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Effect of 2,4 Dichlorophenoxy Acetic Acid on In Vitro Callogenesis of Cocoa (*Theobroma cacao* L.)

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Abstract

Cocoa (*Theobroma cacao* L.) development using modern breeding techniques can be facilitated by propagation of planting material through somatic embryogenesis. Various factors that may affect embryogenesis are the composition of culture medium and culture condition. Hormone commonly used to initiate the formation of callus is auxin with type 2.4-D (2.4 Dichlorophenoxy acetic acid). The aim of this study was to determine the effect of the addition of 2.4-D hormones on the process of cocoa embryogenesis. The treatments were arragged in factorial combination in completely randomized design, which consisted of two factors. The first factor was the concentration of auxin 2,4-D 25 %, 50 %, 75 %, and 100 %; and the second factor was cocoa clones; Sulawesi 01 and Sulawesi 02. The result showed that the addition of 2.4-D hormone up to 100% on somatic embryogenesis of cocoa for Sulawesi 01 clone was not significantly different from Sulawesi 02 clone for all parameters. While on the addition of 2.4-D, there was significant difference between Sulawesi 01 and 02. Cocoa embryogenic callus using the addition of 2.4-D (25%-100%) was significantly different from control. Increased concentrations of 2,4-D hormone which is applied onto media would inhibit the formation of the somatic embryo. Addition of 2.4 D 25%, encouraged towards non-embryogenic callus.

Keywords: 2.4 Dichlorophenoxy acetic acid, embryogenic callus, somatic embryos, cocoa, medium culture, hormone

INTRODUCTION

Tissue culture technique is an alternative technology that able to provide uniform mass production seedlings, low cost and rapid clonal multiplication system. The improvement of *Theobroma cacao* using modern breeding techniques can be greatly facilitated by multiplying elite material using somatic embryogenesis. It is now possible to produce somatic embryos and plantlets from a large number of genotypes (Maximova *et al*., 2000). Development of multiplication method through somatic embryogenesis has been widely performed in many experiments, especially reproduction using flower part explants as petal and staminode (Tan *et al*., 2000). Multiplication is an important key that must be reached in the production of seedlings (Supriati *et al*., 2006). However, development of unsynchronous embryos, low rate germination and inefficient plantlet formation is a major obstacle in the application of somatic embryogenesis in mass production of superior plants (Tahardi *et al*., 2003).

Important factors affecting embryogenesis are the composition of culture

medium and culture conditions (Ferrie & Keller, 2007). Efficiency of somatic embryogenesis and plant regeneration using staminode and petal explant showed significant increase. All genotypes tested produced embryos. Considering that increase, clonal propagation of cocoa can be applied through somatic embryogenesis and for large scale propagation from a limited planting materials (Guiltinan, 1997).

Tissue culture media are differentiated into basal medium and additional medium. Basal medium contains basic macro and micro nutrients, source of energy and vitamins and the amount and type of which depends on the purpose of culture. Treatment medium composed of vitamins, complex organic compounds and plant growth regulators. Plant growth regulators especially auxin and cytokinin are major organic substances that may control morphogenesis process in tissue culture. Plant hormones are part of genetic regulation and serve as precursors. Environmental stimulation triggers the formation of plant hormones. From the point of view of evolution, plant hormones are part of adaptation process and self defense of plants to maintain their viability. The sensitivity of tissues to plant growth regulators added in the medium is determined by concentration of growth regulator in tissue (Starling *et al*., 1986). The lower levels of endogenous substances, the greater exogenous substances that should be added (Sugiri, 2005). The role of auxin in somatic embryogenesis are for the initiation of somatic embryogenesis (Chugh & Khurana, 2002), embryogenic calli induction (Chithra *et al*., 2005; Dudits *et al*., 1995; Vargas *et al*., 2005), embryogenic calli proliferation (Huan *et al*., 2004), and induction of somatic embryos (Shinoyama *et al*., 2004).

