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Enhanced Plantlet Regeneration in Two Cacao (*Theobroma cacao*) Clones from Immature Inflorescence Explants

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***Abstract. Theobroma cacao* L. (cacao) is a major tropical crop, grown for its oil-rich seed, which is used in the manufacture of chocolate, its derivatives, and cosmetics. Cacao is cultivated mainly by smallholders and represents a significant export commodity for some developing countries such as Côte d'Ivoire. It is conventionally propagated by seeds, grafting, and cuttings. Somatic embryogenesis offers an alternative method for propagation where large-scale production of planting materials is possible. In the current study, the effect of different concentrations of 2, 4-dichlorophenoxyacetic acid (2, 4-D) and kinetin on induction of somatic embryogenesis and plantlet regeneration in two cocoa clones (coded as C1 and C14) were evaluated. Flowers were collected early in the morning, sterilized, explants excised and cultured on Driver, and Kuniyuki Walnut (DKW) media supplemented with different concentrations of 2, 4-D (9, 10, and 20 μM) and kinetin (0.5, 1, 2.5, 5, 10, and 25 μM) in separate experiments. The frequently used media in somatic embryogenesis of cacao [DKW supplemented with 0.022 μM thidiazuron (TDZ) and 9 μM 2, 4-D] was used as a control. The results of the study showed that explants cultured on media supplemented with 10 μM 2, 4-D and 5 μM kinetin produced the highest (28.0 ± 1.1) mean number of embryos/explant in C1 and this was a 9-fold increase in the number of embryos compared with the control. Explants cultured on media supplemented with 10 μM 2, 4-D and 2.5 μM kinetin produced the highest (7.0 ± 4.0) mean number of embryos/explant in C14 whereas the explants cultured on media supplemented with 20 μM 2, 4-D and 2.5 μM kinetin gave the highest (22.0 ± 1.7) mean number of embryos in clone C1 and C14. The regenerated embryos were germinated and successfully weaned in the green house with a survival rate of 70% being recorded. The paper describes an improved protocol compared with previous work in terms of embryo recovery and survival rate of the elite clones of cocoa through somatic embryogenesis. The results of the current study confirm that somatic embryogenesis of cacao clones is genotype dependent.**

Cacao (*Theobroma cacao*, Malvaceae) is a crop of major importance for the livelihoods of small-scale farmers and ecosystems in many tropical regions. About 72% of the world's cocoa is produced in Africa, with Côte d'Ivoire being the top producer at 43% of global production (ICCO, 2015). It is produced by smallholders and five to six million rural households gain substantial income from cacao production (ICCO, 2012; WCF, 2014). Cacao is a major source of income for developing countries, especially in Côte d'Ivoire, the world's largest cocoa producer, which grows more than one-third of the world's supply. The production of cocoa in Côte d'Ivoire in 2013–14 went more than 1.7 million tons (Conseil Café Cacao, 2014). However, the country has been experiencing reduction in production because of different reasons, key among them are non-improved, ageing trees, which are susceptible to diseases and insects. Research in the country has developed high-yielding hybrid varieties and clones, but their availability is limited. Propagation and promoting access of high-performing clones to farmers to rehabilitate their old cacao orchards is of paramount importance and is key to enhancing cocoa productivity in Côte d'Ivoire. The conventional propagation by seeds poses a challenge because there is a high degree of segregation for many traits because they are highly heterozygous and thus variable in terms of agronomic performance. For this reason, clonal propagation systems such as rooted cuttings and grafting have been applied for multiplication of elite varieties (Eskes, 2005). However, these methods have some disadvantages including intensive labor and associated costs, generally low propagation rates, and an undesirable bush-like growth pattern which can occur (Figueira and Janick, 1995). Plant regeneration through somatic embryogenesis provides an alternative approach for propagation of plants. Somatic embryogenesis depends on the ability of somatic plant cells to dedifferentiate and are reprogrammed along an embryonic developmental pathway (Fehér, 2015). The main advantages of this method include the possibility of rapidly producing uniform plants of high genetic value and the clonal production of orthotropic plants with normal dimorphic architecture and taproot formation. In addition, somatic embryogenesis ensures the production of disease-free stock materials making quarantine procedures for international exchange of germplasm easy (Quainoo et al., 2012). The use of somatic embryogenesis in cacao has the potential to contribute significantly to the efforts in crop improvement, germplasm conservation, and rapid distribution of new improved varieties. This method might also facilitate genetic engineering (Corredoira et al., 2002). Propagation of elite cacao varieties through somatic embryogenesis has been achieved in a large number of genotypes, but low efficiencies and genotype dependence still present major constraints to its propagation at a commercial level (Li et al., 1998; Maximova et al., 2002, 2008). The commonly used media is the DKW (Driver and Kuniyuki, 1984) basal salts supplemented with 2, 4-D and TDZ (Ajijah et al., 2014; Li et al., 1998; Maximova et al., 2002, 2008; Quainoo and Dwomo, 2012). Lopez-Baez et al. (2000) reported the use of two auxins (2, 4-D or 2, 4, 5-T) and two cytokinins (kinetin or 2 isopentyladenine) on modified Murashige and Skoog (1962) salts. Recently, some workers have reported success in inducing cacao embryogenic cultures using kinetin and 2, 4-D (Ajijah et al., 2016; Kouassi et al., 2017). Although Kouassi et al. (2017) reported success in inducing embryogenic cultures in four of the elite cacao clones in Côte d'Ivoire, the responses were low. For the somatic embryogenesis technology to be adopted for the propagation of the Ivorian cacao clones, there is a need to work out protocols with high multiplication rates. The present study was aimed at exploring the option using various concentrations of 2, 4-D and kinetin to improve the induction of cacao somatic embryos and plantlet regeneration in the Ivorian elite cacao clones.

