Influence of Carbohydrates on Callus Proliferation During Somatic Embryogenesis in Pineapple [*Ananas Comosus* (L.) Merr. (Bromeliaceae) Var. Cayenne Smooth Cultivar CI 16]

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Abstract

The improvement of pineapple (*Ananas comosus* var. Smooth Cayenne) by means of *in vitro* culture is less studied in Côte d'Ivoire despite the importance of this plant for this country's economy. Our work consisted in highlighting nature and concentration effects of carbohydrates on the proliferation of calli in pineapple as a prelude to efficient embryogenesis. Callus proliferation was carried out from the base of pineapple vitroplants leaves. Thirty (30) explants were cultured on the tested culture medium. MS medium (micro- and macro elements of Murashige and Skoog) supplemented with vitamin Gamborg B_5 was used as base medium to which were added 0.05 mg/L BAP, 3 mg/L picloram, 2 mg/L glycine, 1,000 mg/L glutamine, 100 mg/L casein hydrolyzate and 30 g/L carbohydrate. Sucrose was tested at different concentrations (20, 25, 30, 35 and 40 g/L). The results revealed that callus proliferation is strongly influenced ($p < 0.0001$) by nature and concentration of carbohydrate. Sucrose with the highest dry matter content

(61.34 mg) has a higher callogenic potential than the other studied carbohydrates. The concentration of 30 g/L sucrose significantly improved the calli proliferation in pineapple. Galactose and maltose were less favorable to proliferation.

Keywords: Pineapple, embryogenesis, *in vitro* culture, carbohydrate, sucrose

Introduction

In Côte d'Ivoire, the agricultural sector occupies a prominent place in the national economy. It represents 33% of the current GDP and was at the origin of the "Ivorian economic miracle" of the years 1970-1980 (Anonymous 1, 2008). This economic boom was made possible due to a special agricultural policy in favor of the coffee-cocoa binomial. Today, with regard to the international price cuts of those main export products, it is necessary to proceed to the diversification of crops to ensure the economic stability of this country whose economy is mainly based on agriculture. The culture of pineapple (*Ananas comosus* var. Cayenne smooth) was part of this policy of crop diversification. Indeed, it has occupied an important place in the Ivorian economy (0.6% of the national GDP and 1.6% of the agricultural GDP) (OCAB, 2000; Adomon, 2007; MINAGR, 2013). The couple pineapple - banana has therefore generated an annual turnover of 145 billion FCFA (Nouza, 2011). It is not only a source of employment but also an important source of foreign exchange earnings. For more than a decade, Côte d'Ivoire was the leading supplier of fresh pineapple in the European market (97 %). Today, this sector is affected by many problems that consequently led to a sudden drop in production, about 90.4 % in 2014 as compared the production in 1999 (Anonymous 2, 2015). To these problems one should add the issue of the acidity and aging of the orchard (Tanoh, 2008). The economic importance of pineapple crop for Côte d'Ivoire triggered us in giving a high worth study priority in order to find solutions to the problems that undermine this sector. Thus, to renew the orchard and clean up the fruit, several cultivation methods were used without real success. However, biotechnology that covers many fields ranging from tissue culture concepts, genetic engineering to biotechnology applications could be an interesting tool. Several *in vitro* pineapple regeneration tests were carried out (Firoozabady and Gutterson, 2003; Be and Debergh, 2006; Danso *et al*., 2008; Yapo *et al*., 2011). The culture media developed during this work very often include sugars as sources of carbon. Indeed, since *in vitro* tissues are generally heterotrophic due to the absence of chlorophyll assimilation, it is necessary to provide them with a carbon source. Moreover, that carbon source becomes a sort of energy, necessary for tissue growth and maintains an osmotic pressure of the culture medium (Zryd, 1988). Carbohydrates most commonly used as a source of

