Vol. 22(12), pp.347-359, December 2023
DOI: 10.5897/AJB2023.17568
Article Number: 452A62C71547
ISSN: 1684-5315

http://www.academicjournals.org/AJB

ACADEMIC JOURNALS
expand your knowledge

African Journal of Biotechnology

Full Length Research Paper

Preferential expression of somatic embryogenesis in five elite genotypes of *Theobroma cacao* (L.) associated with explant type and protocols used

N'goran Poh Konan Georges[1,4]*, Minyaka Emile[2], Kouassi Kan Modeste[3], Kone Siaka[1], Djeni N'dede Theodore[4], Kouamé Christophe[1] and N'zi Jean-Claude[3]

[1]World Agroforestry Centre (ICRAF), Côte d'Ivoire Country Program, Cocody Mermoz, 08 BP 2823 Abidjan, Côte d'Ivoire.
[2]Department of Biochemistry, Faculty of Sciences, University of Douala, P. O. Box 24157 Douala, Cameroon.
[3]Laboratoire de Physiologie Végétale, Université Félix Houphouët-Boigny, Abidjan, Côte d'Ivoire.
[4]Laboratoire de Biotechnologie et Microbiologie des Aliments, Unité de Formation et de Recherche en Sciences et Technologie des Aliments (UFR-STA), Université Nangui Abrogoua, 02 BP 801 Abidjan, Côte d'Ivoire.

Received 30 March, 2023; Accepted 11 July, 2023

This study aims to identify the suitable subculturing time, culture media (protocols) and explants type suitable for somatic embryogenesis expression in five elite genotypes of *Theobroma cacao* L. Thus, petals and staminodes explants extracted from immature flower buds of genotypes (C1, C8, C9, C14, and C16) are first grown in different media (CIM and CDM) to assess the subculturing time. Then, successive transfers in EDM medium at every 4 weeks intervals until 24 weeks. To assess culture media preference, these genotypes were grown in four different protocols (I, II, III, and IV) for callus induction. They were then successively subcultured (four times at 4 weeks intervals) into EDM. About the subculture time, embryo expression is earlier in C1, C9, C14, and C16 genotypes (2nd and 4th weeks) than C8 genotype (12 and 16th weeks). However, C1, C14, and C16 (with staminodes explants) prefer protocol IV, while their petal explants prefer protocol II. Also, C9 staminodes explants prefer protocol III while the C9 and C8 petals explants prefer protocol I. This study has identified suitable explants and protocols for somatic embryo production in the tested genotypes. Moreover, this work has laid out a new strategy to overcome recalcitrance to the somatic embryogenesis of *T. cacao* genotypes.

Key words: Explants, genotypes, somatic embryogenesis, subculture time, *Theobroma cacao* L.

INTRODUCTION

Theobroma cacao L is a cross-pollinating tree, native to the American tropics, which significantly contributes to the economies of many developing countries on the Asian, African, and Latin American continents (Myeki et al., 2022). Cocoa beans are of particular interest to the global chocolate industry (Ackah and Dompey, 2021; Perez et al., 2021). Chocolate, recognized as a healthy and nutritious food, is mainly considered medicine for

*Corresponding author. E-mail: georges.ngoran@yahoo.fr.

heart diseases (Decroix et al., 2018; Meier et al., 2017; Correia et al., 2016). Due to the continuously increasing global demand for cocoa, the sustainable intensification of its production in West Africa is considered crucial (Kongor et al., 2018).

Côte d'Ivoire is the world's leading producer and exporter of cocoa beans, with a global market share of around 41%. It is expected to produce 2.2 million tons in 2021/2022, compared with 2.248 million tons in 2020/2021. However, climate change, pest infestation, soil degradation and aging plantations are the main factors contributing to the decline in production. Also, these orchards, which are mostly degraded, require replanting (Kouame et al., 2016). As a result, the country needs strategies to maintain its production and its leading position.

Furthermore, the International Agroforestry Research Center, as part of the Vision for Change (V4C) project in 2010 initiated a program whose aim was to revitalize the cocoa sector throughout Côte d'Ivoire. This has resulted in well-trained producers, higher yields, better quality production and a profitable cocoa economy that benefits the whole community. During this program, elite cocoa genotype plants were selected (Jane et al., 2016; Kouamé, 2015). The performance of these genotypes grafted onto mature plants in the field has resulted in yields exceeding 2 t/ha in the fourth year of production, and some of these genotypes show tolerance to the cocoa swollen shoot disease. In addition, the quality of cocoa beans has been determined by many factors, such as size, number, color, degree of bitterness, acidity and flavor (Kouame et al., 2016). Except that the propagation of these genotypes by the classical method is difficult to achieve due to the heterogeneity of the seeds. In fact, cocoa has various propagation systems, such as seeds, grafting, and cuttings that provide planting material for plantations (Batista et al., 2015). However, these systems are difficult to implement due to the heterogeneity of seeds, the poor architecture of plants obtained after cuttings, and the need for large quantities of grafted plants with the desired genetic and sanitary quality (Garcia et al., 2016; Batista et al., 2015). Therefore, the development of rapid and highly efficient systems to facilitate the vegetative propagation of cocoa is considered necessary. Therefore, the somatic embryogenesis approach offers an alternative strategy to produce elite planting material (in quality and quantity) for sustainable cocoa production (Isah, 2019).

Somatic embryogenesis is one of the methods of cocoa propagation with multipurpose potential ranging from genetic improvement to mass production of elite plants and germplasm conservation (Garcia et al., 2016). In *T. cacao*, several somatic embryogenesis protocols using the mineral solution, Driver and Kuniyaki (DKW, 1984), added with different growth regulators have been developed. According to Kouassi et al. (2017), the effect of combining dichlorophenoxyacetic acid (2,4-D) with kinetin is more embryogenic than with thidiazuron (TDZ). Also, reports of Li et al. (1998) showed that a concentration of 22.7 nM of TDZ combined with 2,4-D was found to be optimal for the efficient induction of somatic embryos of 19 cocoa genotypes. These protocols aimed to define an ideal medium for the optimization of somatic embryo induction. However, variability (recalcitrance and low somatic embryo expression) in genotype response is reported. Genotypes have variability in response to the protocols (Garcia et al., 2016). In previous work, research teams (Kouassi et al., 2018; Kahia et al., 2017; Garcia et al., 2016; Li et al., 1998) had conducted investigations with a specific protocol to question somatic embryogenic expression in *T. cacao* genotypes. This approach (use of a specific somatic embryogenesis protocol applied to different genotypes from a specific experimental area) resulted in specific genotypes and protocols used. To overcome the recalcitrance constraints and improve the embryogenic response of *T. cacao* genotypes, an original approach would be to test different somatic embryogenesis protocols on a set of genotypes.

Various protocols for somatic embryogenesis of *T. cacao* are in use in laboratories (Kouassi et al., 2018; Kahia et al., 2017; Li et al. 1998). The main point of divergence of these different protocols is the constitution of the culture medium at the induction stage (of somatic embryogenesis). This phase corresponds to the state of dedifferentiation or disorganization of the current genetic program and the establishment of a new genetic program expression should lead to the formation of somatic embryos.

