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Carbon source and lifting of recalcitrance to the induction of somatic embryos in cocoa (*Theobroma cacao* L.)

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Abstract

In cocoa trees, genetic improvement has allowed the development of elite high-producing varieties. Unfortunately, the plant's natural method of multiplication comes up against certain difficulties which do not allow sufficient improved material to be available and to have homogeneous plantings. This work aimed to develop a method for mass production by *in vitro* culture of improved materials deemed recalcitrant to somatic embryogenesis. Different concentrations of glucose, sucrose and fructose were used to induce the production of embryos of four improved and recalcitrant genotypes to somatic embryogenesis coded C8, C14, C15 and C16. The plant material consisted of petal and staminode explants. The best results which are characterized by levels of callus embryogenesis and the number of somatic embryos of 30% and 20, respectively, were obtained with glucose at a concentration of 30 g/L. This study has shown that it is possible to produce somatic embryos in these improved varieties, thus removing 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 genetic improvement of the cocoa tree is carried out by several methods including reciprocal recurrent selection or selection assisted by markers (Micheli *et al.*, 2009; Tahi *et al.* (2017)). Its purpose is to disseminate among cocoa farmers materials with high productivity potential, resistance and tolerance to diseases with satisfactory physical and organoleptic characteristics.

However, the plant's natural mode of reproduction does not make it possible to have sufficient improved material available and to create homogeneous plantations. To overcome this difficulty, several methods have been considered. Classical techniques for multiplying plant material, such as budding, cuttings and grafting, have been proposed. Unfortunately, this vegetative horticultural propagation has several drawbacks which do not allow their easy application. (Koné *et al.*, 2019). To overcome all these difficulties and obtain homogeneous material in sufficient quantity, *in vitro* culture by somatic embryogenesis has been considered. It is a method that makes it possible to produce a given genotype in a sufficient quantity and a homogeneous manner.

Moreover, according to Traoré *et al.* (2006), Bamba (2014) and Kouassi *et al.* (2017a), the development of a system of rapid mass propagation and producing planting material faithful to the starting genotype, such as somatic embryogenesis, is an effective means of genetic improvement of the cocoa tree. 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 (Issali *et al.*, 2012; Kouassi *et al.*, 2017a). Regenerators, i.e. plants regenerated from somatic embryos, in addition to being genetically identical to each other, behave like seedlings (Tan and Furtek, 2003; Traoré *et al.*, 2006; Jane *et al.*, 2017; Kouassi *et al.*, 2018). However, this method presents some difficulties at the level of the cocoa tree because of the recalcitrance of certain genotypes. This recalcitrance is expressed by the variation in the rate of somatic embryos from one genotype to another, often with very low or zero rates of somatic embryos. Overcoming this recalcitrance requires optimizing existing protocols or developing new ones. The work of Koné *et al.* (2019; 2021) has shown that auxins and mineral salts are taken individually can overcome this recalcitrance.

The general objective of this study is to test the effect of different concentrations of carbon sources on lifting the recalcitrance of recalcitrant genotypes to allow their mass production and large-scale popularization.

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 culture 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) have consisted of the mineral solution of DKW (Driver and Kuniyuki Walnut, 1984).

Induction medium: Different concentrations of glucose, fructose and sucrose 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 20, 30 and 40g /L. A total of 9 induction media varying in nature and concentration of carbon sources were prepared.

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 (ie 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 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, 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 concentrations of carbon sources on callus production in different genotypes

Table 1 shows the percentages of calli produced by the explants of staminodes and petals after 28 days on the culture media containing different concentrations

of carbon sources. Statistical analyzes revealed significant differences between the percentages of calli produced by the explants in the presence of different concentrations of carbon sources (P < 0.001). Responses of staminodia and petal explants to callogenesis varied depending on cocoa genotype, type and concentration of carbon sources.

Table 1. Percentage of calli produced from explants of staminodes and petals depending on the concentrations of carbon sources and the genotype used.

