European Journal of Biotechnology and Bioscience
ISSN: 2321-9122; Impact Factor: RJIF 5.44
Received: 26-03-2020; Accepted: 27-04-2020; Published: 12-06-2020
www.biosciencejournals.com
Volume 8; Issue 2; 2020; Page No. 19-26

Mineral salts and lifting of recalcitrance to the induction of somatic embryos in cacao

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Abstract
In the cocoa tree (*Theobroma cacao* L.), certain elite genotypes have shown in standard study conditions an absence or a very low response to the induction of somatic embryos. This is the case of the recalcitrant genotypes C8 and C15, partially recalcitrant genotypes C14 and C16. This study aims to improve the production of somatic embryos of recalcitrant genotypes. Staminodes and petals excised from the immature buds of the five genotypes C1, C8, C14, C15 and C16 were used as plant material. The C1 genotype is embryogenic under standard conditions. These floral explants were cultured on different media containing different type and concentration of mineral salts such as potassium sulphate (K_2SO_4) at concentrations 18, 27, 36 and 45 mM and magnesium sulphate ($MgSO_4$) whose concentrations used are 5, 10, 15 and 20 mM.
Callus induction was obtained in the five (05) genotypes at percentages ranging from 50 to 100% with staminodes and from 61 to 100% with petals on all media. The transfer of the callogenic explants on the developmental medium allowed the induction of embryogenic callus and somatic embryos after 84 days only with petal explants.
The percentage of embryogenic callus (PEC) and the number of somatic embryo (NSE) obtained with different concentrations of mineral salts ranged from 0 to 42.63% and from 0 to 23.53 respectively. The non-recalcitrant genotype C1 performed well on all media.
The most important PEC and NSE were obtained with two mineral salts concentrations, 27 mM K_2SO_4 and 15 mM MgSO4 for all genotypes. The use of 27 mM K_2SO_4 give for C1: 41.53% and 21.76%, for C8:35.50% and 19.10, for C14: 39.03% and 19.53, for C15: 40.50% and 20.70 and for C16; 39.50% and 19.96 of PEC and NSE respectively. The use of 15 mM $MgSO_4$ give for genotype C1: 42.63% and 23.53), for C8: 36.63% and 19.15, for C14: 40.68% and 20.08, for C15; 40, 81% and 20.10 and for C16: 40.01% and 20.98. Potassium sulphate (K_2SO_4) at 27 and magnesium sulphate ($MgSO_4$) at 15 mM are the concentrations of the most suitable mineral salts to overcome recalcitrance of cocoa genotypes.

Keywords: recalcitrant, cocoa, mineral salts, somatic embryogenesis

Introduction
The cocoa tree (*Theobroma cacao L.),* a species of the humid tropics, is mainly grown for its beans, which produce the cocoa powder used to make chocolate (Koné *et al*., 2019) [13].
Global demand for cocoa continues to increase due to economic growth and public awareness of the benefits for chocolate consumption.
The work done on the cocoa bean has shown that it has virtues on the stimulation of the immune system, the improvement of digestion and cellular detoxification. Cocoa products also have antimicrobial and antioxidant properties beneficial to human health (Sarmadi *et al.,* 2012; Yapo *et al*., 2013) [22, 24].
Cocoa production is important for many cocoa-producing countries in order to maintain export stability and ensure the continuity of supply of industrial raw materials for the chocolate industries.
Cocoa plays an important economic role as a source of foreign exchange for many tropical countries, including Côte d'Ivoire. It is grown by about 6 million farmers globally, and livelihoods of more than 40 million people depend on cocoa (ICCO, 2012; Conseil Café-Cacao, 2014; Beg *et al.* 2017) [9].
However, demand remains unfulfilled despite the fact that its world production has been increasing considerably for several years (ICCO, 2016) [10]. This is due to the fact that in the agricultural sector, cocoa farming is affected by several pathogens, to which is added the aging of orchards, high cost of inputs, use of poor-quality plant material, inappropriate agronomic techniques. To overcome these constraints, and increase production, elite genotypes, high producers have been created for extension to allow the creation and renewal of plantations (Sonwa, 2002) [22]. However, the mode of reproduction of the plant, sexed and allogane, does not make it possible to obtain a homogeneity of the improved material. Since cocoa is naturally pollinated, seed cocoa planting material generally has a very heterogeneous genetic background. As a result, the agronomic performance of seed-derived cocoa plantations is highly variable (Li *et al.*, 1998) [15]. To overcome these difficulties, traditional vegetative propagation techniques of plant material, such as cuttings and grafting, have been advised.
These techniques are not very competent (Figueira and Janick, 1993) [8] because of the poor root and air systems of plants associated with slow processes. One of the appropriate solutions to all these problems is somatic embryogenesis. This technique is used to carry out the propagation of desirable cocoa genotypes. However, this