In Côte d'Ivoire, genotypes (C1, C8, C9, C14, and C16) of *T. cacao* with good agronomic performance in the field had been selected (Kahia et al., 2017). These genotypes were previously studied and showed that C1 and C14 are the most embryogenic, and C8 is recalcitrant with a culture medium using 2,4-dichloro phenoxy acetic acid (2,4-D) and kinetin (KIN) as growth regulators (Kouassi et al., 2018). Furthermore, our research team (Kahia et al., 2017) had developed culture media to induce somatic embryos at very high rates in C9, C1, and C14 genotypes. Also, a recent study according to Henao Ramírez et al. (2018) showed a different capacity of response to genotype induction depending on the subculture time. Considering subculture time, which is a period of tissue cellular incubation in the culture medium, these authors showed on one hand that the time of subculture as usual use proves that certain treatments (hormone, medium of culture) were better than the other. On the other hand of subculture time of the tissues cellular of genotypes was limited at a specific period that may be conducive or non-conducive to somatic embryogenesis. All these approaches show different embryogenic genotypic responses. But no combined study of the protocols has yet been conducted to improve the response of *T. cacao* genotypes to somatic

Table 1. Origin and field performance of five elite cacao genotypes.

Genotype	Origin	Performance (ton/ha) (2011-2015)
C1	Côte d'Ivoire	2.3
C16	Côte d'Ivoire	4
C14	Côte d'Ivoire	2.8
C9	Trinidad	2,3
C8	Trinidad	1,8

Data of vision for change project.
Source: Authors

embryogenesis.

This work will examine the performance of somatic embryogenesis expression according to explants of a pool of *T. cacao* genotypes (C1, C8, C9, C14, and C16) in a pool of protocols and in subculture time (while calli were transferred in EDM medium at every 4 weeks intervals until 24 weeks instead of the 6 weeks usually used), in order to identify which protocol or subculture time is best suited to which type of explant of a genotype.

MATERIALS AND METHODS

Source genotypes for explants

The explants in this study consisted of staminodes and petals explants of immature flower buds of five elite cocoa genotypes with codes C1, C8, C9, C14, and C16. These genotypes are obtained by grafting and kept in the genomic bank in the experimental field of the International Research Centre for Agroforestry in Adiopodoume (Côte d'Ivoire). In this experimental field, the average annual rainfall is 1320 mm. The temperature varies from 22 to 35°C. The origin and field performance of these genotypes are presented in Table 1.

Experimental design

The experiment conducted from January to May 2021, consisted of evaluating the protocol(s) and subculture time best suited for the expression of somatic embryos of five genotypes (C1, C8, C9, C14, and C16) of *T. cacao*.

Staminodes and petals explants of the flower buds (after surface sterilization) of five genotypes were simultaneously seeded on induction media (of variable composition between the four protocols tested) for 14 and 28 days, respectively for protocols I and II, III, and IV. At the end of the 14 and 28 days in the induction media, the explants were either:

(1) subcultured in the maintenance medium (for 14 days) followed by 6 subcultures at 28-day intervals in the somatic embryo expression medium, the protocol I (Table 2);
(2) subcultured 4 times at 28-day intervals in the somatic embryo expression medium (protocols II, III, IV) (Table 2).

Culture medium

Protocol I

Three culture media were used, CIM_1 (primary callus induction medium), CDM (secondary callus development medium), and EDM (somatic embryo development medium). CIM_1 medium was prepared by mixing 100 ml of macroelements and 10 ml of microelements from Driver and Kuniyaki Walnut (DKW) with 1 ml of vitamin (100 mg/ml myoinositol + 2 mg/ml thiamine-HCL + 1 mg/ml nicotinic acid + 2 mg/ml glycine) from DKW, 20 g/l glucose, 250 mg/l glutamine, 100 mg/l myoinositol, 2 mg/l 2,4-Dichlorophenoxyacetic acid, 0.00 mg/l thidiazuron and 2g/l phytagel. The pH of the mixture was adjusted to 5.8.

The CDM medium used consists of 2.3 g of McCown's, 1 ml of Gamborg's vitamin solution (100 mg/ml myoinositol + 10 mg/ml thiamine-HCL + 1 mg/ml nicotinic acid + 1 mg/ml pyridoxine), glucose (20 g/l), 2,4-D (2 mg/l), 6-benzyl aminopurine (0.005 mg/l), and 2.2 g/l phytagel. The pH of the mixture was adjusted to 5.7.

EDM_1 medium was obtained by mixing 100 ml of macroelements, 10 ml of microelements, 1 ml of DKW Vitamin supplemented with 30 g/l sucrose, and 2 g/l phytagels. The pH of the mixture was adjusted to 5.8 (Table 2).

Protocol II

Two types of culture media were used: the callus induction medium (CIM_2) and EDM (somatic embryo development medium). The CIM_2 consisted of DKW macroelements (100 ml/l), DKW microelements (10 ml/l), DKW vitamin (1 ml), Glucose (20 g/l), Myoinositol (100 mg/l), Glutamine (250 mg/l), 2,4-D (4.5 µM), Kinetine (1.125 µM), Glucose (30 g/l), and Phytagel (2 g/l). The somatic embryo expression medium used was identical to that of Protocol I (Table 2).

Protocol III

As in protocol II, two types of culture media were used: the callus induction medium (CIM_3) and the somatic embryo expression medium. The CIM_3 is made up of Glucose (20 g/l), Myoinositol (100 mg/l), Glutamine (250 mg/l), 2,4-D (20 µM), Kinetine (2.5 µM), Glucose (30 g/l) and Phytagel (2 g/l). The somatic embryo expression medium used was identical to that of Protocol I (Table 2).

Protocol IV

In this protocol, two types of culture media were also used: the callus induction medium (CIM_4) and the somatic embryo expression medium. The CIM_4 consists of Glucose (20 g/l), Myoinositol (100 mg/l), Glutamine (250 mg/l), 2,4-D (20 µM), Kinetine (2.5 µM), Sucrose (34.2 g/l) and Phytagel (2 g/l). The somatic embryo expression medium used was identical to that used in Protocol I

Table 2. Culture phases and protocols.

Phase	Biological processes	Types of culture media	Constitution of culture media			
			Protocol I (control)	Protocol II (Kouassi et al., 2017)	Protocol III (Kahia et al., 2017)	Protocol IV (experimental)
Induction (dedifferentiation)	Disorganization of the current genetic program and establishment of a new genetic program	Primary callus induction medium	DKW mineral complex Vitamin de DKW (1 ml/l), Myoinositol (100 mg/l), Glutamine (250 mg/l), 2,4-D (2 mg/l), TDZ (0,005 mg/l), Glucose (20 g/l), Phytagel (2 g/l)	DKW mineral complex Vitamin de DKW (1 ml/l), Myoinositol (100 mg/l), Glutamine (250 mg/l), 2,4-D (4.5 µM), Kinetin (1,125 µM), Glucose (30 g/l), Phytagel (2 g/l)	DKW mineral complex Vitamin de DKW (1 ml/l), Myoinositol (100 mg/l), Glutamine (250 mg/l), 2,4-D (20 µM), Kinetin (2.5 µM), Glucose (30 g/l), Phytagel (2 g/l)	DKW mineral complex Vitamin de DKW (1 ml/l), Myoinositol (100 mg/l), Glutamine (250 mg/l), 2,4-D (20µM), Kinetin (2.5 µM), Saccharose (34,2 g/l), Phytagel (2 g/l)
Maintenance	Maintenance of the new genetic program	Secondary callus induction medium	McCown's (2.3 g/l), Vitamin de Gamborg (1 ml/l), Glucose (20/l), 2,4-D (2 mg/l), BAP (0.05 mg/l), Phytagel (2.2 g/l)	-	-	-
Expression (differentiation)	Expression of the new genetic program	Somatic embryo expression medium	DKW mineral complex Vitamin de DKW (1 ml/l), Saccharose (30 g/l), Phytagel (2 g/l)	DKW mineral complex Vitamine de DKW (1 ml/l), Saccharose (30 g/l), Phytagel (2 g/l)	DKW mineral complex Vitamine de DKW (1 ml/l), Saccharose (30 g/l), Phytagel (2 g/l)	DKW mineral complex Vitamine de DKW (1 ml/l), Saccharose (30 g/l), Phytagel (2 g/l)

Source: Authors

(Table 2).