Genotypes	Carbon source	Concentration (g/l)	Percentage of Callogenic Explant (PCE) (%)	
			Staminodes	Petals
C8	Glucose	20	98.42 ± 4.29 [a]	100 [a]
		30	97.09 ± 1.22 [a]	100 [a]
		40	98.55 ± 2.81 [a]	100 [a]
	Fructose	20	97.69 ± 4.24 [a]	98.37 ± 6.15 [a]
		30	97.87 ± 3.72 [a]	97.80 ± 3.89 [a]
		40	98.08 ± 5.17 [a]	100 [a]
	Sucrose	20	97.66 ± 1.92 [a]	100 [a]
		30	99.02 ± 1.27 [a]	97.99 ± 1.42 [a]
		40	52.88 ± 1.11 [d]	82.66 ± 2.27 [b]
C14	Glucose	20	100 [a]	100 [a]
		30	100 [a]	100[a]
		40	98.14 ± 1.98 [a]	100[a]
	Fructose	20	98.04 ± 0.95 [a]	97.01 ± 2.42 [a]
		30	97.39 ± 2.48 [a]	97.08 ± 2.35 [a]
		40	99.88 ± 1.11 [a]	97.26 ± 5.03 [a]
	Sucrose	20	89.21 ± 8.87 [ab]	96.96 ± 3.79 [a]
		30	96.09 ± 1.22 [a]	97.56 ± 2.88 [a]
		40	90.16 ± 5.68 [ab]	90.21 ± 1.08 [ab]
C15	Glucose	20	97.64 ± 8.46 [a]	98.91 ± 2.08 [a]
		30	96.16 ± 5.68 [a]	98.65 ± 2.06 [a]
		40	100 [a]	100 [a]
	Fructose	20	74.00 ± 9.05 [c]	90.39 ± 0.27 [ab]
		30	89.66 ± 10.18 [ab]	89.08 ± 8.35 [ab]
		40	88.50 ± 11.37 [ab]	83.37 ± 14.45 [b]
	Sucrose	20	100 [a]	100 [a]
		30	100 [a]	100[a]
		40	50.55 ± 0.07 [d]	61.27 ± 04.05 [c]
C16	Glucose	20	96.40 ± 4.32 [a]	97.42 ± 4.29 [a]
		30	95.33 ± 2.90 [a]	97.09 ± 1.22 [a]
		40	96.27 ± 3.19 [a]	96.55 ± 2.81 [a]
	Fructose	20	74.00 ± 9.05 [c]	89.01 ± 2.42 [ab]
		30	82.66 ± 10.18 [b]	90.08 ± 2.35 [ab]
		40	80.55 ± 14.37 [b]	83,26 ± 5,03 [b]
	Sucrose	20	97.66 ± 1.92 [a]	98 ± 3.19 [a]
		30	85.02 ± 1.27 [ab]	90.99 ± 1.42 [ab]
		40	54.88 ± 1.11 [d]	60.37 ± 04.05 [c]
Stastical tests		P	< 0,001	< 0,001
		F	34,25	33,68

In the same column, the averages followed by the same letter are statistically equal (test of Newman-Keuls to the threshold of 5 %).

In the C14 genotype, regardless of the implant used, the different concentrations of glucose, fructose and sucrose induced high and statistically identical percentages (90 to 100%) of embryogenic calli. With the C8 genotype, different concentrations of glucose, fructose and sucrose improved the rate of callus induction from explants of petals and staminodes. On the other hand, the addition of sucrose at 40 g /l in the culture medium significantly reduced to 52.88% the percentage of embryogenic calli induced from

explants of staminodes of this genotype. Concerning the C15 and C16 genotypes, the percentages of calli induced from the explants of staminodes and petals were significantly improved with the different concentrations of glucose, fructose and sucrose. However, when sucrose was used at a concentration of 40 g /L, the callus induction percentages were average with staminodes (50.55% ± 0.07 and 54.88% ± 1.11, respectively) and petals (61.27% ± 04.05 and 60.37% ± 04.05, respectively). Overall, the carbon sources improved the callogenesis of the different genotypes studied C8, C14, C15 and C16. However, the addition of sucrose at a high concentration of 40g /L resulted in a reduction in the percentages of calli produced by explants of genotypes C8, C15 and C16. In these genotypes, the percentages of embryogenic calli varied from 50% to 100% with the staminodes and from 61% to 100% with the petals.