method presents some difficulties in the cocoa tree because of the recalcitrance of certain genotypes. This recalcitrance is expressed by the variation of the somatic embryo rate from one genotype to another, often with very low or zero somatic embryo levels. The lifting of this recalcitrance requires the development of a new protocol. Moreover, several studies on the somatic embryogenesis of cacao have revealed a variation of callogenic and embryogenic responses according to genotypes. The work on somatic embryogenesis developed by Li *et al.* (1998) [15], without being exhaustive, have shown a variation of the responses of 19 cocoa genotypes to somatic embryogenesis. Used as explants, the response of staminodes to callogenesis ranged from 1% to 100% and the number of somatic embryos per explant ranged from 1 to 46 during this work.

Another somatic embryogenesis protocol was developed by Lopez-Báez *et al.* (2001) [16] using an induction medium comprising the macro and microelements of Murashige and Skoog (1962) [20] (MS) supplemented with different types and concentrations of phytohormones (2,4-dichlorophenoxyacetic acid or 2,4-acid, 5-trichlorophenoxyacetic, and kinetin), out of 12 different genotypes. Once again, a variation of the responses according to the genotypes was found.

The elite genotypes, from which genotypes C1, C8, C14, C15 and C16 originate, are characterized by good genotypic performance (high productivity) and resistance to diseases. However, low levels of embryogenic callus were produced by theses genotypes with C8 (0.03%), C14 (14.32%) Kouassi *et al*., 2018) [15] and C16 (8.85%) (Kouassi *et al*., 2017b) [13]. To improve somatic embryo production, it is important to develop new protocols which can increase induction of somatic embryos rates. The objective of the present study is to improve the production of somatic embryos of the recalcitrant genotypes of *Theobroma cacao*. To achieve this goal, two types of explants, petals and staminodes, and differents concentrations of mineral salts were used.

Materials and Methods

- **Plant material**

The plant material consisted of staminodes and petals excised from immature flower buds of the cocoa genotypes coded C1, C8, C14, C15 and C16. The flower buds were taken from the field of cocoa experimentation of the International Center for Agroforestry Research (ICAFR) of Abidjan (Côte d'Ivoire). Five cocoa genotypes were chosen according to their response to somatic embryogenesis. The genotype coded C8 originated from Trinidad and Tobago is recalcitrant to somatic embryogenesis. Genotypes coded C15, recalcitrant, C14 and C16, partially recalcitrant and C1 which is embryogenic, are from Côte d'Ivoire.

- **Methods**
- **Collection and disinfection of flower buds**

In the morning before 9 am, 4 to 5 mm long flower buds were collected and placed in jars and stored in a cooler containing ice and sent to the laboratory. Then, buds were disinfected under a laminar flow hood in sterile conditions, first by soaking them in a 1% (w/v) calcium hypochlorite solution, followed by three rinses in sterile distilled water. After that, they were re-dipped in 70% alcohol solution for 30 seconds and rinsed thoroughly three times with sterile distilled water. Finally, they were immersed a second time in the same solution of calcium hypochlorite 1% (m/v) with three drops of Tween 20 for 10 min and then rinsed thoroughly three times with sterile distilled water.

- **Isolation and culture of explants**

Petals and staminodes were isolated from disinfected flower buds after dissection with a scalpel blade. Petals and staminodes were placed on the callus induction medium with 15 samples per explant (15 petals and 15 staminodes) and per petri dish under a laminar flow hood for sterile conditions.

- **Composition of culture media**

The media used to induce callus (IC medium) and their development into somatic embryos (ED medium) are composed of macro and micro elements of DKW (Driver & Kuniyuki, 1984) [6]. For testing their effect on callus induction and their ability to induce embryogenic callus, two mineral salts: potassium sulphate (K_2SO_4) and magnesium sulphate ($MgSO_4$) were added to DKW medium at different concentrations 18, 27, 36 and 45 for potassium sulphate (K_2SO_4) and 5, 10, 15 and 20 mM for magnesium sulphate ($MgSO_4$). Thus, a total of eight induction media varying by the nature and concentration of mineral salts were prepared. Cytokinin used was kinetin at 1.162 µM. The control medium contained 9 mM potassium sulphate (K_2SO_4) and 3 mM magnesium sulphate ($MgSO_4$). Four (4) weeks after culturing in callus induction medium, explants were transferred to embryo development medium (ED medium) described by Li *et al*., (1998) [15]. The transfer of the explants on this medium was done every 28 days.