After preparation, all culture media were autoclaved at 121°C, under a pressure of 1 bar for 20 min (Sulzer autoclave).

Disinfection of flower buds

The immature flower buds harvested early in the morning (before 9 am) were marked according to genotype and transported in a cooler to the *in vitro* culture laboratory. These flower buds were then disinfected by immersion in a 1% (w/v) calcium hypochlorite solution, to which a few drops of tween-20 were added. After a contact time of 40 min with the 1% (w/v) calcium hypochlorite solution, the flower buds were rinsed three consecutive times with sterile distilled water at 2 min intervals.

Seeding and subculturing

Under the laminar flow hood, the disinfected immature flower buds were dissected with forceps and a scalpel blade in a Petri dish. Following this dissection, the petals explants and staminodes explants were isolated and used as explants.

Petals and staminodes explants of the five genotypes were grown simultaneously and separately in the four induction media at a rate of 25 staminodes explants and 25 petals explants per Petri dish. Each Petri dish was considered an experimental unit. However, for each genotype depending on the type of explant (staminode or petal), 20 experimental units were performed. The experiment was organized with a completely randomized design with two factors (genotype and explant type). Thus, a total number of 1000 explants (500 petals explants and 500 staminodes explants) per genotype and induction medium were used as explants for primary somatic embryogenesis. After seeding, the cultures were incubated in the dark (at 26 ± 1°C) for 14 days. At the end of the 14 days of incubation: (a) The cultures were transferred to a secondary callus induction medium and incubated for 14

days under the same conditions as before. After 14 days in the secondary callus induction medium, the explants were transferred to the embryo development medium (EDM) and the cultures were incubated for 28 days. At the end of the 28 days, five additional subcultures (at 28-day intervals) were then grown on EDM and incubated under the same experimental condition Protocol I).
(b) The cultures were transferred to the EDM and incubated for 28 days in the dark. Three successive transplants at 28-day intervals were then made on the same medium and cultures were incubated under the same conditions (protocols II, III, and IV). The test was replicated three consecutive times under the same experimental conditions.

Evaluation of responses to somatic embryogenesis

For each genotype, the percentage of callogenic explants and embryogenic callus is evaluated by counting the number of explants producing callus and the number of callus-bearing embryos. The parameters assessed are expressed by the formulae:

(1) Percentage of callogenic explants (PCE) (Garcia et al., 2016):

$$PEC=NEC/NTE\times100 \quad (1)$$

where NEC= number of explants yielding a callus and NTE= total number of explants grown.

(2) Percentage of embryogenic callus (PEC) (Garcia et al., 2016):

$$PCE=NCE/NEC\times100 \quad (2)$$

where NCE= total number of embryogenic callus and NEC= number of explants yielding callus.

(3) Number of embryos: The total number of embryos produced was counted every 4 weeks until 24 weeks on EDM medium. Also, the number of embryos produced is recorded per subculture time. The average number of embryos produced per genotype was calculated at each subculture.

(4) Embryogenesis efficiency (EE) (Garcia et al., 2016): EE values are a measure of the net efficiency of a given genotype at the various stages and conditions of culture. EE is calculated according to the formula:

$$EE=NPSE/NTE \quad (3)$$

where NPSE= number of primary somatic embryos produced and NTE= total number of explants grown.

Statistical analysis

Statistical analyses were performed using R software (Version 4.2.0). The experimental values obtained were all subjected to an analysis of variance (ANOVA). When the ANOVA indicated that at least one mean was different from the others, a post hoc Tuckey HSD or Kruskal-Wallis test at the 5% threshold was adopted to separate the means.

RESULTS

Morphologies of primary somatic embryos

Staminodes and petals explants of the five cocoa genotypes (C1, C8, C9, C14, and C16) produced calli in culture. Irrespective of genotype, calluses were observed between days 10 and 14 of culture in the callus induction medium in both experiments. In the embryo expression medium, callogenesis was followed by the differentiation of somatic embryos at different developmental stages (globular, heart, torpedo, and cotyledonary). However, some embryos showed malformations and others were translucent (Figure 1).

Effect of subculture time on response to somatic embryogenesis

Percentage of callus induced

The experimental results are presented in Figure 2. Analysis of this figure shows that all genotype explants (C1, C8, C9, C14, and C16) formed callus from the primary callus induction medium (CIM) and the callus development medium (CDM). The highest percentages, 100%, were obtained with petals explants of the C8 genotype. The lowest rate was observed in petals explants of the C1 genotype (87.13 ± 13.67%). On the other hand, significant differences in percentages were not observed between petals explants of C9 (99 ± 0.72%), C14 (99 ± 0.71%), C16 (96.5 ± 3.96%), C8 (100%), and staminodes explants of C1 (97 ± 0.82%), as well as C14 (98 ± 0.87%). Also, the percentages of staminodes explants producing calli from C8 (93 ± 2.5%) and C9 (93 ± 1.97%) were not significantly different from petals explants producing calli of C16 (88.57 ± 6.51%). Moreover, the percentages of calli derived from petals explants of C1 (87.13 ± 13.67%) and calli from staminodes explants of C16 (88.57 ± 6.51%) were not significantly different.

Percentages of embryogenic callus

The results obtained from the percentages of embryogenic callus of explants of different genotypes are as shown in Figure 3. The analysis shows that of all the callogenic explants, only the petals explants of genotype C1 produced the highest rate of embryogenic callus, that is, 25 ± 2.34%. The lowest rate was 0%, which was observed in C8, C14, and C16 staminodes explants. However, the embryogenic callus of C1 petals explants is significantly different from C8 (5 ± 3.13%), C9 (8 ± 1.76%), C14 (11 ± 3.09%), C16 (7 ± 2.56%) and C1 (7 ± 1.81%), C9 (5 ± 3.13%) staminodes explants.

Embryos produced

Figure 4 shows the average number of embryos produced per explant. The highest average number of embryos per explant was observed in petal explants of