Materials and Methods

Plant materials. Flower buds of about 4–5 mm length were collected (before 9:00 AM) from two elite clones growing in the World Agroforestry Center experimental farm in Adiopodoumé (Côte d'Ivoire). The flower buds were taken to the laboratory and subjected to

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Table 1. Effect of 9 µM 2, 4-D and varying concentrations of kinetin (0.5, 1, 5, and 10 µM) on callus and embryo formation.

Genotypes	2, 4-D (9 µM) + Concentrations of kinetin in µM	Percent callus ± SE	Percent embryogenic callus ± SE	Mean no. of embryos ± SE
C1	Control	94.0 ± 6.0	84.9 ± 3.1 a	3.5 ± 0.5 a
	0.5	100.0 ± 0.0	98.8 ± 1.2 b	4.5 ± 1.3 a
	1	100.0 ± 0.0	100.0 ± 0.0 b	0.7 ± 0.6 a
	5	94.1 ± 5.9 a	0.0 ± 5.0 c	0.0 ± 0.0 a
	10	88.8 ± 2.1 a	0.0 ± 0.0 c	0.0 ± 0.0 a
C14	Control	100.0 ± 0.0 a	90.0 ± 2.0 b	1.5 ± 0.5 a
	0.5	96.6 ± 1.9 a	100.0 ± 0.0 b	6.0 ± 1.7 a
	1	100.0 ± 0.0 a	100.0 ± 0.0 b	5.5 ± 4.2 a
	5	100.0 ± 0.0 a	8.3 ± 8.3 d	0.0 ± 0.0 a
	10	100.0 ± 0.0 a	45.8 ± 5.5 e	6.0 ± 0.6 a

The values represent mean ± SE of at least three replications. Means with different letters are significantly different at 5% (Newman–Keuls test).

two steps of sterilization which involved using 2% (w/v) calcium hypochlorite for 20 min and rinsing four times with sterile distilled water. They were then sterilized a second time using 1% calcium hypochlorite for 10 min and finally rinsed four times. The flowers were then dissected using a sterile scalpel blade and the staminodes and petals extracted.

Media formulation and culture conditions. The explants (staminodes and petals) were cultured on DKW basal salts as described by Driver and Kuniyuki (1984) supplemented with different concentrations of 2, 4-D (9, 10, and 20 µM) and kinetin (0.5, 1, 2.5, 5. 10, and 25 µM) in separate experiments. The control was based