Most commonly used hormone to initiate the formation of callus is auxin or 2.4 D (2.4 dichlorophenoxyacetic acid). George & Sherington (1984) suggested that for callogenesis, rhizogenesis and morphogenesis of roots and shoots from callus culture, a balance of auxin and cytokinin is normally required. Development of tissue culture technique needs to find optimum concentration and balance or interactions between the two plant growth regulators given preferential treatment in the media, and their concentration in plant tissues. Addition of exogenous auxin and cytokinin affects the level of endogenous growth regulator in cells which trigger morphogenesis. High concentration of 2,4-D in media results in greater swelling of callus, whereas treatment with balanced of kinetin and 2,4-D enhance the formation of embryogenic callus (Sugiri, 2005). The aim of this study was to determine the effect of 2.4-D hormone on cocoa embryogenesis.

MATERIALS AND METHODS

This research was carried out in the Laboratory of Biotechnology, Indonesian Coffee and Cocoa Research Institute. Cocoa clones used in this study were Sulawesi 01 and Sulawesi 02.

Medium used in this research was the modified Murashige-Skoog (MS), for solid medium using 30 g/L gelrite. Explant used is friable embryogenic callus. Embryogenic callus characteristics of each clones are different. Therefore, the success of multiplication process depends on the selection of embryogenic calli. Cultures were incubated under dark condition at a temperature of $25 \pm 1^{O}C$. One month after treatment, the percentage of embryogenic callus formation was observed. The frequency of somatic embryos formation can be influenced by genotype and composition of culture medium (Da Silva *et al*., 2003).

Experiment was arranged in factorial combination which were established in

completely randomized design, which consisted of two factors: the first factor was concentration of 2,4-D 25%, 50%, 75% and 100%; and cocoa clones (Sulawesi 01 and Sulawesi 02). Therefore, the total number of combinations was 48 experiment units.

Parameters observed were percentage of embryogenic callus formation, percentage of embryogenic callus expressed into embryo, percentage of biomass and the percentage of non-embryogenic callus. To calculate the number of embryos, the following formula was used:

$$\frac{\text{Number of expressing embryos (heart, torpedo and cotyledon)}}{\text{Number of callis}} \times 100\%$$

Data analysis used Fissher test to see the effect of treatments then continued by Duncan multiple range test at the 5% level.

RESULTS AND DISCUSSION

Effect of 2,4-D

Embryogenic callus formation was observed 3 weeks after callus induction. Embryogenic callus was selected based on color and structure of the callus. The presence of non embryogenic callus and somatic embryo at globular phase were observed on the surface.

Sulawesi 01 clone was not different with Sulawesi 02 clone for all parameters (Figure 1). This means that a wide variety of the addition of 2,4-D treatment up to 100% on somatic embriogeniesis of cocoa had the same effect on both clones. According to Teixeira *et al*. (2004) high frequency of embryogenic formation was determined by 2,4-D level and also the types and concentration of sugars. Besides, different genotype may have different embryogenic response. Auxin used for callus induction was 2,4-D which is known can induce cell proliferation but hinder differentiation of dicotyl. In addition, this compound is effective to induce somatic embryogenesis and callus induction of cereal crops (monocotyl). The weakness of auxin was genetic stability. Keeping callus on medium containing auxin for longtime cause increased genetic diversity. Study of Kumar (2007) showed that to induce embryogenic callus, cultures were incubated at room temperature 25 ± 2°C and 16 hours photoperiod under low lighting flouroscence (5 µmol m-2 s-1) in the first 55 days, then sub-cultured to medium containing 4.5 µM 2.4-D and 17.6 µM benzyl amino purin (BA). The formation of embryogenic callus, significantly influenced the interaction between auxin treatment and explant position (Scherwinski-Pereira, 2010). The same results were obtained on a culture of oil palm (Steinmacher *et al*., 2007). Besides the study also demonstrated that explant position on plant and concentration of auxin were important factors affecting mytosis cycle of the cell and induced somatic embryogenesis on that species.

Embryogenesis Development

According to (Geldner *et al*., 2001; Feher *et al*. 2003), use of plant growth regulators such as auxin and cytokinin in growth medium to induce embryogenic potential was important for activation and regulation of cell division and differentiation. Use of exogenus 2,4-D as auxin in high concentration may induce somatic cells into embriogenic cells on various vegetative parts. Application of 2,4-D in wide scale is based on the fact that the use of 2,4-D in the above specified concentrations, the subtance can play double roles, directly act as auxin or replacing endogenous IAA, and become stressing factor (Feher *et al*., 2003).