- **Culture conditions**

The pH of the media was adjusted to 5.8 for IC medium and 5.7 for ED medium using 1N NaOH or HCl solutions. Media was solidified with 2 g/L phytagel before being sterilized by autoclaving for 20 min at 121 ° C and 1 bar. After sterilization, these culture media were dispensed due to 15 ml in sterile Petri dishs of 90 mm diameter under a laminar flow hood. Cultures were incubated on continuous darkness conditions in the culture chamber at 24 ± 1 ° C with a relative humidity of 70%. Petri dishes were arranged on rows according to a completely randomized dispositif experimental.

- **Variables used to assess the success of somatic embryogenesis**

Twenty-eight (28) days after induction, percentage of callogenic explants (PCE) was evaluated. Eighty four (84) days after induction, percentage of embryogenic calli (PEC) and average number of somatic embryos (NSE) were evaluated on ED medium. These three parameters were calculated according to the following formulas:

- induction evaluation was made on medium induction of callus (IC), after 28 days of culture;
- somatic embryos were assessed on ED medium by the percentage of embryogenic calli 84 days after explants induction.

The percentage of callogenic explants (PCE), the percentage of embryogenic calli (PEC) and the mean number of somatic embryos (NSE) per explant were given respectively by the following formulas:

$$PCE = \frac{\text{Number of explants that induced calli}}{\text{Total number of explants cultured}} X\ 100$$

$$PEC = \frac{\text{Number of callus that induced embryos}}{\text{Number of explants that induced callus}}\ X\ 100$$

$$NSE = \frac{\text{Number of induced embryos}}{\text{Number of callus that induced embryos}}$$

- **Statistical analysis of the data**

Results were subjected to analysis of variance (ANOVA) with Statistica 7.1 software. For unequal numbers, analysis of variance across the generalized linear model (GLM) was adopted. When a significant difference was observed between averages, the Newman-Keuls multi-range test at a 5% threshold was used to separate the averages. Rate evaluation was based on a transformation Arc sin (p = proportion) before performing ANOVA tests.

Results

- **Evaluation of callus induction in the various media after 28 days**

Table 1 shows the percentage of callus induction from staminode and petal explants after 28 days of culture on the various induction media.

The analysis of the results of this table after the statistical analyzes shows that with the C1 and C14 genotypes, there is no significant difference between the callus induction percentages from the staminode and petal explants on the medium supplemented with different types and concentrations of mineral salts. These callus induction percentages were between 90 and 100% for C1 and between 92 and 100% for C14. Moreover, these percentages are identical statistically to that of the control medium. The contribution of mineral salts did not improve or reduce the responses to the callogenesis of genotypes C1 and C14.

With the C8, C15 and C16 genotypes, mineral salts improved the response to callogenesis. A beneficial effect of using potassium sulfate mineral salts and magnesium sulfate was clearly observed on the induction of callus for genotype C15.

However, the addition of $MgSO_4$ mineral salt at a concentration of 5 mM resulted in a reduction in callus induction percentages of genotypes C8 (staminodes 75.08% ± 0.17) and C16 (the staminodes 74.33% ± 1.01). These percentages are statistically identical to that of the control in genotype C16 at the explant staminode 75.38% ± 20.02.

In these genotypes, callus percentages ranged from 74% to 100% with staminodes and from 92% to 100% with petals. The petals yielded calli at slightly higher percentages than staminodes in all genotypes.

- **Evaluation of calli and somatic embryos in the various media after 84 days**

The calli obtained, transferred to the embryonic development medium (EDM medium), gave embryos 84 days after the initiation of the cultures. The percentage of embryogenic callus (PEC) and the average number of embryos (NSE) produced per explant are shown in Table 2. Only the petal explants induced somatic embryos. The percentage of embryogenic callus (PEC) of the different genotypes ranged from 0 to 42.63% ± 0.19 and the average number of embryos (NSE) from 0 to 23.53 ± 0.09 with all of the salts minerals. No embryogenic or embryo callus was induced on the media containing 5 and 20 mM $MgSO_4$ and 45 mM K_2SO_4 for the genotype C8 and on media containing 5 mM $MgSO_4$ and 45 mM K_2SO_4 for the genotypes C14, C15 and C16. Only the genotype C1 produced embryogenic calli and embryos on all media used.