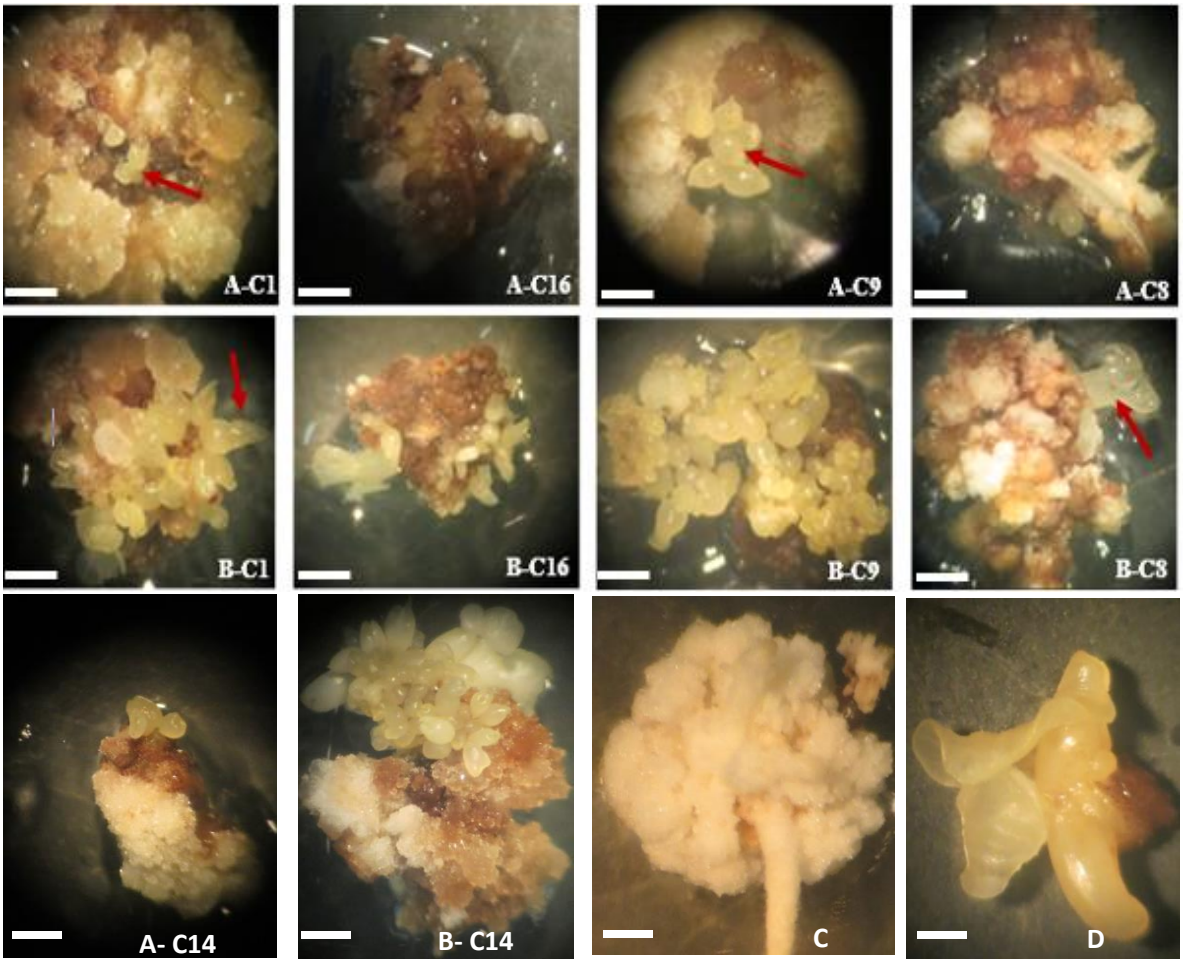

Figure 1. Primary somatic embryogenesis of *T. cacao* (beginning of somatic embryo productions of genotypes: C1 (A-C1), C16 (A-C16), C9 (A-C9), C8 (A-C8) C14 (A-C14). Maximum observed somatic embryo production of the genotypes: C1 (B-C1), C16 (B-C16), C9 (B-C9), C8 (B-C8) C14(B-C14). The red arrows show the different stages: globular (A-C1), heart (A-C9), torpedo (B-C1), and cotyledonary (B-C8). Non-embryogenic callus (C), translucent malformed embryo (D).
Source: Authors

the C1 genotype with a value of 28 ± 3.4. However, staminode explants of the C8, C14, and C16 genotypes did not produce any embryos. Thus, they constitute the lowest number of embryos. On the other hand, the petals explants of the C1 genotype are significantly different from the petal explants of C8 (4 ± 1.3), C9 (15 ± 3.3), C14 (17 ± 2.9), C16 (10 ± 2.1), and the staminodes explants of C9 (15 ± 3.3), C1 (4 ± 1.5).

Evolution of embryo production by subculture time

For this study, the evolution of somatic embryo production was assessed through the estimation of the number of embryos produced by subculture time.

The results obtained were presented in Figure 5. The analysis of the figure indicates that the petals explants of genotypes producing the most embryos from the beginning (2nd and 4th weeks) are C1 with an average of 2. While the genotypes of C9, C14, and C16 produce on average 1 embryo between the second and fourth weeks of subculture in the embryonic development medium (EDM). Embryo production was therefore rapid or early for these four genotypes. Furthermore, their number of embryos increased with subculture time (4 weeks interval) and reached a maximum between the 8 and 12th weeks of subculture with an average of 19 for C1, 7 for C9, 11 for C14, and 8 for C16. Thereafter, production decreased and stopped between the 16 and 20th weeks of subculture with an average of 3 embryos for C1, 2 for C9, 2 for C14, and 1 for C16. However, the first embryos of the C8 genotype appeared between the 12 and 16th weeks with an average of 2 embryos. The appearance of the first C8 embryos is therefore late compared to the

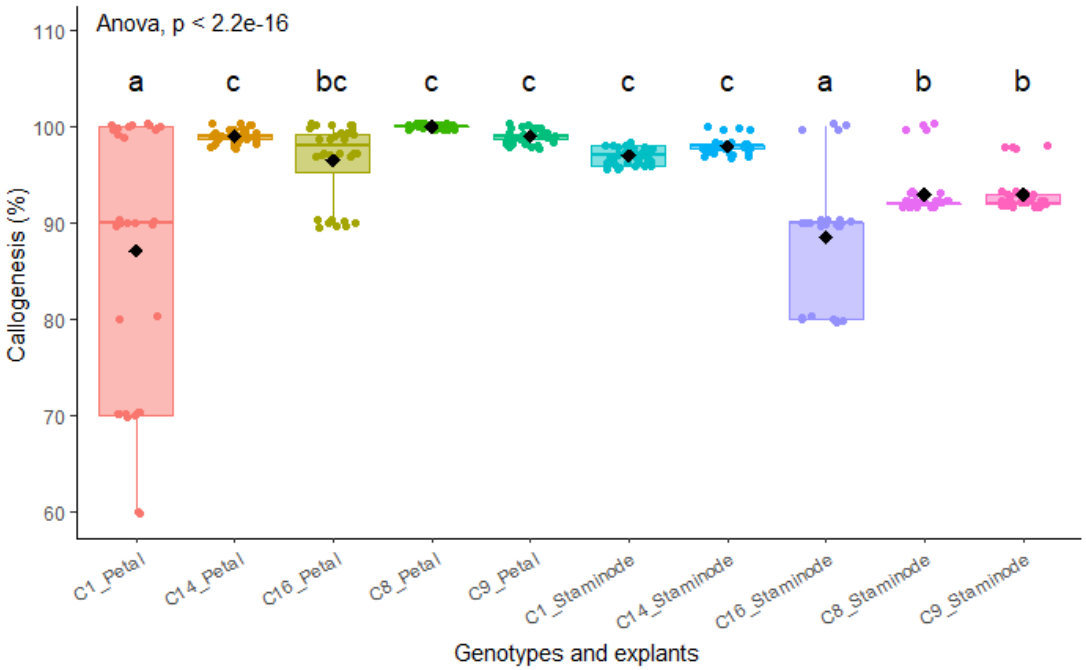

Figure 2. Percentage of callus induction in staminodes and petals of elite genotypes of *T. cacao*.
Source: Authors

