁻¹ + ascorbic acid 50 mg.L⁻¹)

Media C3 (adenin 0.025 mg.L⁻¹ + ascorbic acid 100 mg.L⁻¹)

Media C4 (adenin 0.0125 mg.L⁻¹ + ascorbic acid 100 mg.L⁻¹)

Media C5 (adenin 0.025 mg.L⁻¹ + ascorbic acid 150 mg.L⁻¹)

Media C6 (adenin 0.0125 mg.L⁻¹ + ascorbic acid 150 mg.L⁻¹)

The study was arranged according to a factorial completely randomized design

(CRD), the first factor was four cocoa clones (MCC 01, MCC 02, Sulawesi 03, and ICCRI 09) and the second factor was seven types of media with three replications. The total treatment combinations were 84. Further, analysis of variance will be carried out using DMRT at 5% level.

Observation Method

Several parameters were observed in this study. Observation of secondary embryogenic callus percentage was carried out started at eight weeks after culture on secondary embryogenic callus initiation media (SEC).

$$\% \text{ embryogenic callus} = \frac{\text{total embryogenic callus}}{\text{total explants}} \times 100$$

Percentage of secondary somatic embryos was observed when ± 3 g of embryogenic callus clusters were cultured on the expression medium for somatic embryo formation. Observations were carried out qualitatively using a microscope and quantitatively by counting the number of embryos produced by each embryogenic callus cluster. Observations were made at four weeks after culture in embryo regeneration medium.

$$\% \text{ somatic embryos} = \frac{\text{total somatic embryos}}{\text{total explants}} \times 100$$

RESULTS AND DISCUSSION

Embryogenesis Process

The process of embryogenesis in cocoa is secondary somatic embryogenesis, where the resulting primary somatic embryo is re-initiated to produce secondary somatic embryos. At this initiation stage, there are regeneration constraints that are mainly caused by the oxi-

dation of phenolic compounds which causes browning in the explants (Figure 2). Browning is one of the obstacles in in-vitro culture caused by the accumulation of phenolic compounds due to polyphenol oxidase (PPO) oxidation (Jones & Saxena, 2013). Browning in explant tissue is the main obstacle at this stage. In addition, variations in the content of endogenous hormones also affect the rate of regeneration of embryogenic callus. Based on Pancaningtyas (2015) the highest content of phenolic compounds was at the embryogenic callus stage. The addition of organic acids in in-vitro culture media was carried out to overcome the inhibition of phenolic compounds so that the explants could increase their regeneration to produce somatic embryos.

In the first secondary embryogenesis observation (3 weeks after initiation), ICCRI 07, Sulawesi 03, and ICCRI 09 clones showed a higher percentage of embryogenic callus growth compared to the other clones (3.0%, 2.10%, and 1.80%). Meanwhile, in the MCC 01 clone, all treatments did not affect the regeneration of embryogenic callus. In the further regeneration of secondary somatic embryogenesis, Sulawesi 03 clones showed a higher percentage of secondary somatic embryo formation both at the globular and torpedo stage and even at shoot growth at the 9th week of observation (Figure 2). Based on these data, genotype differences greatly influence the initiation of secondary somatic embryogenesis. The effect of genotypes had a significant effect



Figure 1. Browning on callus biomass culture of cocoa (before treatment) (A & B). Embryogenic callus after the addition of ascorbic acid treatment (Observations using an Olympus microscope *SZ61* equipped with a DP25 camera) (C & D). Bar = 3,75 mm