However, the percentage of embryogenic callus (PEC) and the average number of embryos (NSE), were improved in the presence of 27 mM K_2SO_4 (41.53% ± 2.59 and 21.76 ± 2.50), 10 mM $MgSO_4$ (39.97% ± 0.40 and 19.87 ± 0.04), 15 mM $MgSO_4$ (42.63% ± 0.19 and 23.53 ± 0.09) and 20 mM $MgSO_4$ (40, 03% ± 0.09 and 20.53 ± 1.04) for the genotype C1.

For the genotype C8, the highest PEC and NSE were produced on media enriched with 27 mM K_2SO_4 (35.50% ± 1.09 and 19.10 ± 1.89) and 15 mM MgSO4 (36.63% ± 0.59 and 19.15 ± 0.89)

For the C14 genotype, high PEC and NSE are obtained with media containing 27 mM K_2SO_4 (39.03% ± 1.59 and 19.53 ± 0.39) and 10 mM $MgSO_4$ (39.78% ± 0, 64 and 19.67 ± 1.63), 15 mM $MgSO_4$ (40.68% ± 0.93 and 20.08 ± 0.14), and 20 mM $MgSO_4$ (39.83% ± 1.02 and 19.87). ± 0.19).

Concerning the genotype C15, the high values of PEC and NSE were obtained on media supplemented with 27 mM of K_2SO_4 (40.50% ± 0.04 and 20.70 ± 0.21), of 10 mM (39.01% ± 0.04 and 19.98 ± 0.08) and 15 mM $MgSO_4$ (40.81% ± 0.03 and 20.10 ± 0.15).

For the genotype C16, high PEC and NSE were obtained on media containing 27 mM K_2SO_4 (39.50% ± 0.04 and 19.96 ± 0.21), 10 mM $MgSO_4$ (39.18% ± 0), 05 and 19.80 ± 0.45) and 15 mM $MgSO_4$ (40.01% ± 0.06 and 20.98 ± 0.02).

The highest PEC and NSE were obtained with media supplemented with 27 mM K_2SO_4 in all genotypes with C1 (41.53% ± 2.59 and 21.76 ± 2.50), C8 (35.50% ± 1, 09 and 19.10 ± 1.89) C14 (39.03% ± 1.59 and 19.53 ± 0.39), C15 (40.50% ± 0.04 and 20.70 ± 0.21), C16 (39.50% ± 0.04 and 19.96 ± 0.21) as well as with the medium enriched with 15 mM $MgSO_4$: C1 (42.63% ± 0.19 and 23.53 ± 0.09), C8 (36.63% ± 0.59 and 19.15 ± 0.89), C14 (40.68% ± 0.93 and 20.08 ± 0.14), C15 (40, 81% ± 0.03 and 20.10 ± 0.15) and C16 (40.01% ± 0.06 and 20.98 ± 0.02). Figure 1 shows embryogenic calli developed by the C8 et C15 genotype petals induced on medium supplemented with 15 mM of MgSO4 and 27 of mM K_2SO_4

Table 1: Percentage of callus produced from staminode and petal explants as a function of mineral salt concentrations and genotype used

Genotypes	Mineral Salt	Concentration (mM)	Percentage of Callogenic Explant (PCE) (%)	
			Staminodes	Pétales
C1	Control (K_2SO_4 + $MgSO_4$)	9 +3	90.80 ± 0.01 ^{ab}	93.34 ± 0.06 ^{ab}
	K_2SO_4	18	93.76 ± 0.02 ^{ab}	94.43 ± 0.08 ^{ab}
		27	100 ^{a}	100 ^{a}
		36	98.10 ± 02.00 ^{a}	100 ^{a}
		45	94.94 ± 0.37 ^{ab}	94.02 ± 0.07 ^{ab}