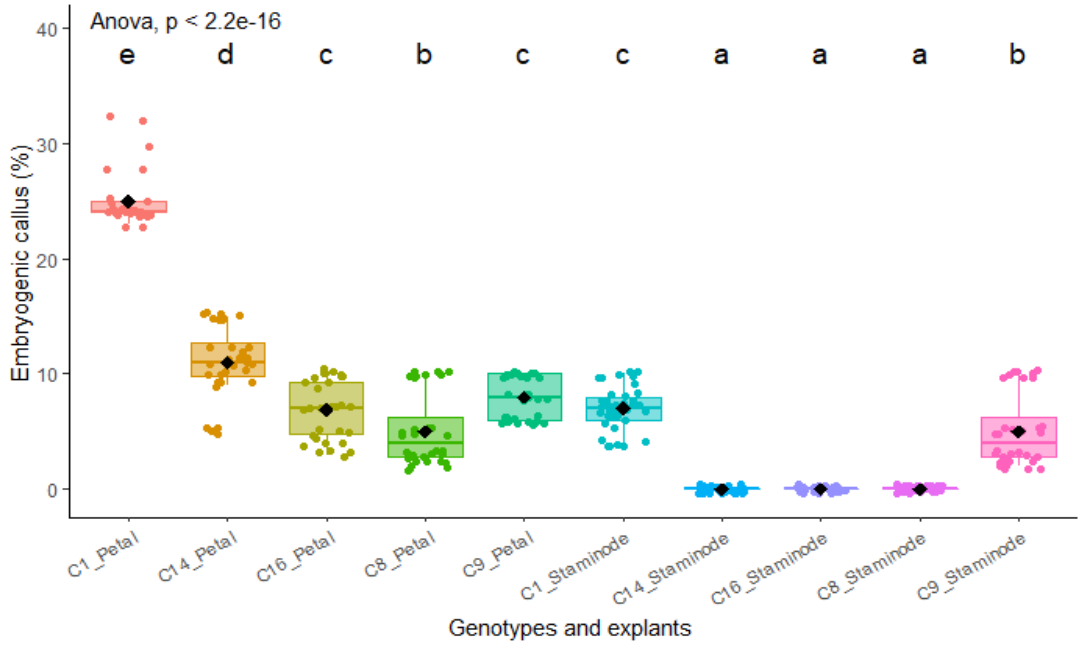

Figure 3. Percentages of embryogenic callus of elite genotypes of *T. cacao*.
Source: Authors

other genotypes. Nevertheless, the number of C8 embryos varied very little before reaching a maximum in the 16 and 20th weeks of subculture, with an average of 3 embryos. This production decreased thereafter and stopped between the 20 and 24th weeks of subculture.

The evolution of embryo production from staminode

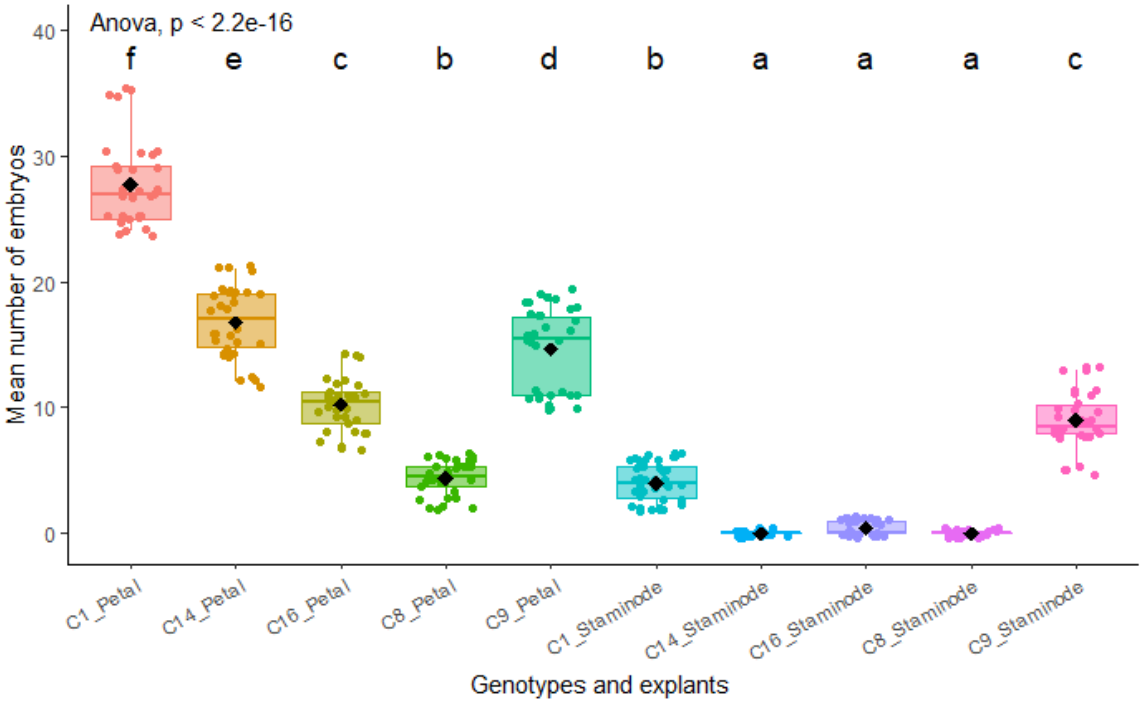

Figure 4. Number of embryos produced per explant according to elite genotypes of *T. cacao*.
Source: Authors

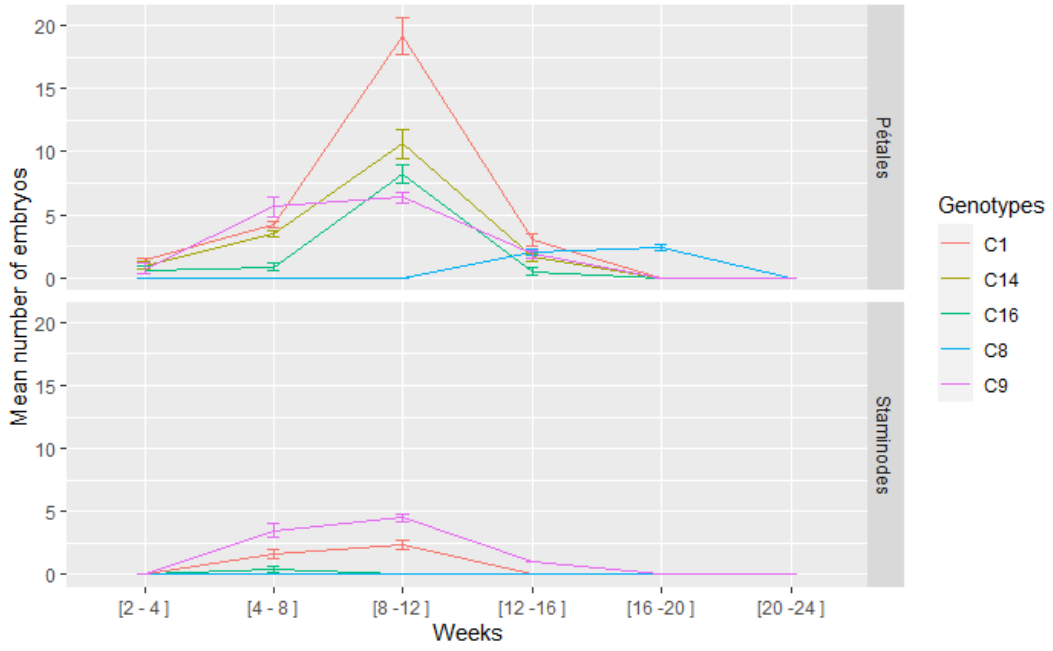

Figure 5. Evolution of embryo production according to subculture time of elite genotypes of *T. cacao*.
Source: Authors

explants was observed between the fourth and eighth week of subculture. The genotype that produced the most embryos was C9 with 4 embryos. During the same period, the C1 and C16 genotypes produced 3 embryos,