Table 2. Somatic embryos induction as a function of carbon source and genotype concentrations.

Genotypes	Carbon source	Concentration (g/l)	Somatic embryo induction by petals explants	
			Induction rate embryogenic calli	Mean number of somatic embryos
C8	Glucose	20	14.16±0.14 bc	04.11±0.20 bc
		30	25.02±0.10 ab	16.16±3.5 ab
		40	00.00±0.00 c	00.00±0.00 c
	Fructose	20	00.00±0.00 c	00.00±0.00 c
		30	00.00±0.00 c	00.00±0.00 c
		40	00.00±0.00 c	00.00±0.00 c
	Sucrose	20	00.00±0.00 c	00.00±0.00 c
		30	00.00±0.00 c	00.00±0.00 c
		40	00.00±0.00 c	00.00±0.00 c
C14	Glucose	20	35.73±01.01 a	18.16±0.07 a
		30	38.29±1.12 a	20.75±0.17 a
		40	00.00±0.00 c	00.00±0.00 c
	Fructose	20	26.07±2.40 ab	14.90±0,04 ab
		30	27.61±4.60 ab	15.11±1,69 ab
		40	0.00±0.00 c	0.00±0.00 c
	Sucrose	20	38.16±02.5 a	19.10±0.12 a
		30	39.64±01.02 a	21.27±0.05 a
		40	00.00±0.00 c	00.00±00.00 c
C15	Glucose	20	36.09±0.07 a	19.03±0.06 a
		30	39.60±2.02 a	20.19±0.07 a
		40	00.00±0.00 c	00.00±0.00 c
	Fructose	20	24.07±0.04 ab	14.04±0.34 ab
		30	25.61±01.0 6ab	15.11±0.19 ab
		40	00.00±0.00 c	00.00±0.00 c
	Sucrose	20	25.33±0.02 ab	14.20±0.25 ab
		30	26.93±0.05 ab	14.50±0.03 ab
		40	00.00±0.00 c	00.00±0.00 c
C16	Glucose	20	36.73±0.05 a	19.59±0.04 a
		30	38.27±02.08 a	20.07±0.08 a
		40	00.00±0.00 c	00.00±0.00 c
	Fructose	20	23.10±0.05 ab	10.20±0.01 b
		30	22.11±0.04 ab	09.90±0.12 b
		40	00.00±0.00 c	00.00±0.00 c
	Sucrose	20	28.16±01.05 ab	15.90±0.02 ab
		30	26.16±02.03 ab	15.90±0.12 ab
		40	00.00±0.00 c	00.00±0.00 c
	Statistical tests	P	< 0,001	< 0,001
		F	9,31	10,68

In the same column, the averages followed by the same letter are statistically equal (test of Newman-Keuls to the threshold of 5%).

In all genotypes, petal explants resulted in somewhat higher percentages of calli than staminode explants. Fig. 1A illustrates an example of calli developed from explants of petals and staminodes of the C15 genotype at a concentration of 30 g /L of glucose.

Effect of concentrations of carbon sources on embryo production in different genotypes

Table 2 shows the levels of embryogenic calli and the average number of somatic embryos produced from explants of petals of the C8, C14, C15, and C16 cocoa

genotypes at the different concentrations of carbon sources following analysis of the variance with $P < 0.001$. Staminode explants from all cocoa genotypes did not develop embryogenic calli in the presence of all carbon sources tested. In contrast, petal explants of all genotypes produced embryogenic calli and somatic embryos at varying rates depending on the type and concentration of carbon sources. Fig. 1B, 1C and 1D illustrate an example of embryogenic calli developed from explants of petals of genotypes C15, C14 and C15 at a concentration of 30 g /L of glucose.

For the C8 genotype, the medium was supplemented with 30 g /L of glucose-induced high percentages of embryogenic calli (25.02% ± 0.10) and mean number of somatic embryos (16.16% ± 3.5). On the other hand, a low percentage of embryogenic calli (14.16% ± 0.14) and mean number of somatic embryos (04.11 ± 0.20) were obtained on the medium containing 20 g /L of glucose. No induction of PEC and NSE was observed on a medium supplemented with 40g /L of glucose as well as with other concentrations of other carbon sources.