	$MgSO_4$	5	92.00 ± 0.00 ab	93.80 ± 0.08 b
		10	93.64 ± 0.46 ab	93.00 ± 0.00 ab
		15	100 a	100 a
		20	94.00 ± 0.00 ab	95.00 ± 0.00 ab
C8	Control (K_2SO_4 + $MgSO_4$)	9 +3	93,42 ± 4029 ab	94.29 ± 01.23 ab
	K_2SO_4	18	94,09 ± 1,22 ab	93.29 ± 01.03 ab
		27	100 a	100 a
		36	100 a	100 a
		45	94.87 ± 0,72 ab	94.87 ± 0,72 ab
	$MgSO_4$	5	75.08 ± 0,17 b	84.00 ± 0,00 b
		10	92.66 ± 1,02 ab	94.66 ± 0,00 ab
		15	100 a	100 a
		20	100 a	100 a
C14	Control (K_2SO_4 + $MgSO_4$)	9 +3	92.33 ± 0,04 ab	92.33 ± 0,04 ab
	K_2SO_4	18	92.00 ± 0,00 ab	94.55 ± 0,04 ab
		27	100 a	100 a
		36	93.40 ± 0,10 ab	100 a
		45	93.39 ± 0,08 ab	94.85 ± 0,02 ab
	$MgSO_4$	5	93.88 ± 0,11 ab	92.00 ± 0,00 ab
		10	92.21 ± 0,87 ab	94.00 ± 0,00 ab
		15	100 a	100 a
		20	94,00 ± 0,00 ab	94.55 ± 0,04 ab
C15	Control (K_2SO_4 + $MgSO_4$)	9 +3	94.64 ± 0,06 ab	94.57 ± 0,02 ab
	K_2SO_4	18	93,16 ± 0,68 ab	94.68 ± 0,20 ab
		27	100 a	100 a
		36	100 a	100 a
		45	94.00 ± 00,00 ab	100 a
	$MgSO_4$	5	92.00 ± 00,00 ab	94.95 ± 0,03 ab
		10	93.95 ± 0,09 ab	94.00 ± 00,00 ab
		15	100 a	100 a
		20	100 a	100 a

In the same column, the averages followed by the same letter are statistically equal (test of Newman-Keuls to the threshold of 5%); Average ± standard deviation;K_2SO_4: potassium sulphate;$MgSO_4$: magnesium sulphate; Observations were made after 28 days of culture

Table 2: Percentage of callus produced from staminode and petal explants as a function of mineral salt and genotype concentrations

Genotypes	Mineral Salt	Concentration (mM)	Percentage of Callogenic Explant (PCE) (%)	
			Staminodes	Petals
C16	Control (K_2SO_4 + $MgSO_4$)	9 +3	75.38 ± 20.02 b	100 a
	K_2SO_4	18	90.88 ± 21.80ab	100 a
		27	100 a	100 a
		36	100 a	100 a
		45	100 a	100 a
	$MgSO_4$	5	74.33 ± 1.01b	92.44 ± 1.92ab
		10	100 a	100 a
		15	100 a	100 a
		20	100 a	100 a
Statistical tests		P	<0.001	<0.001
		F	30.31	29.68

In the same column, the averages followed by the same letter are statistically equal (test of Newman-Keuls to the threshold of 5%); Average ± standard deviation;K_2SO_4: potassium sulphate;$MgSO_4$: magnesium sulphate; Observations were made after 28 days of culture.

Table 3: Somatic embryos induction as a function of mineral salt concentrations and genotypes used

Genotypes	Mineral salt	Concentration (mM)	Somatic embryo induction by petal explants	
			Induction rate embryogenic callus	Mean number of somatic embryos
C1	Control (K_2SO_4 + $MgSO_4$)	9+3	30.90 ± 0.40 ab	15.46 ± 0.40 ab
	K_2SO_4	18	32.97 ± 0.44 ab	16.49 ± 0.44 ab
		27	41.53 ± 0.59 a	21.76 ± 0.50 a
		36	30.68 ± 0.64 ab	15.35 ± 0.61 ab
		45	18.39±0.14bc	9.90±1.01bc
	$MgSO_4$	5	25.00±00.00b	12.40±0.22b
		10	39.97 ± 0.40 a	19.87 ± 0.04 a
		15	42.63 ± 0.19 a	23.53 ± 0.09 a
		20	40.03 ± 0.09 a	20.53 ± 1.04 a
	Control (K_2SO_4 + $MgSO_4$)	9 + 3	10.97 ± 0.04 bc	5.22 ± 0.22 bc
	K_2SO_4	18	08.07 ± 0.14 bc	4.22 ± 0.52 bc