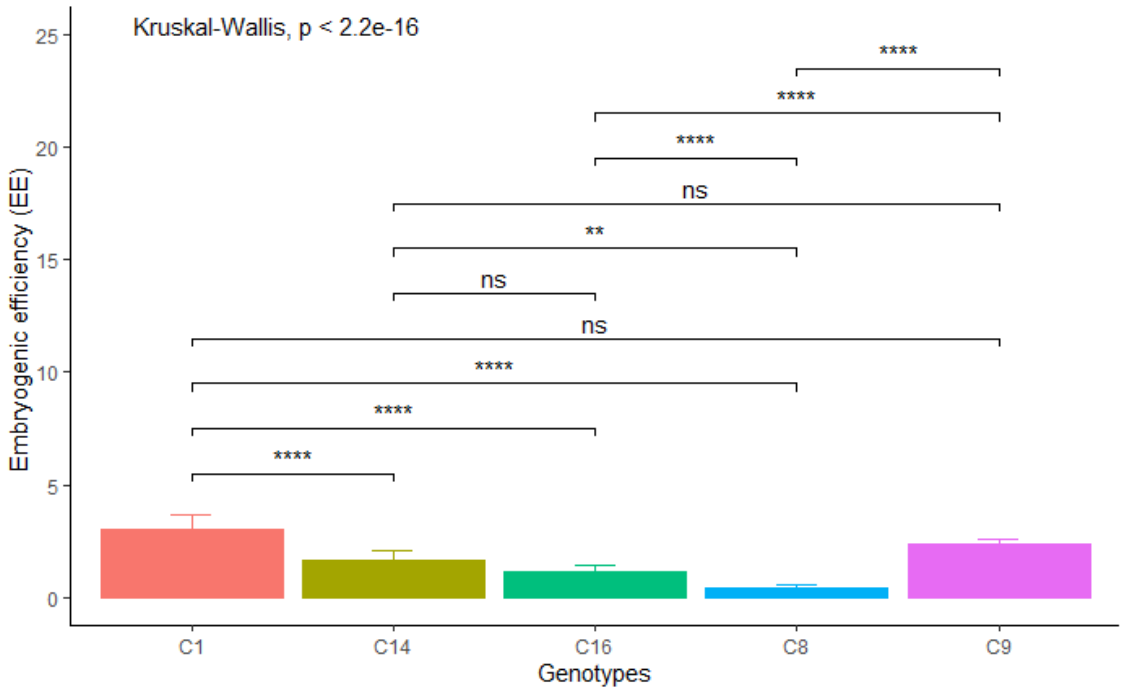

Figure 6. Somatic embryogenesis efficiency according to *T. cacao* elite genotype.
Source: Authors

respectively. Subsequently, embryo production increased for these genotypes and reached a maximum between the 8 and 12th weeks of subculture with average embryos of 3 for C1, 6 for C9, and 1 for C16. These embryo production values then decreased to zero between the 16 and 20th weeks of subculture.

The efficiency of somatic embryogenesis of elite genotypes

About the efficiency of somatic embryogenesis of the genotypes, the results were presented in Figure 6. The analysis shows that the most responsive genotype to embryogenesis is C1 with a somatic embryogenesis efficiency (EE) value of 3.1. In contrast, the least responsive genotype is C8 with an EE of 0.4. However, the EE value of C1 is significantly different from that of the C14 genotype with an EE of 1.7, and the C16 genotype with an EE of 1.1, except that of C9 with an EE of 2.4.

Effect of explant preferential response on somatic embryogenesis

Percentage of callus induced

Petals and staminodes explants were grown on DKW basal medium containing varying combinations of 2,4-D and kinetin. After 28 days of cultivation on the callogenesis induction and expression media, the results obtained were recorded in Table 3. The observed callus induction rate varied between 86 ± 18.6 and 100%. The most callogenic explants (100%) were observed with petals explants of C16, C8 and C9 genotypes for the initial culture media of protocols II, III, and IV. Similarly, staminodes explants of C16 genotypes for the medium of protocols II, C1, C14, C8, and C9 genotypes for culture media of protocols II, III, and IV were the most callogenic (100%). In contrast, the least callogenic explant was with C1 genotype petals explants (86 ± 18.6%). However, there were significant differences between the callogenesis of staminodes and petals explants of C1, C14, C16, C8, and C9 genotypes for the culture medium of protocols I, II, II, I, and IV.

Percentage of embryogenic callus

After 28 days of culture on callogenesis induction and expression medium, explants subcultured at 28-day intervals for 16 weeks on somatic embryo expression media recorded results shown in Table 4. All initial culture media (protocols I, II, III, and IV) did not allow all types of genotype explants to form embryogenic callus. Thus, the most embryogenic explant callus (54 ± 26.9%) was obtained with staminodes explants of genotype C9 for the culture medium of protocol III. As for the medium of protocol II, staminodes explants of C1, C14, C1, 6, and

Table 3. Percentages of callus formed from culture media of protocols I, II, III, and IV.

Genotype	Explants	Percentages of embryogenic callus			
		Protocol I	Protocol II	Protocol III	Protocol IV
C1	Petals	87 ± 13.5^{a}	86 ± 18.6^{a}	99 ± 1.7^{cf}	99 ± 1.7^{cf}
	Staminodes	97 ± 1^{b}	100^{f}	100^{f}	100^{f}
C14	Petals	99 ± 0.7^{c}	90 ± 22^{d}	96 ± 7.6^{d}	96 ± 7.6^{d}
	Staminodes	98 ± 0.9^{cb}	100^{f}	100^{f}	100^{f}
C16	Petals	97 ± 3.9^{b}	100^{f}	100^{f}	100^{f}
	Staminodes	89 ± 6.4^{d}	100^{f}	97 ± 6.5^{b}	97 ± 6.5^{b}
C8	Petals	100^{f}	100^{f}	100^{f}	100^{f}
	Staminodes	93 ± 2.5^{e}	100^{f}	100^{f}	100^{f}
C9	Petals	99 ± 0.7^{cf}	100^{f}	100^{f}	100^{f}
	Staminodes	93 ± 1.7^{e}	100^{f}	100^{f}	100^{f}

Within a column, numbers followed by the same letter are statistically identical at the α = 5% threshold (Tuckey test); Mean ± standard error.
Source: Authors

Table 4. Percentages of embryogenic callus formed from culture media of protocols I, II, III, and IV.

Genotype	Explants	Percentages of embryogenic callus			
		Protocol I	Protocol II	Protocol III	Protocol IV
C1	Petals	25 ± 2.3^{a}	21 ± 10.5^{a}	10 ± 5.1^{b}	7 ± 8.9^{b}
	Staminodes	7 ± 1.8^{b}	0	10 ± 2.8^{b}	21 ± 10.33^{a}
C14	Petals	11 ± 3.1^{c}	10 ± 6.1^{b}	0	0
	Staminodes	0	0	11 ± 4.2^{c}	14 ± 5.4^{d}
C16	Petals	7 ± 2.5^{b}	10 ± 5.1^{b}	6 ± 3.4^{b}	0
	Staminodes	0	0	6 ± 5.2^{b}	12 ± 7.7^{c}
C8	Petals	5 ± 3.1^{d}	0	0	0
	Staminodes	0	0	0	0
C9	Petals	8 ± 1.7^{e}	7 ± 5.3^{c}	6 ± 2.1^{b}	7 ± 5.3^{b}
	Staminodes	5 ± 3.1^{f}	0	54 ± 26.9^{d}	6 ±2.8^{e}

Within a column, numbers followed by the same letter are statistically identical at the α = 5% threshold (Tuckey test); Mean ± standard error.
Source: Authors

C8 genotypes did not form embryogenic callus. Also, the petal explant callus of the C8 genotype was not embryogenic for the culture media of protocols III and IV.

Embryos produced

The results of the embryo production are shown in Table 5. Analysis of this table shows that all genotypes produced embryos from the culture media (protocols I, II, III, and IV) tested. However, significant differences were observed depending on the genotype and the type of explant used. Thus, the culture medium according to genotype and explant that produced the most embryos (88 ± 56) was the culture medium of protocol III, C9 genotype and staminodes explants. On the other hand, for the culture medium of protocol II, the petals explants of C1, C14, and C16 genotypes produced more embryos

Table 5. Number of embryos produced per explant from culture media of protocols I, II, III, and IV.