In the C14 genotype, the percentage of embryogenic calli and the average number of high embryos were obtained in the culture medium containing 20g /L (35.73% ± 01.01 and 18.16 ± 0.07) and 30g /L (38.29% ± 1.12 and 20.75 ± 0.17) of glucose and 20g /L (38.16% ± 02.5 and 19.10 ± 0.12) and 30 g /L (39.64% ± 01.02 and 21.27 ± 0.05) of sucrose. However, no embryogenic callus and no somatic embryos were obtained with each of the carbon sources at a concentration of 40g /L.

For the C15 genotype, the percentages of embryogenic calli and the average number of high embryos were observed only with the concentrations of 20g /L (36.09% ± 0.07 and 19.03 ± 0.06) and 30g /L (39.60% ± 2.02 and 20.19 ± 0.07) glucose. The low percentages of embryogenic calli and the average number of embryos were obtained on media containing 40g/L of glucose and the various concentrations of fructose. However, no embryogenic callus and no somatic embryos were obtained with the concentration of 40g /L at each of the carbon sources. The percentages of embryogenic calli ranged from 0 to 26.93% and the mean number of embryos from 0 to 14.50.

Concerning the C16 genotype, the percentages of embryogenic calli and the average number of high embryos were obtained with the concentrations of 20g /L (36.73% ± 0.05 and 19.59 ± 0.04) and 30g /L (38.27% ± 02.08 and 20.07 ± 0.08) of glucose. These percentages of embryogenic calli and the average number of embryos are significantly higher than those obtained on media supplemented with different concentrations of fructose and sucrose.

Among the different carbon sources used, the concentration of 30g /L of glucose made it possible to obtain the best percentages of embryogenic calli and the average number of embryos in the four genotypes C8, C14, C15 and C16. The lowest percentages were obtained when the sucrose is used at the concentration of 40g /L and generally with the concentrations of fructose.

Discussion

In order to develop a reliable protocol applicable to certain genotypes considered recalcitrant to somatic embryogenesis, four of these genotypes were tested on media with different concentrations of carbon sources.

Calli were obtained with the explant types at high percentages, however, only petal explants allowed the production of embryos in the four genotypes C8, C14, C15 and C16 with varying percentages depending on the genotypes and concentrations used. This shows that the response to somatic embryogenesis depends on the implant. Our study has shown that the protocol used is more suitable for petal explants. These results are consistent with those of Kouassi *et al.* (2017) and Eliane *et al.* (2019) and Koné *et al.* (2019; 2021) who found that the petals were better suited for the production of somatic embryos. For most concentrations of carbon sources, variations were also observed in the production of embryos from one

genotype to another, highlighting a genotype effect. The effect of the genotype on the response to somatic embryogenesis of the cocoa tree has already been reported by several authors such as Kouassi *et al.* (2017), and Eliane *et al.* (2019). For the four genotypes C8, C14, C15 and C16 of cocoa trees, the best percentages of embryogenic calli and an average number of somatic embryos were obtained in the presence of 30g /L of glucose.