carbon are sucrose and glucose (Walker and Parrott, 2001). Maltose may be a good carbon source, since it can improve both the quality and quantity of somatic embryos produced (Saadi, 1991) in some studies of embryogenesis. The effects of other carbohydrates have been tested during the embryogenesis of several species (Unnikrishnan *et al*., 1990; Tremblay and Tremblay, 1995; Kouakou, 1996; Zouzou *et al*., 1997; N'cho, 2006). However, for calli proliferation, the results obtained differ from one sugar to another (Najiba *et al*., 2008)**.** Our objective in this study is to identify the carbohydrate and its appropriate concentration to the proliferation of callus in the pineapple (*Ananas comosus* var. Smooth Cayenne), cultivar CI 16. Thus, six carbohydrates (sucrose, glucose, maltose, mannitol, fructose and galactose) were tested. The choice of these carbohydrates is motivated by the work done by Najiba *et al*. (2008) on the embryogenesis of *Olea europaea*. After selecting the best carbon source, its optimal concentration was identified.

Material and methods

Plant material

In this study, the leaves of young shoots obtained *in vitro* from pineapple (*Ananas comosus* var. Smooth Cayenne, cv. CI 16) suckers were used as plant material. They were harvested from the plantations of CNRA's production station in Anguédédou (Côte d'Ivoire, West Africa) to be carried out at CNRA's central biotechnology laboratory in Abidjan (Côte d'Ivoire).

Methods

Callus induction

Callus induction was performed on MS basal medium (Murashige and Skoog, 1962) containing Gamborg vitamin B_5, supplemented with picloram (3 mg/L), glycine (2 mg/L), glutamine (1,000 mg/L), casein hydrolyzate (100 mg/L) and sucrose (30 g/L). The pH of the culture medium was adjusted to 5.5 with NaOH (1N) or HCl (1N). The culture medium was solidify with 6 g/L of Agar-agar, then sterilized by autoclaving (Autester) for 30 min at 121 °C, under a pressure of 1 bar. After solidification of the culture medium, under a hood, about 5 to 7 mm from the base of the shoots leaves were cut and then deposited on the callus induction medium at the rate of five explants per jar. The jars containing the explants are placed in the culture room for four weeks. The calli obtained served as an explant for the study of calli proliferation.

Effect of carbon sources on callus proliferation

Preparation of the medium

The MS medium (Murashige and Skoog, 1962) was used as basic medium in this study. This medium was used by several authors (Akbar *et al*., 200; Ika and Ika, 2003; Yapo *et al*., 2011) for calli induction in pineapple. It

includes macroelements and microelements (Murashige and Skoog, 1962)**,** added Gamborg B_5 vitamin, supplemented by 0.05 mg/L BAP, 3 mg/L picloram, 2 mg/L glycine, 1,000 mg/L glutamine, 100 mg/L casein hydrolyzate. These concentrations were chosen according to callus induction method (Yapo *et al*., 2011). All culture media have the same composition of mineral elements with the exception of carbohydrate which is the specific element of each medium. The different media were named according to the carbon source used. Thus, carbohydrates such as sucrose; Glucose; Maltose; Mannitol; Fructose and galactose designate the culture media which respectively contain those carbon sources. The media were solidified with 6 g/L agar-agar and 0.75 g/L magnesium chloride. The pH of calli proliferation media was adjusted to 5.5. The culture media were sterilized in an autoclave (Autester) during 30 min at 121 °C, under one barof pressure.

Callus proliferation and culture conditions

Friable and non-brown calli obtained from calli induction phase were weighed. Then, approximately 10 mg of these calli were seeded on to the culture medium at a rate of two cals per jar. The jars containing 10 mL of callus proliferation media were placed in a controlled culture room of 20 m^2 according to the conditions identified above for 6 weeks. Thirty (30) explants were cultured on each tested medium. Each medium is repeated 15 times. A repeat consists of a jar of 2 explants. The best carbohydrate identified in this study is used for further testing.