C8		27	35.50 ± 1.09 [a]	19.10 ± 1.89 [a]
		36	12.00 ± 0.00 [bc]	6.00 ± 0.00 [bc]
		45	00.00±00.00[c]	00.00±00.00[c]
	$MgSO_4$	5	00.00±00.00[c]	00.00±00.00[c]
		10	20.00±00.00[b]	11.40±0.22[b]
		15	36.63 ± 0.59 [a]	19.15 ± 0.89[a]
		20	00.00±00.00[c]	00.00±00.00[c]
C14	Control (K_2SO_4 + $MgSO_4$)	9 + 3	30.05±0.02[ab]	15.01±0.37[ab]
	K_2SO_4	18	32.00 ± 0.00 [ab]	15.68 ± 1.20 [ab]
		27	39.03 ± 1.59 [a]	19.53 ± 0.39 [a]
		36	30.58 ± 1.64 [ab]	14.97 ± 3.64 [ab]
		45	00.00±00.00[c]	00.00±00.00[c]
	$MgSO_4$	5	14.97 ± 0.14 [bc]	7.22 ± 0.20 [bc]
		10	40.68 ± 0.93 [a]	20.08 ± 0.14 [a]
		15	39.78 ± 0.64 [a]	19.67 ± 1.63 [a]
		20	39.83 ± 1.02 [a]	19.87 ± 0.19 [a]

In the same column, the averages followed by the same letter are statistically equal (test of Newman-Keuls to the threshold of 5%); Average ± standard deviation;K_2SO_4: potassium sulphate; $MgSO_4$: magnesium sulphate. Observations were made after 84 days of culture

Table 4: Somatic embryos induction as a function of mineral salt concentrations and genotypes used

Genotypes	**Mineral Salt**	**Concentration (mM)**	**Somatic embryo induction by petal explants**	
			Induction rate embryogenic callus	Mean number of somatic embryos
C15	Control (K_2SO_4 + $MgSO_4$)	9 + 3	31.97 ± 0.44[ab]	15.27 ± 0.04 [ab]
	K_2SO_4	18	30.73±0.03[ab]	14.90±0.16[ab]
		27	40.50±0.04[a]	20.70±0.21[a]
		36	32.88±0.05[ab]	14.68 ± 3.64 [ab]
		45	00.00±00.00[c]	00.00±00.00[c]
	$MgSO_4$	5	00.00±00.00[c]	00.00±00.00[c]
		10	39.01±0.04[a]	19.98±0.08[a]
		15	40.81±0.03[a]	20.10±0.15[a]
		20	18.48±0.05[bc]	09.50±0.40 [bc]
C16	Control (K_2SO_4 + $MgSO_4$)	9 + 3	30.07±1.44 [ab]	14.07 ± 1.04 [ab]
	K_2SO_4	18	32.73±0.03[ab]	15.80±0.16[ab]
		27	39.50±0.04[a]	19.96±0.21[a]
		36	33.01±0.07[ab]	15.28±0.08[ab]
		45	00.00±00.00[c]	00.00±00.00[c]
	$MgSO_4$	5	00.00±00.00[c]	00.00±00.00[c]
		10	39.18±0.05[a]	19.80±0.45[a]
		15	40.01±0.06[a]	20.98±0.02[a]
		20	26.88±0.05[b]	13.80±0.45[b]
Tests statistiques	P		<0.001	<0.001
	F		9.11	11.01

In the same column, the averages followed by the same letter are statistically equal (test of Newman-Keuls to the threshold of 5%); Average ± standard deviation;K_2SO_4: potassium sulphate; $MgSO_4$: magnesium sulphate. Observations were made after 84 days of culture