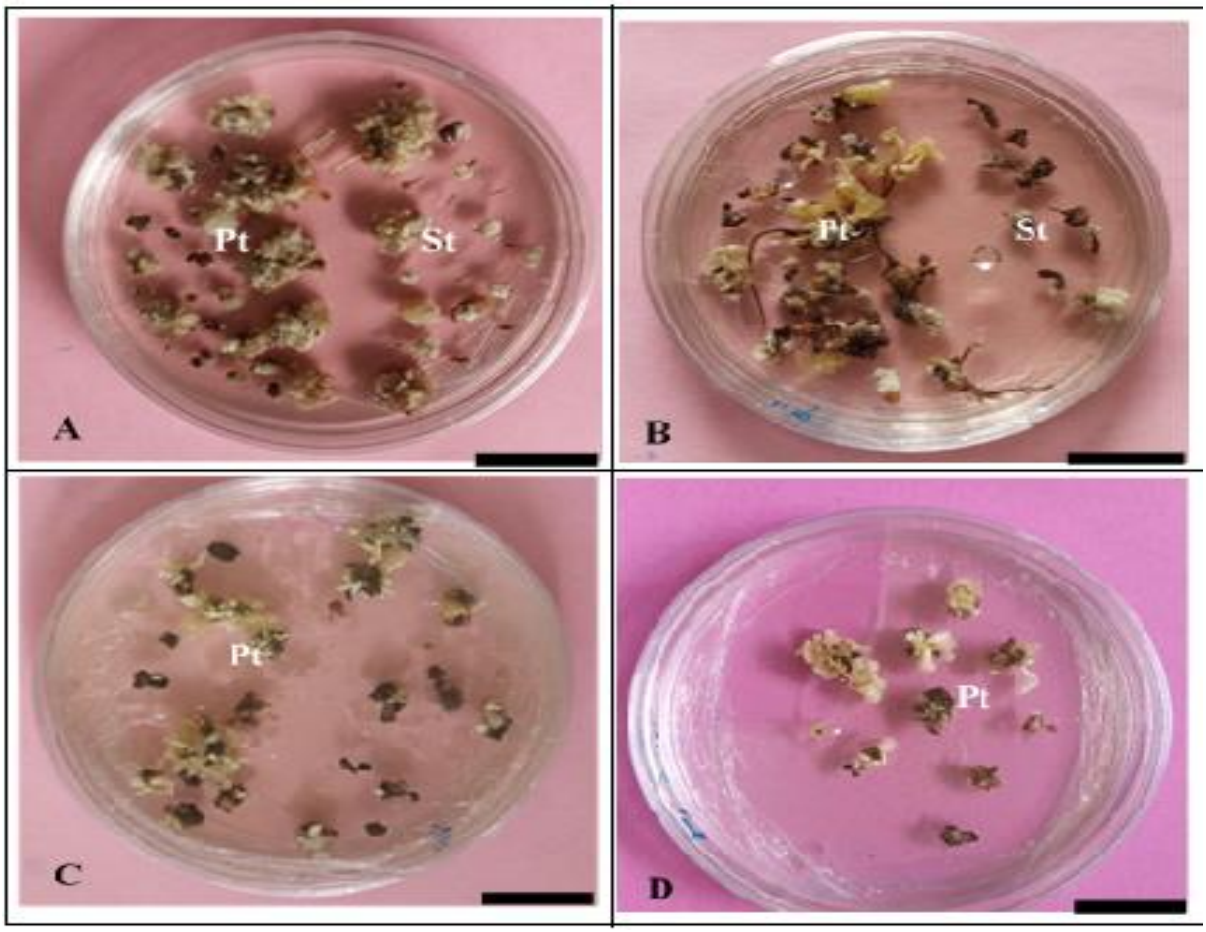

Fig. 1. Embryogenic calli from flower explants in media supplemented with 30g/L of glucose.
A- Callogenic explant of genotype C15 on EDM medium supplemented with 30g/L of Glucose. Pt- Petals; St- Staminodes. Bar = 1cm. B- Embryogenic calli of genotype C15 on EDM medium supplemented with 30g/L of Glucose. Pt- Petals; St-Staminodes. Bar = 1cm. C- Embryogenic calli of genotype C14 on EDM medium supplemented with 30g/L of Glucose. Pt- Petals. Bar = 1cm. D- Embryogenic calli of genotype C8 on EDM medium supplemented with 30g/L of Glucose. Pt- Petals. Bar = 1cm.