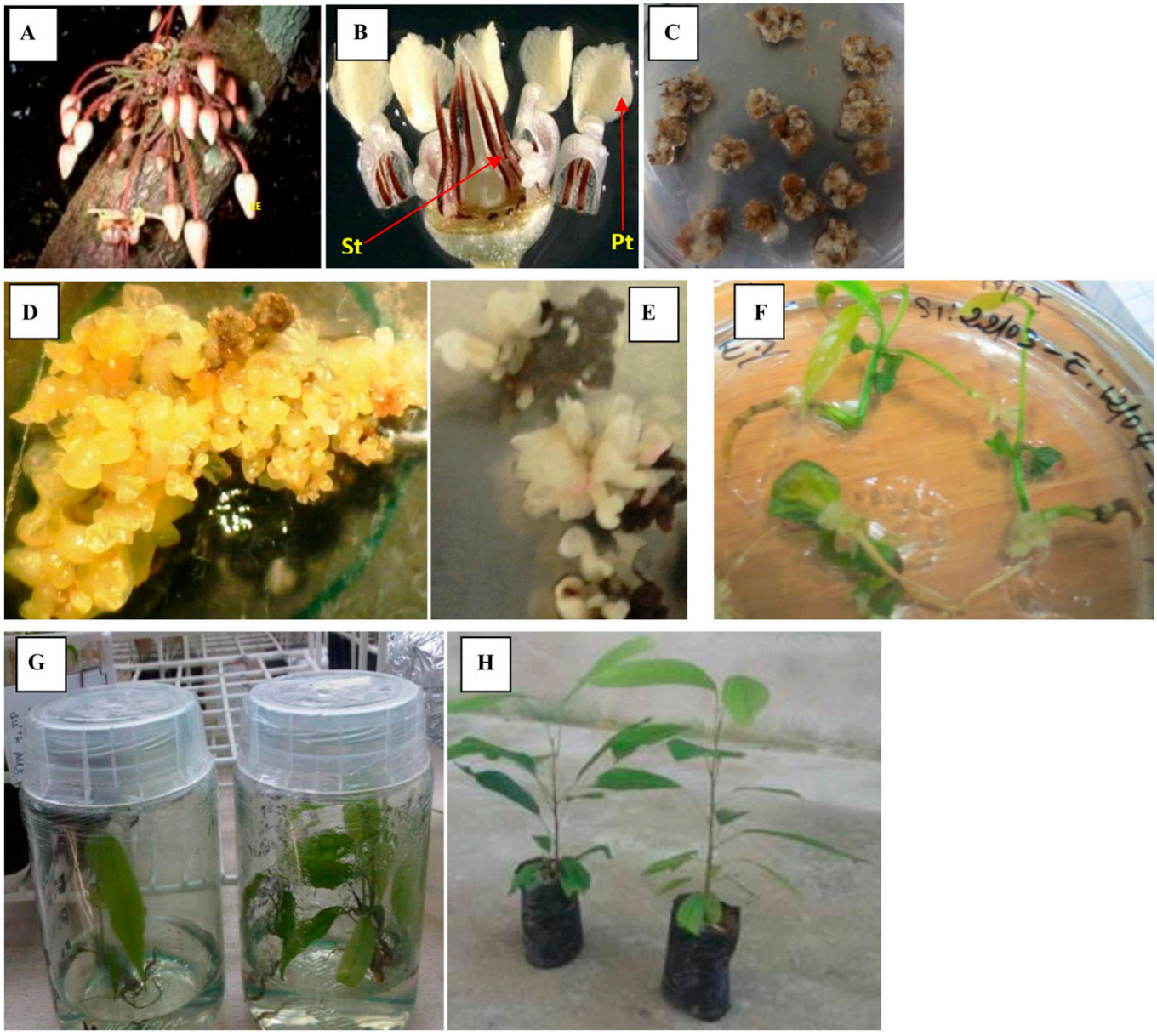

Fig. 1. Formation of somatic embryos from flower explants. (**A**) Flower on a cacao tree. (**B**) The opened flower showing the explants: (Pt) Petals and Staminodes (St). (**C**) Callus formed from the explants. (**D**) Embryos derived from the embryogenic callus. (**E**) Embryos at different stages. (**F**) Germination of embryos after 3 months. (**G**) Germination after 4 months. (**H**) Weaned plantlet.

on the protocol developed by Li et al. (1998). The pH of the media was adjusted to 5.8 using 0.1 M HCl or 0.1 M NaOH and gelled with 0.2% (w/v) phytagel before autoclaving it at 1.06 kg·cm^{-2} and 121 °C for 20 min.

The explants were cultured in the media under evaluation and incubated in a dark room maintained at 25 ± 2 °C. After 28 d on induction medium, the explants were transferred onto embryo development medium as described by Li et al. (1998) and subcultured at 4-week intervals for 3 months. The regenerated embryos were germinated in a growth room maintained at 25 ± 2 °C, cool white fluorescent light intensity of 33 mmol·m^{-2}·s^{-1}, and 16-h photoperiod.

