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Use of Im medium to overcome recalcitrance to somatic embryogenesis of improved cocoa genotypes (*Theobrama cacao* L.)

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Abstract

In vitro recalcitrance is the inability of plant cells, tissues and organs to respond to tissue culture manipulations. In cocoa (*Theobroma cacao* L.), recalcitrance is a limiting factor in tissue culture for the production of somatic embryos. This study aims to improve the production of somatic embryos by *In Vitro* culture of improved genotypes deemed recalcitrant to somatic embryogenesis. "Im"medium was used to induce the production of embryos of four enhanced and recalcitrant somatic embryogenesis genotypes encoded C8, C14, C15 and C16. The plant material consists of explants of petals and staminodes. The best results, which are characterized by levels of cal embryogenesis and the number of somatic embryos of 16% and 07, respectively, were obtained in the C14 genotype. This study showed that it is possible to produce somatic embryos in these improved varieties, thus eliminating the recalcitrance of these genotypes to somatic embryogenesis. The availability of improved material consisting of identical plants will allow the creation of more homogeneous plantations with high yields.

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Introduction

The cocoa tree (*Theobroma cacao* L.) is a tree that comes from the Amazon basin. This plant with multiple virtues is mainly grown for its beans. They are the main raw material for the chocolate industry (Koné *et al.*, 2021).

Despite its importance, cocoa farming is facing enormous difficulties, which can be explained by the attacks of several pathogenic and parasitic agents, the aging of the orchards, the high cost of inputs, the non-availability, and the under-use of plant material. improved (Koné *et al.*, 2021).

To overcome these difficulties, several methods have been considered. Conventional techniques for the multiplication of plant material, such as cuttings and grafting, have been proposed. Unfortunately, these vegetative horticultural propagation techniques have several drawbacks that do not allow their easy application (Koné *et a*l., 2021).

To overcome all these difficulties and obtain homogeneous material in abundant quantity, in vitro culture by somatic embryogenesis was considered. It is a method that makes it possible to produce a given genotype in sufficient quantity and in a homogeneous manner.

This method of in vitro propagation has advantages over conventional methods of propagation and large-scale production of woody plants while ensuring stability and genetic integrity (Kouassi *et al.*, 2017a; Eliane *et al.*, 2019; Koné *et al.*, 2019). Regenerators, i.e., plants regenerated from somatic embryos, in addition to being genetically identical to each other, behave like seedlings (Jane *et al.*, 2017; Kouassi *et al.*, 2018).

However, this method presents some problems in the cocoa tree because of the recalcitrance of certain genotypes. This recalcitrance is reflected in the variation in the rate of somatic embryos from one genotype to another, often with very low or even zero somatic embryo rates. Overcoming this recalcitrance requires optimizing existing protocols or developing new ones. The works of Koné *et al.* (2019; 2021) have shown that mineral salts and carbon sources taken individually make it possible to resolve this recalcitrance. The general objective of this study is to test the effect of the Im medium on lifting the recalcitrance of recalcitrant genotypes to allow their production in sufficient quantity and their large-scale distribution.

Materials and methods

Plant material

The plant material consists of petals and staminodes taken from immature flower buds of four improved cocoa genotypes coded C8, C14, C15 and C16 all partially or very recalcitrant to *in vitro* culture by somatic embryogenesis. The flower buds were taken from Adiopodoumé in the experimental field of the International Agroforestry Research Center (ICRAF) of Abidjan (Côte d'Ivoire).

Methods

Collection and disinfection of flower buds

In the morning before 9 a.m., 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.

Composition and preparation of cultural media

Two types of medium were used to induce calli and somatic embryos during this experiment. The callus induction media (medium I) and the embryo development medium (EDM medium) consisted of the mineral solution of DKW (Driver and Kuniyuki Walnut, 1984). Induction medium: Different

concentrations of glucose, mineral salts, and auxin were added to the induction medium to test their influence on the ability of explants to induce calli and embryos. The concentrations used for each of the three carbon sources are 30 g/L, mineral salts are 27 mM de K_2SO_4 and 15 mM de $MgSO_4$ and 18 µM of 2, 4, 5-trichlorophenoxyacetic acid (2, 4, 5T). In total, a single medium was prepared comprising the different concentrations mentioned above.