Genotypes	Explants	Number of embryos per explant			
		Protocol I	Protocol II	Protocol III	Protocol IV
C1	Petals	19 ± 4[a]	24 ± 23[a]	5 ± 3[b]	0
	Staminodes	2 ± 1[c]	0	5 ± 1[b]	13 ± 3[d]
C14	Petals	11 ± 3[e]	17 ± 9[b]	5 ± 2[b]	0
	Staminodes	0	0	4 ± 3[b]	9 ± 0.0[g]
C16	Petals	8 ± 2[f]	18 ± 8[b]	3 ± 2[c]	0
	Staminodes	0	0	2 ± 1[c]	6 ± 4[h]
C8	Petals	2 ± 1[c]	0	0	0
	Staminodes	0	0	0	0
C9	Petals	6 ± 1[b]	4 ± 2[c]	3 ± 1[c]	0
	Staminodes	5 ± 1[b]	0	88 ± 56[i]	4 ± 1[b]

Within a column, numbers followed by the same letter are statistically identical at the α = 5% threshold (Tuckey test); Mean ± standard error.
Source: Authors

than the other media (protocols I, III, and IV). Similarly, for the protocol IV culture medium, staminodes explants of C1, C14, and C16 genotypes produced more embryos than the protocols I, II, and III culture media. However, the C8 and C9 genotypes produced more embryos with petals explants from the protocol I medium.

DISCUSSION

Explant responses to somatic embryogenesis in cocoa are genotype-dependent. To select a pool of genotypes associated with somatic embryogenesis performance, five genotypes (C1, C16, C9, C14, and C8) were tested on different culture media. The results in the study of the effect of subculture time on somatic embryogenesis response showed that petals and staminodes explants of C1, C8, C9, C14, and C16 genotypes induced callus at rates ranging from 87.13 to 100%. Similar observations were made by Kahia et al. (2017) and Osorio Montoya et al. (2022). According to their reports, *T. cacao* flower bud explants yield high percentages of callus during cocoa somatic embryogenesis. Our results show a success that could also be explained by the nutrient composition of the culture medium and the effect of the hormonal balance used (Daouda et al., 2019; Osorio Montoya et al., 2022). The hormonal balance (2,4-D / TDZ) used in the culture medium is the best condition to obtain callus (Boutchouang et al., 2016). Indeed, auxin (2,4-D) and cytokinin (TDZ) facilitate cell division and protein synthesis that affects callus and somatic embryo formation (Garcia et al., 2016). In contrast, the significant differences between the callus percentages of the different genotypes would be due to a genotype effect. This can be explained either by the concentration of endogenous phytohormone of the explant type of genotypes such as indole-3-acetic acid or by the sugar concentration of the explant which influences the callus formation process (Grzyb et al., 2017).

Furthermore, calli from petals explants of the C1 genotype were found to be more embryogenic than calli from petals explants and staminodes explants of other genotypes. Similar results were reported in the somatic embryogenesis of *T. cacao* by Garate-Navarro and Arévalo-Gardini (2017), in which all explants gave callus. However, not all were able to produce embryogenic calli. These observed differences in response could be explained either by the physiological state of the explant donor tree or by the accumulation of ethylene and carbon dioxide produced in the dark by the plant tissues in the Petri dishes during *in vitro* cultivation, which would hinder cell growth and regeneration (Boutchouang et al., 2016). Likewise, excessive hydrogen peroxide (H_2O_2) accumulation in calli may lead to the loss of embryogenic potential of calli (Peng et al., 2020). This variability in the response to somatic embryogenesis would therefore also be attributed to the effect of genotypes. However, the influence of genotype in the response to somatic embryogenesis in cocoa has also been reported in several previous works (Kouassi et al., 2017; Dangou et al., 2002).

The study carried out here on the evolution of embryo production according to the time of subculture of the genotypes has made it possible to highlight the effect of subculture time on the response to somatic embryogenesis of *T. cacao*. Indeed, there is an early

production of embryos by the C1, C9, C14, and C16 genotypes between the 2nd and 4th weeks in the EDM medium, and a late production of embryos by the C8 genotype between the 12 and 16th weeks in the EDM medium. These results may be linked to the plant gene, which has an immediate impact on the recalcitrance of certain tissues and genotypes concerning their totipotency (Daouda et al., 2019). Prolonged subculture on the EDM medium of *T. cacao* is, therefore, necessary to overcome recalcitrance or improve the response of certain genotypes. Indeed, according to Ren et al. (2022) during *in vitro* culture, the long-term subculture of some plant genotypes, the high endogenous content of cytokinin like indol-3- acetic acid in tissues cellular is beneficial to the vigorous growth of embryogenic calli and the high potential for somatic embryogenesis. In addition, the efficiency of somatic embryogenesis showed that the C1 genotype had a better production of embryos than the C14, C16, C8, and C9 genotypes. These results would also reflect the strong dependence on the genotypic effect. The genotypic differences observed in the somatic embryogenesis response probably reflect either endogenous soluble sugar provides indispensable energy as a carbon source, either genetic variation in the concentration of endogenous phytohormones, or the type of endogenously produced compounds such as polyamines, ethylene, phenolic compounds, auxin, or the synergy of endogenous phytohormones with exogenous phytohormones supplied in a medium (Ren et al., 2022; Peng et al., 2020).

Furthermore, the study carried out on the preferential effect of explants on somatic embryogenesis response identified media suitable for somatic embryogenesis responses of elite genotypes. Indeed, the production of embryos from C1 (petals explants), C14 (petals explants), and C16 (staminodes explants) was most effective in protocols II (C1 and C14) and IV (C16). Additionally, protocol I (with staminodes explants) and protocol III (with petal explant) were found to be suitable to obtain embryos from C9. Concerning the C8 genotype, the production of embryos is better only with petals explants in the culture medium of protocol I. The variation in response observed between genotypes would confirm on the one hand the differences in the requirements of endogenous and exogenous phytohormones of the explants of genotypes, and on the other hand, the endogenous storage substances (protein, sugar, and starch) in the explants. In fact, the molecular components (phytohormones, sugar, protein, lipids) in plant organs are influenced by their genetic origin, physiological status, and environmental factors. Environmental changes affect various biochemical reactions, often disrupting the balanced distribution of metabolites in cells (Shah et al., 2019). Moreover, The results of the present study are in line with those of Garcia et al. (2016) who showed that any variation in endogenous auxin levels would likely impact embryogenic capacity and other underlying response variations from one genotype to another. Also, Peng et al. (2020) showed that endogenous components like soluble protein, starch, soluble sugar, and superoxide dismutase were involved in the development of embryogenic calli in the pin. Their results indicate that somatic embryogenesis involves energy storage, and antioxidant enzymes cooperate to regulate the occurrence and development of embryos. This discovery of protocols adapted to genotype pools *in vitro* culture of *T. cacao* could be used for large-scale production for commercial purposes.

Conclusion

The first step of this study was to assess the effect of subculture time on the response to somatic embryogenesis. It was found that among the five genotypes tested, the C1, C9, C14, and C16 genotypes produced embryos early while the C8 genotype produced embryos late. Also, the C1 genotype has a higher somatic embryogenesis efficiency than the C8, C9, C14, and C16 genotypes.