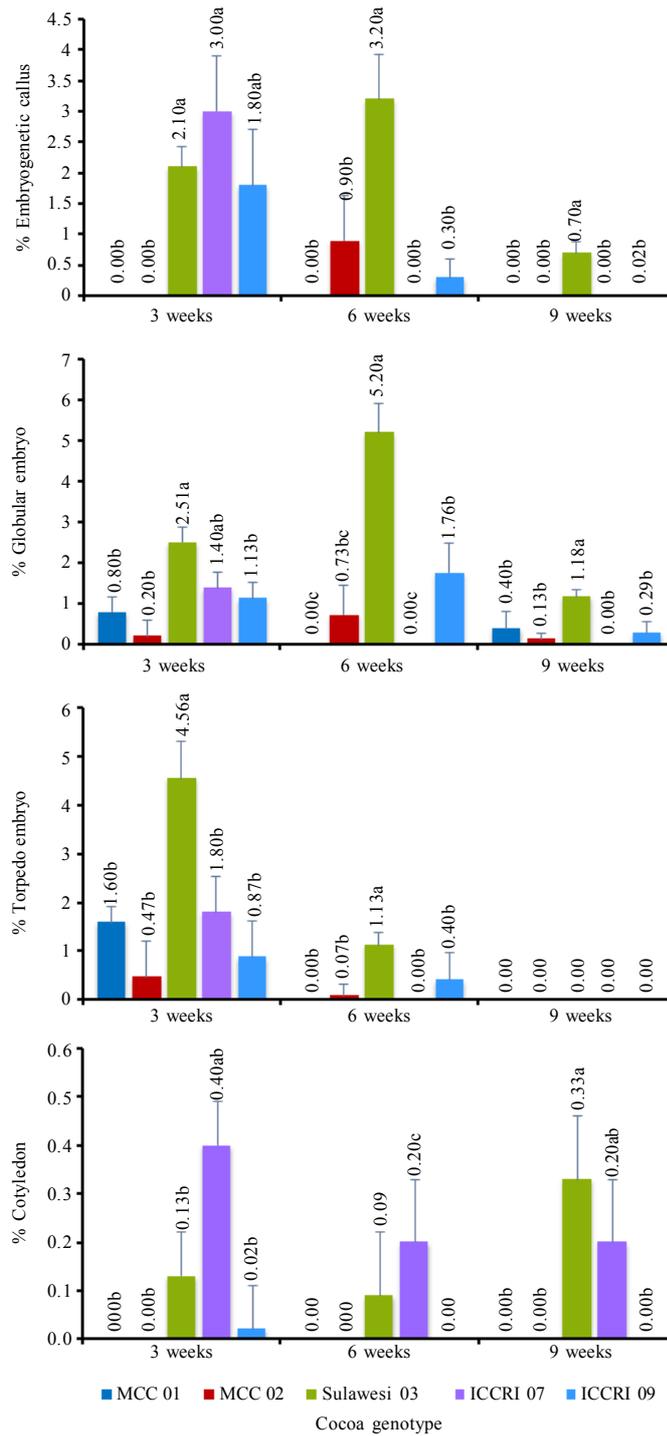


Figure 2. Response of some cocoa clones to the addition of organic acids and hormones 2,4,5-T at the initiation stage of secondary embryogenesis