Data collection. Four weeks after initiation, the percent explants with callus were evaluated. This was calculated as (the number of explants producing calli/total number of explants) × 100. About 3 months after initiation, the percentage of embryogenic calluses were evaluated by calculating (the number of explants producing embryos/total number of explants) × 100.

Experimental design and data analysis. The experiments were laid out in a completely randomized design. Ten petri dishes, each containing 25 explants (250 explants/treatment) were used for all the experiments. Each trial was replicated three times. STATISTICA 7.1 was used to analyze the data. Analysis of variance was performed where applicable, and differences between means were determined by Student–Newman–Keuls multiple range test. The treatments were considered as significant at $P < 0.05$.

Results

Effect of 9 μM 2, 4-D and varying concentrations of kinetin (0.5, 1, 5, and 10 μM) on callus and embryo formation. Culturing the explants on media supplemented with 9 μM 2, 4-D and 0.5 μM kinetin produced the highest mean number of embryos (4.5 and 6.0) in C1 and C14, respectively (Table 1). During the current study, it was observed that there were no embryo formed on explants cultured on media containing 5 and 10 μM kinetin in C1 and in media containing 5 μM kinetin in C14 (Table 1). Figure 1A–H shows the pathway of regenerating the cacao plantlets from the floral explants.

Effect of 10 μM 2, 4-D and varying concentrations of kinetin (2.5, 5, and 25 μM) on callus and embryo formation. Inclusion of 10 μM 2, 4-D and kinetin in the media had a significant ($P < 0.05$) effect on embryo formation. The media supplemented with 10 μM 2, 4-D and 5 μM kinetin produced the highest (98.7%) embryogenic cultures and the highest (28.0) mean number of embryos/explant in C1 (Table 2). This was a 9-fold increase in the number of embryos compared with the control. On the other hand, the C14 explants cultured on media supplemented with 10 μM 2, 4-D and 2.5 μM kinetin produced 7.0 mean number of embryos per explant. It was observed that increasing the concentration of kinetin from 5 to 25 μM was found to inhibit embryo formation in both C1 and C14.

Effect of 20 μM 2, 4-D and varying concentrations of kinetin (2.5, 5, and 25 μM) on callus and embryos formation. Inclusion of 20 μM 2, 4-D and 2.5 μM kinetin in the media had a significant ($P < 0.05$) effect on embryo formation. The explants cultured on media supplemented with 20 μM 2, 4-D and kinetin 2.5 μM produced the highest (95.8 and 97.3) percentage of embryogenic cultures in C14 and C1, respectively, and the highest (22.0) mean number of embryos in both C1 and C14. Increasing the concentration of kinetin from 5 to 25 μM was found to inhibit embryo formation in both C1 and C14 (Table 3).

Discussion

Auxin and cytokinins play a very important role in plant somatic embryogenesis and are required for the activation of somatic cell (Chaudhury and Qu, 2000; Jia et al., 2008; Liu et al., 2008). According to Kintzios et al. (2002), the role of cytokinins is to enhance cell division of pre-embryogenically determined cells. During the current study, kinetin was found to be more effective than TDZ in inducing somatic embryogenesis. These results are contrary to many studies which indicate that TDZ (a phenylurea type) is superior to the adenine types of cytokinins in inducing somatic embryogenesis in several plant species (Aboshama, 2011; Feng and Chen, 2014; Gill and Saxena, 1992; Kahia et al., 2016; Visser et al., 1992). In cacao, TDZ has been used by many workers (Ajijah et al., 2014; Garcia et al., 2016; Li et al., 1998; Maximova et al., 2002; Quainoo and Dwomo, 2012). An attempt to use TDZ during this study led to low responses. However, high percent embryogenic cultures (73% to 100%) were obtained when kinetin was used. This was much higher than those reported by Ajijah et al. (2016) (5.6% to 66.7%) and Kouassi et al. (2017) (13% to 14%) when they used kinetin. Moreover, the mean number of embryos per explant was also higher (3.50–28) in the current study compared with the number (0.7–24) reported by Ajijah et al. (2016). This could probably be explained by the difference in the genetic makeup of the clones used. The work being reported is similar to that of Mayati (2015) who found that kinetin was better than zeatin in somatic embryogenesis of *Hevea brasiliensis* RRIM 2025. However, it conflicts with the work of Chen and Chang (2001), Wongtiem et al. (2011), and Kahia et al. (2016) who reported that kinetin was inferior to other cytokinins evaluated for the induction of somatic embryos in Oncidium 'Gower Ramsey', *Manihot esculenta* Crantz, and *Coffea Arabica*, respectively. During the current study, increasing the concentration of kinetin was found to inhibit induction of somatic embryos. These results are similar to those of Ajijah et al. (2016) who observed that increasing the kinetin: 2, 4-D ratios did not significantly increase the average number of embryos in cacao. However, our results were contrary to those of Abdin and Ilah (2007) who reported that somatic embryogenesis was higher in media with high concentration of kinetin in *Cychorium intybus* L. During the present study, increasing the concentration of 2, 4-D from 9 to 20 μM was found to increase the embryogenic calluses. These results are in contrary to those of Ali et al. (2007) and Xing et al. (2010) who