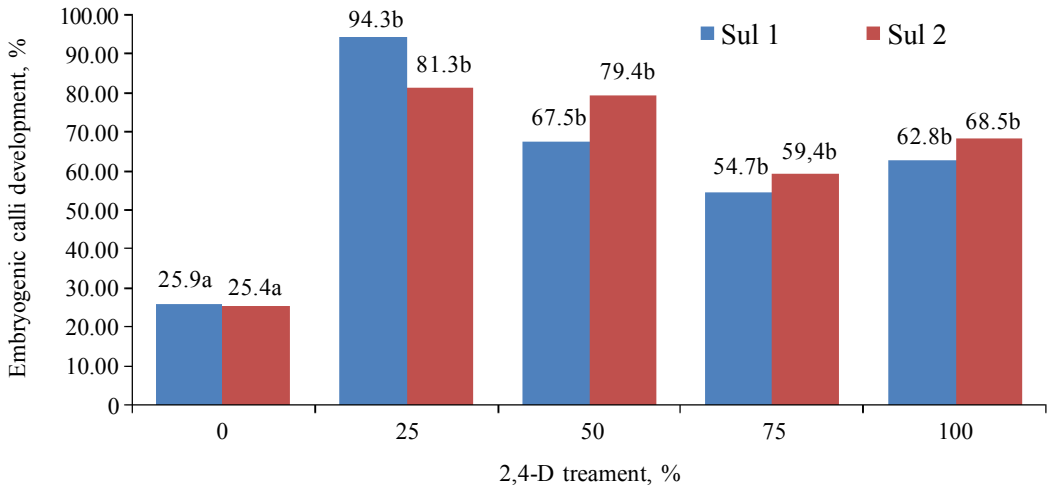

Figure 1. Effect of 2.4-D addition on the percentage of embryogenic callus development of Sulawesi 01 and Sulawesi 02 clones

Da Silva-Guedes (2011) showed that on callus induction the addition of high concentration 2,4-D (450 μM) in medium containing macro and micro nutrients and vitamins together with a 3.0 g/L of activated charcoal gave best results. Meanwhile, 2,4-D concentration levels provided significant difference between Sulawesi 01 and Sulawesi 02 clones (Figure 2). Addition of 2,4-D (25–100%) significantly different compared with control on embryogenic competence of cocoa. Increase of 2,4-D concentration in the medium inhibited formation of normal somatic embryos. Addition of 2.4-D more than 25% encouraged development of non-embriogenic callus. Based on the research by Lopez-Baez *et al*. (1993), only 2,4-D and kinetin were used in the medium for embryogenic induction.

Based on embryogenic parameters, addition of 2,4-D to the medium gave significant influence compared with control. Most of the cocoa embryogenic callus remained in embryogenic phase when 25% and 50% 2,4-D given to the media. In the media with 75% and 100% 2,4-D, some embryos remained in embryogenic phase and most grew into embryonic phase, while in control only a small part remained in embryogenic phase. Addition of 2,4-D in high concentrations can inhibit the formation of somatic embryo and 2,4-D lead to callus formation (Mamun *et al*., 2004; Baskaran *et al*., 2005; Tahir *et al*., 2011). Gandonuo (2005) and Xing *et al*. (2010) stated that lower concentration of 2,4-D gave better response of embryogenic callus. According to Ali *et al*. (2007), a decrease in 2,4-D concentration, the rate of embryogenic callus formation increase. The effect of 2,4-D on a certain concentration is very beneficial, because embryogenic explan can be multiplied in unlimited quantities. It would not happen if the explant in embryogenic phase has evolved into embryo phase. The response to the addition of 2.4-D concentrations depend on each genotype. In general high concentration of 2,4-D increased death rate due to deterioration on cells of explant or lose its ability to further process of embryogenesis.