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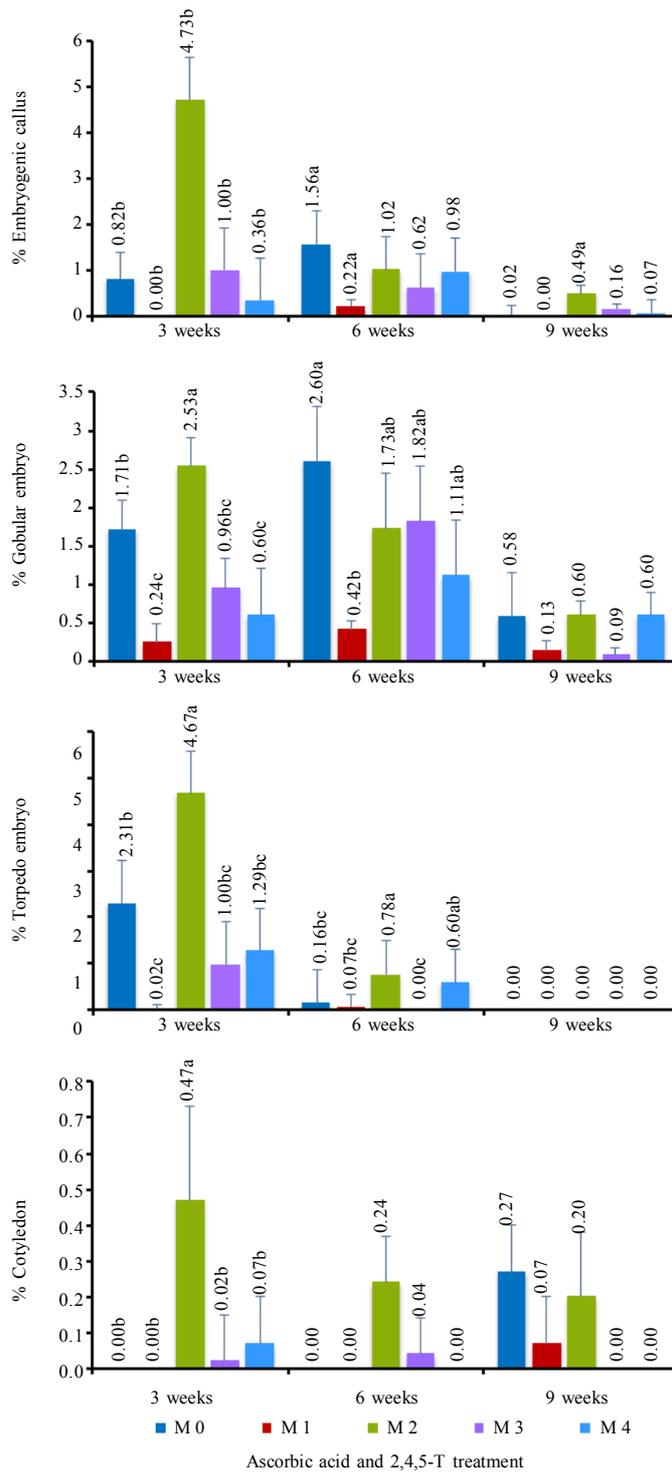


Figure 3. Effect of ascorbic acids and 2,4,5-T concentration on the initiation of secondary embryogenesis
 Notes: M1 (ascorbic acid 100 mg.L⁻¹ + 2,4,5-T 0.5 mg. L⁻¹); M2 (ascorbic acid 100 mg.L⁻¹ + 2,4,5-T 1 mg.L⁻¹); M3 (ascorbic acid 200 mg.L⁻¹ + 2,4,5-T 0.5 mg.L⁻¹); M4 (ascorbic acid 200 mg.L⁻¹ + 2,4,5-T 1 mg.L⁻¹).

on the regeneration response of cocoa secondary somatic embryos. Nonetheless, there is no interaction between genotype and culture media. This is also related to the physiological conditions of the cultured cells or tissues.

The addition of 2,4,5-T hormone combined with ascorbic acid affected the initiation of cocoa secondary somatic embryogenesis. The treatment combination of ascorbic acid 100 mg.L⁻¹ and 2,4,5-T 1 mg.L⁻¹ had a significant effect on the initiation of secondary somatic embryogenesis of cocoa in both embryogenic callus phase (4.73%), globular embryos (2.53%), torpedo embryos (4.67%) and planlets (0.47%) (Figure 3). That treatment medium still showed good results even up to the observation at week nine. In other treatment mediums, embryogenic callus and globular embryos showed an increase in the 6th week. The combination of ascorbic acid 100 mg.L⁻¹ and 2,4,5-T 0.5 mg.L⁻¹ treatment showed the lowest values in all parameters (embryogenic callus, globular embryos, torpedo embryos, and planlets).