In fact, this concentration allowed, like phytohormones and mineral salts taken individually (Koné *et al.*, 2019; 2021), the production of somatic embryos of genotypes considered recalcitrant at high and almost identical percentages. At this concentration, glucose would lead to maintaining a good osmotic pressure, would eliminate the accumulation of phenolic compounds, would provide enough energy to ensure cell divisions and thus allow the induction of somatic embryos, thus leading to the lifting of recalcitrance genotypes. This concentration decreases the genotype effect and shows that it is possible to obtain the formation of somatic embryos in the cocoa tree. In fact, the lifting of the recalcitrance has generally been obtained in certain genotypes of cocoa trees by the use of several combinations of nutrients. Kouassi *et al.* (2017) reported that the combination of 4.5 µM 2,4-D with 20 µl /L of Thidiazuron (TDZ), a cytokinin, induced somatic embryogenesis in some genotypes. These

different results suggest that to overcome the recalcitrant in certain genotypes of cocoa trees a combination of nutrients is often useful when the dose or the nature of the element used is not able to meet the nutrient requirements on its own. Compound carbon sources, used alone without association with other compounds, have created optimal conditions for the production of embryos, as have phytohormones and mineral salts (Koné *et al.*, 2019; 2021). The concentration of 30g /L of glucose is the optimum concentration for lifting the recalcitrance of the genotypes studied. Its use will make it possible to avoid the use of complex media containing several nutrients as shown by the work of Kouassi *et al.* (2017). The effectiveness of glucose as a carbon source has been mentioned in cocoa trees by several authors such as Li *et al.* (1998).

While the concentration of 30g / L of glucose was found to be optimal for the production of somatic embryos, inhibition of embryo production was generally observed with all 20, 30, or 40 g /L concentrations of fructose and sucrose and 40g / L of glucose in the C8 genotype then 40 g /L for all carbon sources in the C8, C14, C15 and C16 genotypes. This inability of the genotypes to produce somatic embryos under these culture conditions could be due either to inefficiency of the carbon source used, as is the case of fructose in this very recalcitrant genotype or to toxicity caused by certain concentrations. Too high, especially 40g /L, regardless of the carbon source. In fact, Gill *et al.* (1993) reported that high concentrations of carbon sources are likely to inhibit embryo formation as has been observed with geranium in which concentrations of 6%, 9%, or 12% of sucrose or glucose or fructose prevented the formation of somatic embryos. Unlike the cocoa tree, some species such as asparagus (5%) and chrysanthemum (12-180%), however, require high percentages of sucrose, glucose, or fructose for the induction and development of somatic embryos (Hitomi *et al.*, 2019). Sucrose is formed from the two hexoses, glucose and fructose, so at very high concentrations, it accentuates cell plasmolysis. With regard to fructose, even if Traoré *et al.* (2006) reported that the use of fructose in the induction medium results in a strong embryogenic response from cocoa tissue, its role in aseptic cultures is controversial. It gave good results for the culture of Castanea sativa (Chauvin and Salesses, 1988), but was inappropriate for Malus Jork 9 (Moncousin *et al.*, 1992) for Prunus cerasus (Borkowska *et al*, 1991) and in particular for the C8 genotype used in our study. Other carbon sources such as lactose, mannitol and sorbitol have also been used in the study of somatic embryogenesis in plants (Brown *et al.*, 1995).

The inhibition of somatic embryogenesis in the cocoa tree would be due, in addition to the insufficiency or the toxicity of certain nutrients used, to a strong secretion of ethylene and polyphenols by the explants of certain genotypes according to Kouassi *et al.* (2017) and Minyaka *et al.* (2017). Polyphenols, through their oxidation, act as inhibitors of metabolic reactions or antagonists of growth substances. The work of Alemanno *et al.* (2003) and Kouassi *et al.* (2017a) carried out on cocoa tree flowers have shown that they synthesize a significant amount of phenolic compounds. Indeed, these compounds are involved in the defense of plants (Minyaka *et al.*, 2017). When the plant is subjected to mechanical injury, simple phenols are synthesized and the peroxidase activity characteristic of lignifying tissues is stimulated. Phenolic secretions and other exudates in plant tissue culture systems inhibit the development of the callogenous implant into an embryo (Alemanno *et al.*, 2003; Kouassi *et al.*, 2017).

Conclusion

The results obtained revealed that the removal of the recalcitrance of genotypes is possible with carbon sources. This lifting of the recalcitrance was obtained with 30 g / L of glucose as a carbon source. Carbon sources as well as mineral salts and phytohormones can allow the mass production of these improved genotypes and meet the demands of producers.

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