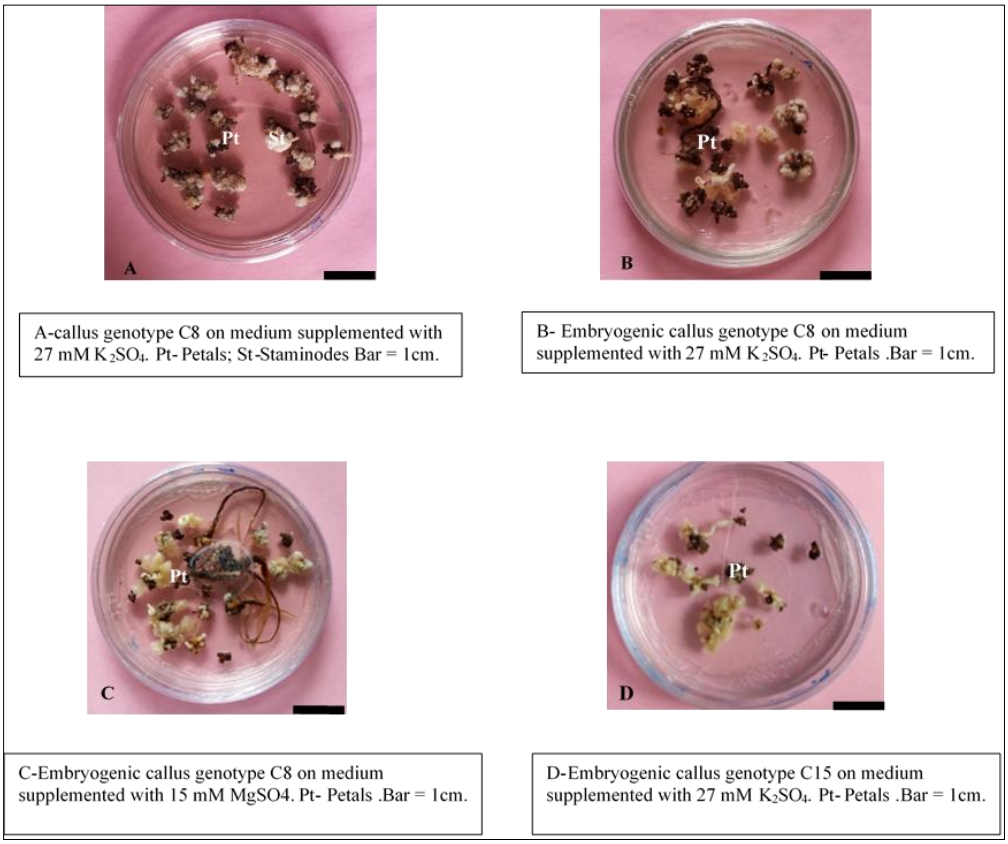

Fig 1: Formation of Embryogenic callus from flower explants.

Discussion

The response of explants of *Theobroma cacao* to somatic embryogenesis is dependent on genotype.

In order to develop, a protocol applicable to certain genotypes deemed recalcitrant to somatic embryogenesis, `two types of explants, staminodes and petals of five (05) genotypes, from the most embryogenic (C1) to at least embryogenic, C14, C16, C15 and C8 were used as plant material. A total of eight (08) induction media with four (04) concentrations of two (02) inorganic salts that are potassium sulfate (K_2SO_4) at concentrations 18, 27, 36 and 45 mM and magnesium sulfate ($MgSO_4$) at concentrations 5, 10, 15 and 20 mM were carried out for the avoidance of recalcitrance in the studied of cocoa genotypes.

The use of mineral salts in the induction medium did not have a negative impact on the callogenesis responses of staminode and petal explants in the five (05) genotypes C1, C14, C15, C8 and C16 in the most cases. The various mineral salts used, improved the responses to callogenesis, particularly in the most embryogenic C1, the partially recalcitrant C14 and the recalcitrant C15 genotypes. A reduction of the rate of callus induction was however observed when the concentration of 5 mM $MgSO_4$ was added to the culture medium with the two types of explant (staminodes and petals) for the most recalcitrant genotype C8 and the partially recalcitrant genotype C16.. These results show that the responses to callogenesis varied according to the genotype of cacao, the type and concentration of the mineral salt used.

The petal and staminode explants of the genotypes tested allowed, however, the induction of callus with levels higher than 74% on all media. These results are similar to those obtained by Minyaka *et al.* (2008) [18] who showed that petal and staminode explants respond to callus production with the mineral salts K_2SO_4 and $MgSO_4$.

In addition, the petals had higher callogenesis percentages than staminodes for the five (05) genotypes evaluated. This response shows that petals respond better to callogenesis than staminodes with mineral salts. Similar results have been obtained by Da Silva *et al*. (2008) [5] and Koné *et al*. (2019) [13] who found that petals are better adapted to callus formation than staminodes in T. cacao. Staminodes and petals could therefore indistinctly of genotype provide high percentages of callogenic explants with a maximum for the petals. These results, however, contradict those of Bahoya (2012) [2]. Indeed, these authors have shown that staminodes of genotypes named genotype 1, genotype 2 and genotype 3 used during their work were more conducive to callogenesis than petals. This shows that the response to callogene in addition to being dependent genotype is also a function of the explant.