Browning intensity in culture is influenced by plant species and varieties, tissues or organs, plant development phase, tissue or organ age, nutrient media, and other tissue culture variables (Huang *et al.*, 2002; Chandra *et al.*, 2005). Based on Jakhar *et al.* (2013), the addition of 100 mg.L⁻¹ ascorbic acid can suppress browning intensity and increase shoot induction in vitro. The addition of ascorbic acid to the culture medium was also used to overcome browning in some in-vitro cultures of banana plants (Ko *et al.*, 2009; Munguatosha *et al.*, 2014).

Embryogenic Callus Regeneration and Somatic Embryo

Browning in tissue greatly affects the decline in-vitro regeneration of callus and organ

cultures, including the process of somatic embryogenesis. The addition of organic acid (ascorbic acid) to the culture media to overcome browning was also given to the embryo regeneration medium to increase the number of somatic embryos produced. In the first observation (3rd weeks), ICCRI 09 clones followed by Sulawesi 03 clones showed the percentage of embryogenic callus formation (2% and 1.7%), globular embryos (4.67% and 3.08%), and torpedo embryos (4.56% and 3.47%) which were higher than the other clones. In Sulawesi 03 clones embryogenic callus still produced a high percentage until the 9th week (Figure 6). MCC 02 clone showed the lowest embryogenesis response for all observation parameters.

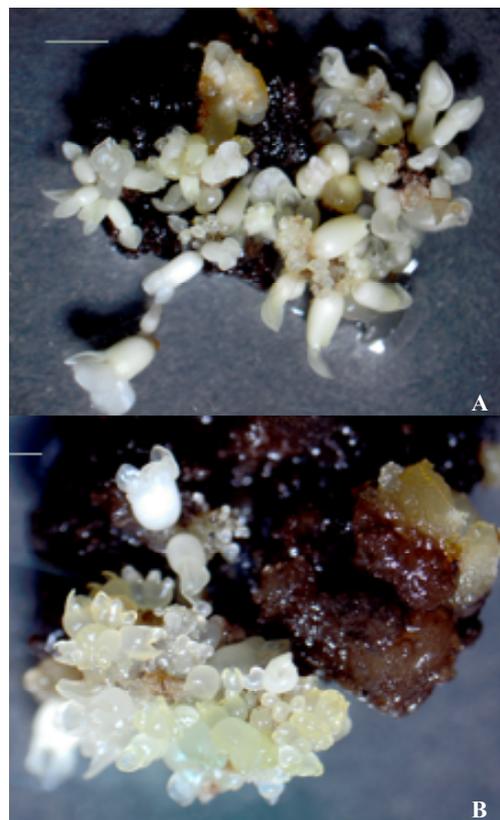


Figure 4. Callus biomass after treatment with C4 medium (Observations using an Olympus microscope SZ61 equipped with a DP25 camera); A. Bar = 3,75 mm, B. Bar = 3 mm