Table 2. Effect of 10 μM 2, 4-D and varying concentrations of kinetin (2.5, 5, and 25 μM) on callus and embryo formation.

Genotypes	2, 4-D (10 μM) + Concentrations of kinetin in μM	Percent callus induction ± SE	Percent embryogenic callus ± SE	Mean no. of embryos ± SE
C1	Control	67.0 ± 1.6 a	24.9 ± 3.1 a	3.5 ± 0.5 a
	2.5	100.0 ± 0.0 b	93.4 ± 3.5 bd	10.0 ± 1.1 b
	5	98.6 ± 1.4 b	100.0 ± 0.0 b	28.0 ± 1.1 c
	25	73.3 ± 4.4 c	0.0 ± 0.0 c	0.0 ± 0.0 a
C14	Control	100.0 ± 0.0 a	90.0 ± 2.0 d	1.5 ± 0.5 a
	2.5	100.0 ± 0.0 a	100.0 ± 0.0 b	7.0 ± 4.0 b
	5	76.2 ± 0.0 b	68.7 ± 0.0 e	1.0 ± 0.0 a
	25	93.6 ± 1.0 a	0.0 ± 0.0 c	0.0 ± 0.0 a

The values represent mean ± SE of at least three replications. Means with different letters are significantly different at 5% (Newman–Keuls test).

Table 3. Effect of 20 μM 2, 4-D and varying concentrations of kinetin (2.5, 5, and 25 μM) on callus and embryos formation.

Genotypes	2, 4-D (20 μM) + Concn of kinetin in μM	Percent callus ± SE	Percent embryogenic callus ± SE	Mean no. of embryos ± SE
C1	Control	94.0 ± 8.5 a	84.9 ± 3.1 a	3.5 ± 0.5 a
	2.5	100.0 ± 0.0 a	97.3 ± 2.7 a	22.0 ± 1.7 b
	5	100.0 ± 0.0 a	93.2 ± 6.8 a	0.0 ± 0.0 a
	25	100.0 ± 0.0 a	95.8 ± 4.2 a	0.0 ± 0.0 a
C14	Control	100.0 ± 0.0 a	90.0 ± 2.0 a	1.5 ± 0.5 a
	2.5	96.3 ± 6.4 a	95.8 ± 4.2 a	22.0 ± 2.3 b
	5	100.0 ± 0.0 a	100.0 ± 0.0 a	3.0 ± 1.1 a
	25	92.8 ± 4.7 a	100.0 ± 0.0 a	0.0 ± 0.0 a

The values represent mean ± SE of at least three replications. Means with different letters are significantly different at 5% (Newman–Keuls test).

reported that lower concentration of 2, 4-D gave better response of embryogenic callus in tobacco and *Erigeron breviscapus*, respectively. During the current study, the embryos were germinated and successfully weaned in the greenhouse with a survival rate of 70% being recorded. This success rate reported in the current study was higher compared with that (54%) observed by Garcia et al. (2016) while working with the Brazilian cacao clone.

The protocol developed in this study was an improvement of that reported previously by Kouassi et al. (2017) while working with the elite cacao clones in Côte d'Ivoire. These workers evaluated the effect of two (0.25 and 0.5 mg/L) concentrations of kinetin and one (1 mg/L) concentration of 2, 4-D on induction of embryogenic cultures in four elite cacao clones. There was a need to evaluate a wider range of kinetin and 2, 4-D concentrations because these authors reported low responses. Furthermore, they did not report on the number of embryos from the embryogenic cultures and yet regeneration of embryos from embryogenic cultures, germination, and regeneration of plantlets is of paramount importance if the in vitro techniques have to be adopted for mass propagation of cacao. In the present study, we describe an enhanced in vitro propagation protocol for the elite Ivorian clones where higher percentage of embryogenic cultures, embryos, and plantlets were obtained than previously reported.

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