Development medium: The embryo development medium (EDM) is the same for all concentrations used. It is devoid of phytohormones and mineral salts and contains 30g /L of sucrose and 1g /L of glucose.
The pH of the medium was adjusted to 5.8 for the induction media (medium I) and 5.7 for the embryo development medium (EDM) using NaOH or 1N HCl solutions. The media were solidified with Phytagel (2g /L). They were then sterilized in an autoclave for 20 min at 121 ° C and 1 bar. After sterilization, these culture media were dispensed at the rate of 15 ml in sterile Petri dishes under a laminar flow hood.

Culture of explants and culture conditions

The methodology adopted during this study is that described by Kouassi *et al.* (2017b). It consisted of inducing calluses on the induction medium from the petals and staminodes from flower buds. The petal and staminode explants were isolated after dissection of the sterilized flower buds using a scalpel blade. These explants were placed on the callus induction medium at the rate of 15 explants per petri dish under a laminar flow hood. Two weeks later (i.e., 28 days after culturing the explants), the growing explants were transferred to the embryo development medium (EDM). After inoculation of the explants on the different culture media, the cultures were incubated in continuous darkness in the culture chamber at a temperature of 24 ± 1 ° C and relative humidity of 70%. Petri dishes were arranged in rows according to a completely randomized arrangement.

Variables evaluated

Twenty-eight (28) days after induction, the percentage of callogenic explants (PCE) was evaluated. Eighty-four (84) days after induction, the percentage of embryogenic calli (PEC) and the mean number of somatic embryos (NSE) were evaluated on EDM 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 EDM 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:

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

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

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

Statistical analysis

Results were subjected to analysis of variance (ANOVA) with Statistica 7.1 software. For unequal numbers, an 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

Effect of media "Im "on callus production in different genotypes

Table 1 presents the percentages of staminode explants and callus petals after twenty-eight (28) days of culture on the "Im" callus induction medium. There are significant differences between the percentages of calogenic explants of the different genotypes of cocoa trees ($p < 0.001$). The C15 genotype presented the best percentages of staminode explants (97.93% ± 0.50) and petals (98.93 ± 0.30)

calogens. The lowest callogenic explant percentages were observed in the C8 genotype (78.18% ± 1.09 for staminode explants and 90.84% ± 0.30 for petal explants). Elsewhere were observed between the percentages of staminode explants and calogenic petals at the 5% threshold. There are significant differences between the percentages of callogenic petal explants of the five genotypes studied. On the other hand, there are no significant differences between the percentages of explants of staminodes and callogenic petals of the C1, C14 and C16 genotypes.

Table 1. Percentage of calli produced from staminode explants and petals of the genotypes used on "Im" medium.

Génotypes		Percentage of callogenic explants (%)	
		Staminodes	Pétales
C1		90,04 ± 0,30 ab	95,84 ± 0,30 ab
C8		78,18 ± 1,09b	90,84 ± 0,30 b
C14		90,08 ± 0,90 ab	95,82 ± 9,05ab
C15		97,93 ± 0,50 a	98,93 ± 0,30 a
C16		90,20 ± 1,75 ab	95,90 ± 10,18 ab
Tests	P	< 0,001	< 0,001
statistiques	F	9,84	9,15

Moreover, the percentages of staminode explants and petals of the C15 genotype were higher than those of the C1, C8, C14 and C16 genotypes. Thus, the percentages of callogenic explants of staminodes (97.93% ± 0.50) and petals (98.93% ± 0.30) of the C15 genotype were followed by those of the explants of staminodes and petals of the genotypes C1 (90.04%± 0.30 and 95.84% ± 0.30), C14 (91.78%± 0.90 and 95.22% ± 9.05), C16 (90.20%± 1 .75 and 95.90% ± 10.18) and C8 (78.18% ± 1.09 and 90.84% ± 0.30). Overall, the percentages of staminode explants and callogenic petals varied from 78% to 97% and 90% to 98%, respectively.

Fig. 1A illustrates an example of calli developed from explants of petals and staminodes of the C8 genotype.

Table 2. Percentage of embryogenic calli and average number of somatic embryos produced by the petal explants of the genotypes used on the medium development of embryos from "Im" medium.