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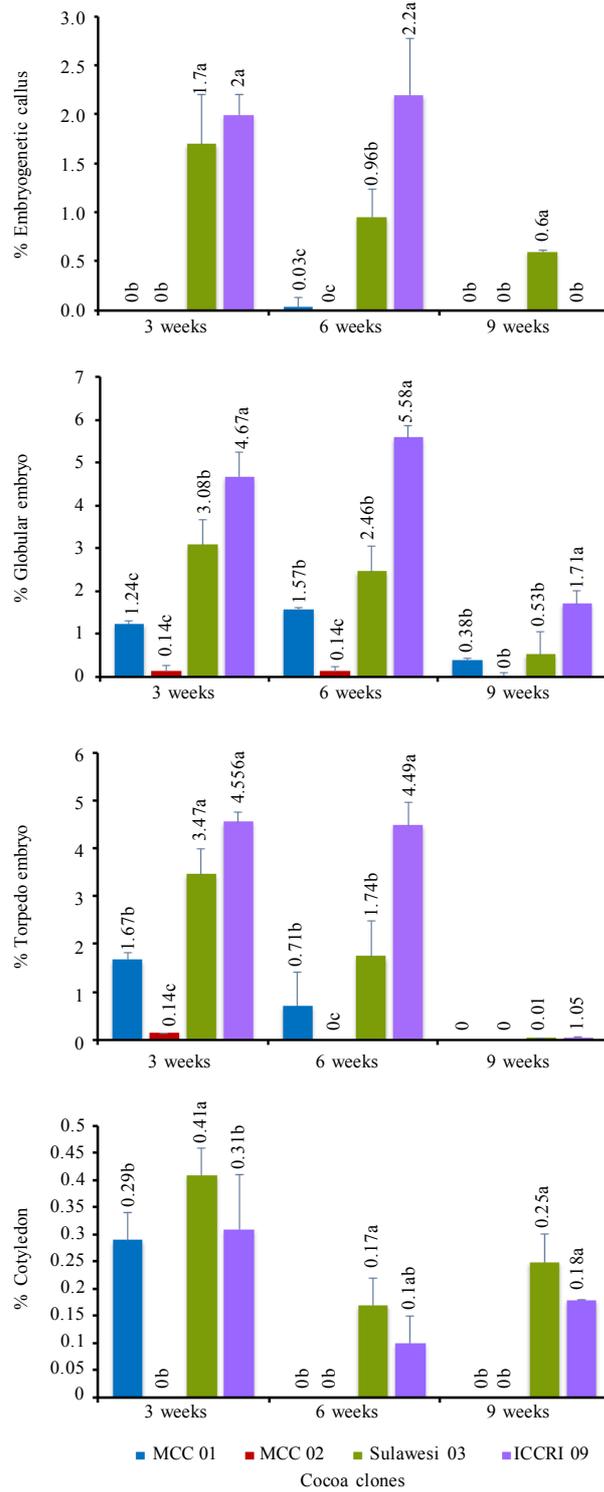


Figure 5. Response of various cocoa clones in terms of embryogenic callus and secondary somatic embryo regeneration

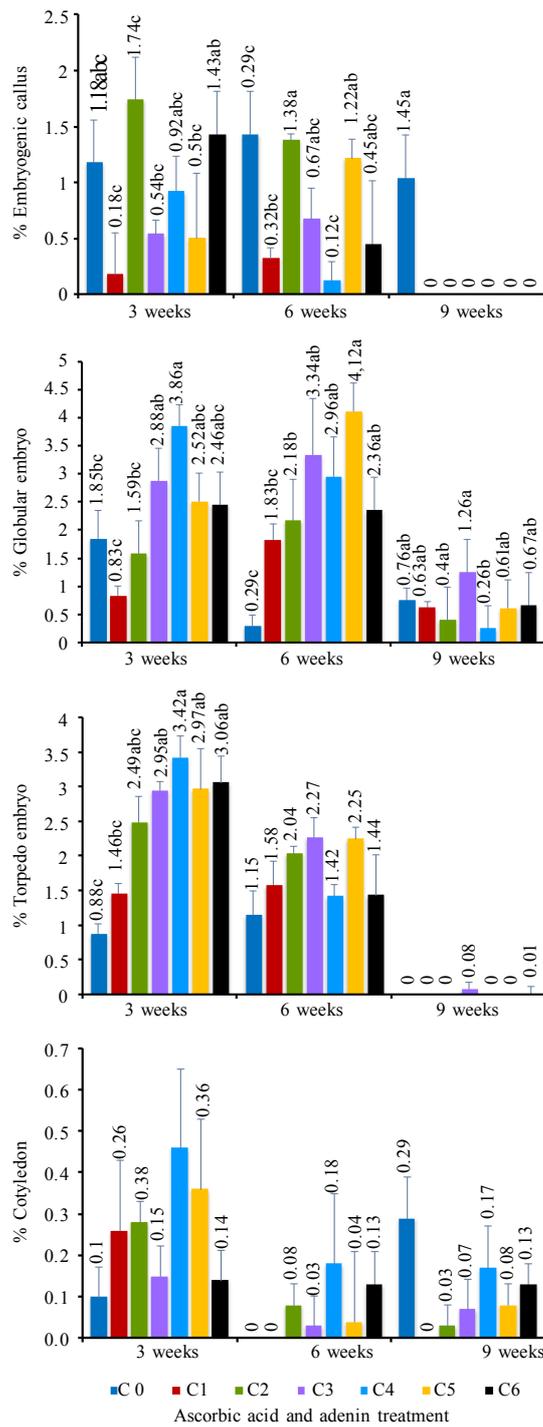


Figure 6. Effect of ascorbic acid and adenine concentration on the regeneration of embryogenic callus and secondary somatic embryos