These authors have worked on genotypes that are different from those used on our study. In our study, the percentage of embryogenic explants and the average number of somatic embryos were obtained with the callus derived from the petal explants in the five (05) genotypes C1, C8, C14, C15 and C16 of cacao and not with staminode explants. This result shows that the protocol developed in this study is more adapted to petal explants and the response to somatic embryogenesis also depends on the genotype. These results are consistent with those of Kouassi *et al*. (2017a) [13] and Eliane *et al*. (2019) which revealed that petals are better adapted to somatic embryo production than staminodes. However, contrary to our observations, numerous studies have reported the superiority of embryogenic performance of staminodes to petals (Bahoya, 2012) [2].

With mineral salts, inhibition of embryo production was observed with the concentration of 45 mM K_2SO_4 for the

genotypes C8, C14, C15 and C16 and with 5 mM $MgSO_4$ for the genotypes C8, C15 and C16. This shows that too low concentrations are sometimes insufficient to lift the recalcitrant however when they are too high they cause toxicity. These concentrations of mineral salts are too high for K_2SO_4 and too low for $MgSO_4$ to allow the lifting of recalcitrance. Inhibition of somatic embryo production of *Theobroma cacao* would be due, in addition to the insufficiency or toxicity of certain compounds used, to a strong secretion of ethylene and polyphenols by the explants of certain genotypes according to Fang *et al*. (2014) [7] and Minyaka *et al.* (2017) [20]. Polyphenols by their oxidation act as inhibitors of metabolic or antagonistic reactions of growth substances. The work of d'Alemanno *et al.* (2008) [1] and Boutchouang *et al.* (2016) [4] conducted on cacao flowers showed that they would synthesize a significant quantity of phenolic compounds. Indeed, these compounds intervene in the defense of plants (Kouassi *et al.*, 2017a; Minyaka *et al.*, 2017) [13, 20]. When the plant is subjected to mechanical injury, simple phenols are synthesized and the peroxidase activity characteristic of the lignifying tissues is stimulated. Phenolic secretions and other exudates in plant tissue culture systems inhibit the development of the callogenic explant in embryos (Kouassi *et al.*, 2017a) [13]. Among the inorganic salts used in the induction medium, the concentration of 27 mM potassium sulfate (K_2SO_4) and of 15 mM magnesium sulfate ($MgSO_4$) taken individually, gave the the most important percentages of embryogenic callus and of average numbers of embryos for all genotypes C1, C8, C14, C15 and C16 regardless of their level of recalcitrance. This response shows that concentrations of 27 mM potassium sulphate (K_2SO_4) and 15 mM magnesium sulphate ($MgSO_4$) compensate the deficiencies in mineral salts, (potassium sulphate (K_2SO_4) and of magnesium sulphate ($MgSO_4$)); in the callus induction of these genotypes and making them able to produce embryos. According to Minyaka *et al.* (2010) [19], a deficiency of potassium sulphate (K_2SO_4) or magnesium ($MgSO_4$) has a negative influence on the production of somatic embryos, resulting in a gradual loss of embryo production during culture and thus explains the fact that these metabolites, K_2SO_4 or $MgSO_4$, are essential for the good development of plants. The use of such concentrations allows compound to fill the deficit of minerals and avoid the development of medium often complex with different minerals.

Conclusion

Results of the current study showed that somatic embryogenesis of *Theobroma cacao* genotypes are genotype and explant dependent. This study set up an improved protocol compared to previous works in terms of embryo production for cocoa.

The results obtained also revealed that the elimination of recalcitrance even for recalcitrant genotypes is possible with certain concentrations of mineral salts. This lifting of recalcitrance was carried out with 27 mM potassium sulfate (K_2SO_4) and 15 mM magnesium sulfate ($MgSO_4$).

These two concentrations gave the best percentages of embryogenic explants and the highest average numbers of embryos for the five genotypes tested. These two concentrations produce embryogenic calluses with the recalcitrant genotypes C8 and C15, partially recalcitrant C14 and C16 at the same level as the most embryogenic C1 genotype.

We can therefore say that the dependence of the genotype on somatic embryogenesis has been mastered for *Theobroma cacao*.

These different defined concentrations constitute the optimal concentrations, which supplement the data of the literature for the lifting of the recalcitrance of the genotype for this species.

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DOI: http://dx.doi.org/10.4314/ijbcs.v7i5.1