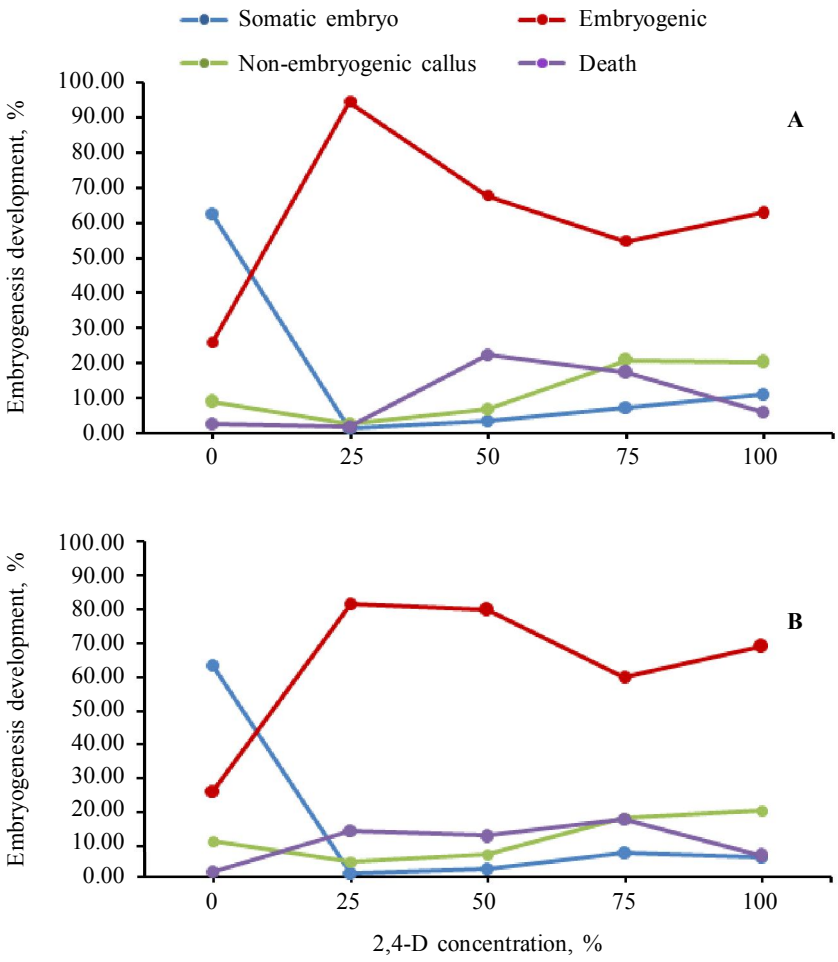

Figure 2. Effects of 2,4-D concentration on embryogenesis development on Sulawesi 01 (A) and Sulawesi 02 (B) clones

In the cluster of control for both clones Sulawesi 01 and Sulawesi 02, there was heterogenity of somatic embryos development. Somatic embryos growing in a cluster is not uniform, where there are variuos phases of development, such as the globular phase and mature phase with bipolar characteristics (Steinmacher, 2007). Ledo *et al*. (2002) also examined the heterogenity of somatic embryo development on *Euterpe edulis* obtained in zygotic embryo culture using 2,4-D.

2.4-D hormone is most widely used because it is the most powerful substance in its activity. Callus which can be regenerated normally has a friabel structure, round form white or yellowish slightly shiny colored that. Callus with these structures is often referred to as embryonic callus (embryogenic). Soft callus contain lots of water, pale white or brownish color usually difficult to be regenerated, and the callus is classified as non-embryonic type.