Notes: Media C0 (Control /expression medium); Media C1 (adenin 0.025 mg.L⁻¹ + ascorbic acid 50 mg.L⁻¹); Media C2 (adenin 0.0125 mg.L⁻¹ + ascorbic acid 50 mg.L⁻¹); Media C3 (adenin 0.025 mg.L⁻¹ + ascorbic acid 100 mg.L⁻¹); Media C4 (adenin 0.0125 mg.L⁻¹ + ascorbic acid 100 mg.L⁻¹); Media C5 (adenin 0.025 mg.L⁻¹ + ascorbic acid 150 mg.L⁻¹); Media C6 (adenin 0.0125 mg.L⁻¹ + ascorbic acid 150 mg.L⁻¹).

The combination of treatments of ascorbic acid and adenine showed a low embryogenic callus responses in MCC 01 and MCC 02 clones. However, MCC 02 clones also did not show a response to globular, torpedo, and shoot growth phase somatic embryo regeneration. This indicates that different plant cultivars show different responses to the addition of organic acids. The treatment combination of adenine 0.0125 mg.L⁻¹ and ascorbic acid 100 mg.L⁻¹) gave the best response to the regeneration of somatic embryos in the globular, torpedo, and shoot growth phases starting 3 weeks after being transferred to regeneration media. Increasing the concentration of organic acids did not affect the increase in the percentage of embryogenic callus.

Dubravina *et al.* (2005) in their research stated that high phenolic compounds in callus can cause explant tissue death. In guava plant micropropagation, browning can inhibit morphogenesis activity (Chandra & Mishra, 2007). The high activity of POD and PPO can suppress or even inhibit the development of somatic embryos in undifferentiated callus (Beruto *et al.*, 1996).

The addition of ascorbic acid antioxidants to in vitro culture media can reduce the occurrence of browning. In this case, ascorbic acid does not interact directly with PPO but inhibits browning by reducing substrate oxidation (Arias *et al.*, 2007). Oxidation due to the exudation of phenolic compounds can cause browning of the culture media which will inhibit nutrient uptake in explant tissues. In the study of Jakhar *et al.* (2019), ascorbic acid treatment of 150-200 mg.L⁻¹ can increase the proliferation of embryogenic callus in *Commiphora wightii* plants.

CONCLUSIONS

Combination of ascorbic acid 100 mg.L⁻¹ and 2,4,5-T 1 mg.L⁻¹ had a significant effect on the initiation of secondary somatic embryo-

genesis of cocoa in both embryogenic callus phase (4.73%), globular embryos (2.53%), torpedo embryos (4.67%) and germination stages (0.47%). ICCRI 07, Sulawesi 03, and ICCRI 09 clones showed a higher percentage of embryogenic callus growth than the other clones. Meanwhile, on MCC 01 clone, all treatments did not affect the regeneration of embryogenic callus. The combination treatment of organic acid and adenine showed a low embryogenic callus response in MCC 01 and MCC 02 clones. However, MCC 02 clones also did not show a response to globular, torpedo, and germination phase somatic embryo regeneration. This indicates that different plant cultivars show different responses to the addition of organic acids. The treatment combination of adenine 0.0125 mg.L⁻¹ and ascorbic acid 100 mg.L⁻¹ gave the best response to the regeneration of somatic embryos in the globular, torpedo, and germination stages, started 3 weeks after being transferred to regeneration medium.

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