The second step was to test the preferential effect of explants on embryo production and showed variability of results concerning the tested genotypes. Indeed, staminodes explants of the C1, C14, and C16 genotypes preferred the culture medium of protocol IV, while their petals explants preferred the culture medium of protocol II. Whereas, staminodes explants of the C9 genotype prefer the culture medium of protocol III and its petals explants prefer the culture medium of protocol I. As for the C8 genotype, its petals explants prefer the culture medium of protocol I. However, its staminodes explants did not produce embryos. This study, therefore, highlighted the somatic embryogenesis response pool of the five elite genotypes of *T. cacao*.

ABBREVIATIONS

2,4-D, 2,4-Dichlorophenoxyacetic acid; **TDZ,** thidiazuron; **EE,** efficiency of embryogenesis; **PEC,** Percentage of embryogenic callus; **PCE,** Percentage of callogenic explants; **KIN,** kinetin; **DKW,** Driver and Kuniyaki; **CIM,** primary callus induction medium; **CDM,** secondary callus development medium; **EDM,** somatic embryo development medium.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The authors are grateful to the ICRAF Côte d'Ivoire's

Somatic Embryogenesis Laboratory staff for their technical support. The financial support offered by Mars Incorporated is highly appreciated.

REFERENCES

Ackah E, Dompey E (2021). Effects of fermentation and drying durations on the quality of cocoa (*Theobroma cacao* L.) beans during the rainy season in the Juaboso District of the Western-North Region, Ghana. Bulletin of the National Research Centre 45(1):1-10.

Dangou JS, Hocher V, Ferrière N, Fulcheri C, Morard P, Alemanno L (2002). Histological and biochemical characterization of *Theobroma cacao* L. endosperm during seed development. Seed Science Research 12(2):91-100.

Batista AF, Dos Santos GA, Silva LD, Quevedo FF, De Assis TF (2015). The use of mini-tunnels and the effects of seasonality in the clonal propagation of Eucalyptus in a subtropical environment. Australian Forestry 78(2):65-72.

Boutchouang RP, Akitio OFZ, Tchouatcheu AGN, Niemenak N (2016). Influence of the position of flower buds on the tree on somatic embryogenesis of cocoa (*Theobroma cacao* L.). International Journal of Plant Physiology and Biochemistry 8(2):7-16.

Correia SI, Alves AC, Veríssimo P, Canhoto JM (2016). Somatic embryogenesis in broad-leaf woody plants: What we can learn from proteomics. In Vitro Embryogenesis in Higher Plants 1359:117-129.

Daouda K, Modeste KK, Oulo NA, Edmond KK (2019). Induction of somatic embryos of recalcitrant genotypes of *Theobroma cacao L*. Journal of Applied Biosciences 133:13552-13560.

Decroix L, Soares DD, Meeusen R, Heyman E, Tonoli C (2018). Cocoa Flavanol Supplementation and Exercise: A Systematic Review. Sports Medicine 48(4):867-892.

Driver JA, Kuniyuki AH (1984). In vitro propagation of paradox walnut rootstock. HortScience 19(4):507-509.

Garate-Navarro MA, Arévalo-Gardini E (2017). Induction of somatic embryogenesis from cocoa farmer field collection of ICT-Peru. International Annals of Science 2(1):6-11.

Garcia C, Corrêa F, Findley S, Almeida AA, Costa M, Motamayor JC, Schnell R, Marelli JP (2016). Optimization of somatic embryogenesis procedure for commercial clones of *Theobroma cacao* L. African Journal of Biotechnology 15(36):1936-1951.

Grzyb M, Kalandyk A, Waligórski P, Mikuła A (2017). The content of endogenous hormones and sugars in the process of early somatic embryogenesis in the tree fern Cyathea delgadii Sternb. Plant Cell, Tissue and Organ Culture 129(3):387-397.

Henao Ramírez AM, de la Hoz Vasquez T, Ospina Osorio TM, Garcés LA, Urrea Trujillo AI (2018). Evaluation of the potential of regeneration of different Colombian and commercial genotypes of cocoa (*Theobroma cacao* L.) via somatic embryogenesis. Scientia Horticulturae 229:148-156

Isah T (2019). Proteome study of somatic embryogenesis in Nothapodytes nimmoniana (J. Graham) Mabberly. 3 Biotech 9(4):1-23.

Jane K, Siaka K, Ngoran G, Christophe K (2016). 4C Technical Brief Series 2: Somatic Embryogenesis. https:// www.worldagroforestry.org

Kahia J, Kone S, Diby L, Ngoran G, Dadjo C Kouame C (2017). Enhanced plantlet regeneration in two cacao (*Theobroma cacao.*L) clones from immature inflorescence explants. HortScience 52(6):892-895.

Kongor JE, De Steur H, Van de Walle D, Gellynck X, Afoakwa EO, Boeckx P, Dewettinck K (2018). Constraints for future cocoa production in Ghana. Agroforestry Systems 92(5):1373-1385.

Kouame C (2015). Quelle Diversification pour une production durable du cacao ? Expérience du projet V4C Panel sur la Diversification Projet Vision for Change (V4C). April. ICRAF Côte d'Ivoire Country Program. https:// www.worldagroforestry.org

Kouame C, Kouassi J, Kouassi A, Gyau A, Kahia J, Diby L (2016). Land use change and farmer's perceptions of deforestation and land degradation: A case study in the South-West Region of Côte d' Ivoire. November 120 p.

Kouassi MK, Kahia J, Kouame CN, Tahi MG, Koffi EK (2017). Comparing the effect of plant growth regulators on callus and somatic embryogenesis induction in four elite *Theobroma cacao* L. genotypes. HortScience 52(1):142-145.

Kouassi MK, Koffi EK, Silue O, Tahi MG, Toure M, Konan EPK (2018). Comparison of systems combining auxins with thidiazuron or kinetin supplemented with polyvinylpyrrolidone during embryogenic callus induction in three *Theobroma cacao* L. genotypes. I International Journal of Biological and Chemical Sciences 12(2):804-811.

Li Z, Traore A, Maximova S, Guiltinan MJ (1998). Somatic embryogenesis and plant regeneration from floral explants of cacao (*Theobroma cacao* L.) using thidiazuron. *In Vitro* Cellular & Developmental Biology-Plant 34:293-299.

Meier BP, Noll SW, Molokwu OJ (2017). The sweet life: The effect of mindful chocolate consumption on mood. Appetite 108:21-27.

Myeki LW, Bahta YT, Matthews N (2022). Exploring the Growth of Agricultural Productivity in AFRICA: A Färe-Primont Index Approach. Agriculture 12(8):1236.

Osorio Montoya T, Henao Ramírez AM, de la Hoz Vasquez T, Urrea Trujillo AI (2022). Propagation of IMC67 Plants, Universal Cacao (*Theobroma Cacao* L.) Rootstock via Somatic Embryogenesis. International Journal of Fruit Science 22(1):78-94.

Peng C, Gao F, Wang H, Shen H, Yang L (2020). Physiological and biochemical traits in Korean pine somatic embryogenesis. Forests 11(5):577.

Perez M, Lopez-Yerena A, Vallverdú-Queralt A (2021). Traceability, authenticity and sustainability of cocoa and chocolate products: a challenge for the chocolate industry. Critical Reviews in Food Science and Nutrition 62(2):475-489.

Ren Y, Yu X, Xing H, Tretyakova IN, Nosov AM, Yang L, Shen H (2022). Interaction of Subculture Cycle, Hormone Ratio, and Carbon Source Regulates Embryonic Differentiation of Somatic Cells in *Pinus koraiensis.* Forests 13(10):1-12.

Shah D, Sajjad N, Ali R, Nazir N, Hassan S, Shah S (2019). Sugar regulates plant growth and development under in vitro conditions. Plant Signaling Molecules pp. 257-268.