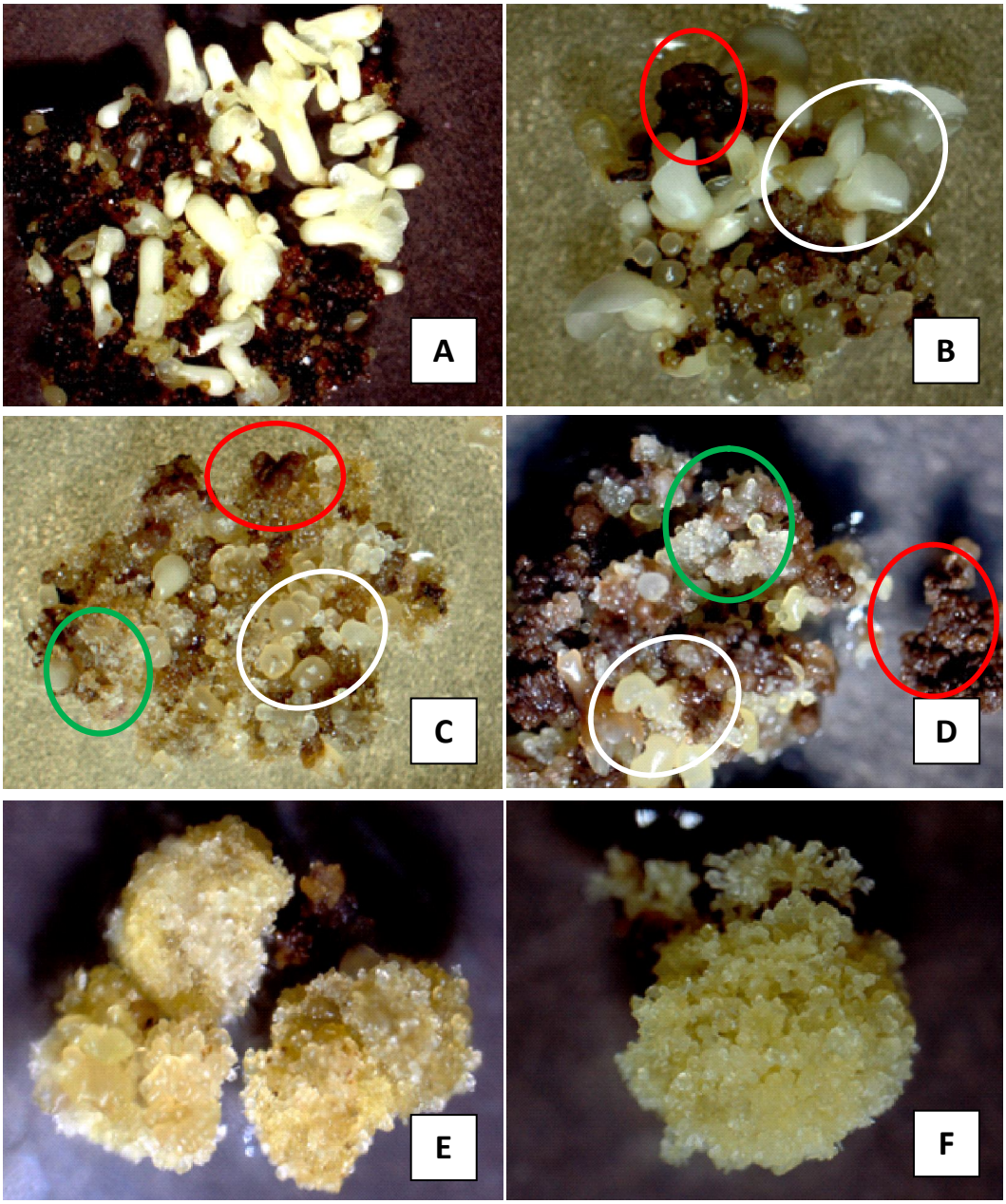

Figure 3. Somatic embryos development phases; Sulawesi 01 control (A), Sulawesi 02 control (B). The addition of 25% 2,4-D Sulawesi 01 clone (C). The addition of 25% 2,4-D Sulawesi 02 clone (D). Embryogenic calli Sulawesi 01 clone (E). Embryogenic calli Sulawesi 02 clone (F); Red cyrcle show non-embryogenic calli, green cyrcle show embryogenic calli and white cyrcle show somatic embryos.

CONCLUSION

Addition of 2,4-D on somatic embryogenesis of cocoa up to 100% for clone of Sulawesi 01 showed gave no significant difference compared with Sulawesi 02 clone for all parameters. Among 2,4 D concentrations, there was significant difference either in Sulawesi 01 clones or Sulawesi 02 clones. On the whole cocoa embryogenic callus on medium using 25%–100% 2,4-D was significantly different from control. Increasing concentration of 2,4-D applied in planting medium inhibit the formation of somatic embryo. Addition of 2.4-D more than 25%, encourage development toward non-embryogenic callus.

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