Génotypes		Percentage of embryogenic calli (%)	Average number of somatic embryos
C1		17,16±0,14 a	08,17±0,04 a
C8		04,16±0,13 b	1,09±0,20 b
C14		16,07±2,30 a	07,90±0,04 a
C15		09,07±0, 04 ab	03,04±0,34 ab
C16		10,11±0,14 ab	04,90±0,12 ab
Tests statistiques	P	< 0,001	< 0,001
	F	5,23	4,05

Effect of "Im" media *on embryo production in different genotypes*

The levels of embryogenic calli and mean numbers of somatic embryos developed on the embryo development medium (EDM) from the calli produced on the "Im" medium are presented in Table 2 after the analysis of variance. The results showed that only the petal explants produced embryogenic calli and somatic embryos. Significant differences ($p < 0.001$) between the levels of embryogenic calli were observed, as well as with the average numbers of somatic embryos of the different genotypes of cocoa trees. Only petal explants of all genotypes produced embryogenic calli and somatic embryos at varying

rates. Figs 1B, 1C, 1D and 1E illustrate an example of embryogenic calli developed from petal explants of the C8, C14, C15 and C16 genotypes.

The C14 genotype had the highest percentages of embryogenic calli (16.07% ± 2.30) and the average number of embryos (07.90 ± 0.04). The low percentages of embryogenic calli (04,16 % ± 0,13) and the average number of embryos somatic (04,16 % ± 0,13) were obtained at the genotype C8.

A significant difference was observed both between the percentage of embryogenic callus of the four genotypes and between their mean somatic embryo number.

On the other hand, no significant difference was observed between the different percentages of embryogenic calli as well as the different values of the average number of somatic embryos of the C15 genotypes comprising 09.07% ± 0.04 and 03.04 ± 0, 34 and C16 holding 10.11% ± 0.14 and 04.90% ± 0.12.

Discussion

The technique of somatic embryogenesis is not fully mastered in cocoa and the response of explants remains dependent on genotypes. In order to develop a reliable protocol suitable for genotypes recalcitrant to somatic embryogenesis, four genotypes C8, C14, C15 and C16 were tested on the "Im" induction medium. During this study, high percentages of calli were obtained independently of the type of explant and the culture medium. These results show that staminodes and petals as well as the "Im" medium are adequate for the in vitro induction of *Theobroma cacao* calli. By some authors reported that Theobroma cacao floral parts, petals and staminodes respond positively to the induction of callogenesis (Koné *et al*., 2021; Sandra *et al*., 2023).

Callogenesis monitoring showed that the C15 genotype presents the best percentage of callogenic explants, whatever the type of explant, compared to the other genotypes. This genotype had good callus development compared to the other genotypes. These results reveal that the callogenesis response in T. cacao would be strongly linked to the genotype, as mentioned by many authors (Koné *et al*. (2019; 2021). In addition, the types of explants cultured (staminodes or petals) have shown significantly different percentages of callogens. Petals showed significantly different percentages of callus for the genotypes studied, suggesting that there is also an inter and intra-genotypic explant effect. Petal explants excelled in their response to the formation of callus, mainly due to the time it took for them to form. This result is consistent with that described by d'Eliane *et al*. (2019) and Koné *et al*. (2019; 2021), where excellent callus formation was reported for petals of Ivorian cocoa genotypes.

It is possible that the structure of the staminodes, being concave, does not allow full contact with the culture medium, and this undoubtedly influences the response to the process of callogenesis, limiting development on the surface in direct interaction with the medium. Of culture taking longer to develop completely, the reverse occurs in the petals where the major part is in contact with the medium. Results were opposed to ours obtained by García (2016; 2021) and Sandra *et al*. (2023), who reported better callus formation of staminodes than petals of Venezuelan and Colombian cocoa genotypes.

A reduction in the percentage of calluses was, however, observed in the C8 genotype, unlike the other genotypes. This work shows once again that the responses to callogenesis vary according to the cocoa genotype. The genotype effect during callogenesis has been stated by several authors, including Koné *et al*. (2019; 2021). This reduction could be explained by low water absorption or competition between nutrients, which can lead to a drop in the percentage of calluses in the C8 genotype.

The transfer of the calluses in the embryo expression medium (ED) allowed the production of embryos only in the petal explants of the four genotypes C8, C14, C15 and C16 with variable percentages according to the genotypes. This result shows that the protocol

used is better suited to petal explants than to staminode explants. This variability in the response could indicate the existence of an effect of the explant on the embryogenic potential of the cocoa tree. These results are consistent with those of Eliane *et al.* (2019) and Koné *et al.* (2019; 2021), who found petals to be better suited for somatic embryo production than staminodes.

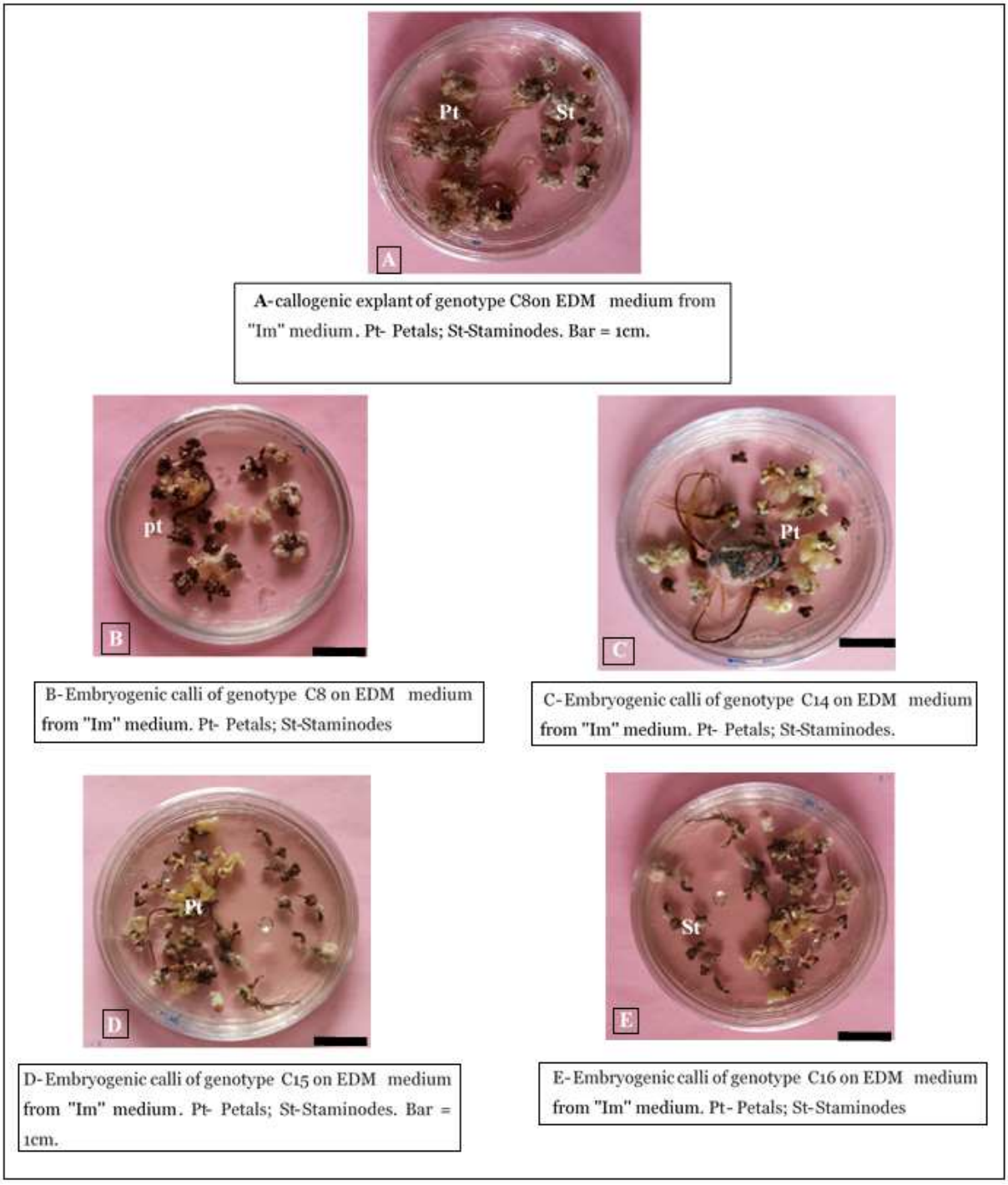

Fig. 1. Embryogenic calli from flower explants in on EDM medium from "Im" medium.

The C8 genotype gave percentages of embryogenic calli (04.16% ± 0.13) and mean somatic embryo number (1.09 ± 0.20). Contrary to our results, Kouassi *et al.* (2017) mentioned that the C8 genotype was incapable of producing embryos at the concentration of 4.5 µM of 2-4-D used. These different results suggest that to overcome recalcitrance in certain genotypes of cocoa trees, a

combination of nutrients is often useful when the dose or the nature of the auxin used is not capable of meeting the nutrient needs alone. It is, therefore, useful to combine them in the same medium to increase the production of somatic embryos or remove recalcitrance.

The C14 had better embryogenic powers than the C8, C15 and C16 genotypes. This shows once again that somatic embryogenesis in cocoa is genotype-dependent, as reported by Koné *et al.* (2019; 2021).

The percentages of embryogenic calli and the average number of somatic embryos of the C8 and C14 genotypes on the "Im" medium were higher than those obtained on the culture media containing different concentrations of antioxidants, according to the work of Kouassi et al. (2017). This shows that it is useful to combine certain nutrients when the antioxidant dose is not capable of ensuring the nutrient needs alone. It is, therefore, essential to associate them in the same medium to increase the production of somatic embryos.

Conclusion

The results obtained revealed that the elimination of the recalcitrance of the genotypes is possible with the nutrients gathered in the same medium. This lifting of recalcitrance was obtained with medium "Im". This medium can enable the mass production of these improved genotypes and meet grower demands.

References

Driver JA, Kuniyuki AH. 1984. In *vitro* propagation of paradox walnut root stock. HortScience **19,** 507-509.

Eliane MT, Modeste KK, André SB, Edmond KK, Mongomaké K. 2019. Effect of Water Stress Induced by Polyethylene Glycol 6000 on Somatic Embryogenesis in Cocoa (*Theobroma cacao L.*). Agricultural Sciences **10,** 1240-1254.

Garcia C, Furtado AA, Costa M. 2019. Abnormalities in somatic embryogenesis caused by 2,4-D: an overview. *Plant Cell, Tissue and Organ Culture*, **137(2),** 193-212. http://dx.doi.org/10.1007/s11240-019-01569-8

Garcia C, Corrêa F, Findley S. 2016. Optimization of somatic embryogenesis procedure for commercial clones of Theobroma cacao L. African Journal of Biotechnology **15(36),** 1936-1951. http://dx.doi.org/10.5897/ajb2016.15513

Jane K, Siaka K, Lucien D, Georges N. 2017. Enhanced plantlet regeneration in two cacao (*Theobroma cacao*) Clones from Immature Inflorescence Explants. HORTSCIENCES, **52(6),** 892–895. http://dx.doi.org/10.21273/HORTSCI11844-17

Koné D, Kouassi KM, N'Nan AO, Kouablan KE. 2019. Induction of somatic embryos of recalcitrant genotypes of theobroma. Journal of Applied Biosciences **133,** 13552–13560.

Koné D, Kouassi KM, N'Nan AO, Kouablan KE. 2019. Use of mineral salts to remove recalcitrance to somatic embryogenesis of improved genotypes of cacao (Theobrama cacao L.). African Journal of Biotechnology **20(1),** 33-42.

Koné D, Kouassi KM, N'Nan AO, Kouablan KE. 2019. Carbon source and lifting of recalcitrance to the induction of somatic embryos in cacao (Theobroma cacao L.).International journal of biosciences **19(1),** 75-84.

Kouassi KM, Manlé TE, Koné D, Soumahoro AB, Koné T, Kouablan KE, Koné M. 2017a. Effect of antioxidants on the callus induction and the development of somatic embryogenesis of cocoa [*Theobroma cacao* (*L.*)]. Australian Journal of Crop Sciences **11(1),** 25-31. https://doi.org/10.21475/ajcs.2017.11.01.pne174

Kouassi KM, Kahia J, Kouame NC, Tahi GM, Kouablan KE. 2017. Comparing the effect of plant growth regulators on callus and somatic embryogenesis induction in four elite *Theobroma cacao L.* Genotypes. Hortsciences **52(1),** 142–145. http://dx.doi.org/10.21273/HORTSCI110.92-16

Kouassi KM, Kouablan KE, Silué O, Tahi GM, Touré M, Konan KP. 2018.Comparison of systems combining auxins with thidiazuron or kinetin supplemented with polyvinylpirrolidone during embryogenic callus induction in three *Theobroma cacao* L. genotypes. Internatinal Journal Biological and Chemical Sciences **12(2),** 804-811. https://doi.org/10.4314/ijbcs.v12i2.15

Sandra MMN, Ana MHR, Aura IUT. 2023. Propagation of the Colombian genotype of cacao (*Theobroma cacao* L.) *CNCh-12* by somatic embryogenesis. Revis Bionatura **8(1),** 26. http://dx.doi.org/10.21931/RB/2023.08.01.16.bd268