Effect of sucrose concentration on callus proliferation

The aim of this study is to determine the best concentration of sucrose identified in the previous study as the adequate carbohydrate for callus growth in pineapple. For this purpose, only the concentration of sucrose varies; those of other components of these media being already known. In this study, different concentrations of sucrose (20, 25, 30, 35 and 40 g/L) were tested. As in the previous study, under a laminar flow hood, about 10 mg of healthy and friable calli were and seeded on the proliferation culture medium. Thirty (30) explants were used on each of these media. Each medium tested is repeated 15 times. A repeat consists of a jar of two explants. The jars containing the media-seeded explants were randomly placed in a culture chamber under the same conditions as in the previous experiments. After 6 weeks, the dry weight of the calli which best expresses the quantity of material produced, was evaluated.

Statistical analysis

Statistical analysis were carried out through one-way ANOVA using General Linear Model procedure in Statistica 7.1 software and standard

deviation for each mean was worked out. In case of a significant difference between averages in a given parameter, Newman Keuls comparison test is used at $p \leq 0.05$ for averages classification. This enabled us to identify variables (fresh weight and dry weight) for which there is a significant difference between the tested parameters (nature and content of carbon source).

Results

Effect of carbohydrate nature on callus proliferation

After six weeks of culture, the evaluation of callus proliferation on the six studied media showed a difference in the intensity of callus proliferation according to the used carbon source (Figure 1). The fresh weight of the calli was significantly influenced by the carbon source ($p<0.0001$). These results revealed that glucose induced the highest fresh material weight (106.1 mg) followed by mannitol (103.41 mg) and sucrose (101.07 mg), which were not significantly different, then maltose (73.17 mg); galactose (73.53 mg) and finally fructose (70.21 mg) (Table 1). Statistical analysis also revealed that dry weight was very significantly influenced by the carbon source ($p<0.0001$). Unlike the fresh weight of calli, the largest dry matter weight (61.34 mg) was obtained with sucrose. The other types of sugar had statistically identical mean dry matter weights. All carbon sources resulted in greenish-colored calli with the exception of galactose and mannitol, which produced brown calli.

From these observations, it appears that the sucrose which enabled us to obtain the highest amount of dry matter is the most favorable carbon source for callus proliferation in pineapple. The dry matter weight of the calluses, having made it possible to assess the quantity of cells formed, will therefore be used for the rest of the work.

Table 1. Evaluation of carbon source effect on callus proliferation in pineapple

Nature of carbon source (30 g/L)	Parameters		
	Fresh weight (mg ± s)	Dry weight (mg ± s)	Color
Glucose	106,1 ± 3,54a	16,61 ± 2,35b	greenish
Sucrose	103,07 ± 5,12ab	61,34 ± 5,73a	greenish
Mannitol	101,41 ± 4,51ab	11,23 ± 3,58b	greenish
Galactose	73,53 ± 2,35b	17,17 ± 2,01b	brownish
Maltose	73,17 ± 5,12b	11,7 ± 3,49b	brownish
Fructose	70,21± 2,32c	19,25 ±0,34b	greenish

Effect of sucrose concentrations on callus proliferation

The results of sucrose concentrations effect on callus proliferation are shown in Figure 2. The proliferation of calli was very significantly affected ($p<0.0001$) by the concentration of sucrose. Indeed, the dry weight of calli increases from 26 to 93 mg for sucrose concentrations ranging from 20 to 30

g/L and then decreases sharply (from 93 to 31 mg) for concentrations above 30 g/L. These results revealed that sucrose at 30 g/L induced the formation of the largest amount of dry matter (93 mg), followed by 25 g/L (62 mg) and 35 g/L (58 mg). These sucrose contents (25 and 35 g/L) induced statistically identical amounts of dry matter (respectively 62 and 58 mg). The concentrations 20 and 40 g/L obtained the lowest amounts of dry matter (respectively 26 and 31 mg).

This study makes us identify the sucrose concentration of 30 g/L as the optimal concentration for a good calli proliferation in pineapple, as a prelude to efficient embryogenesis.

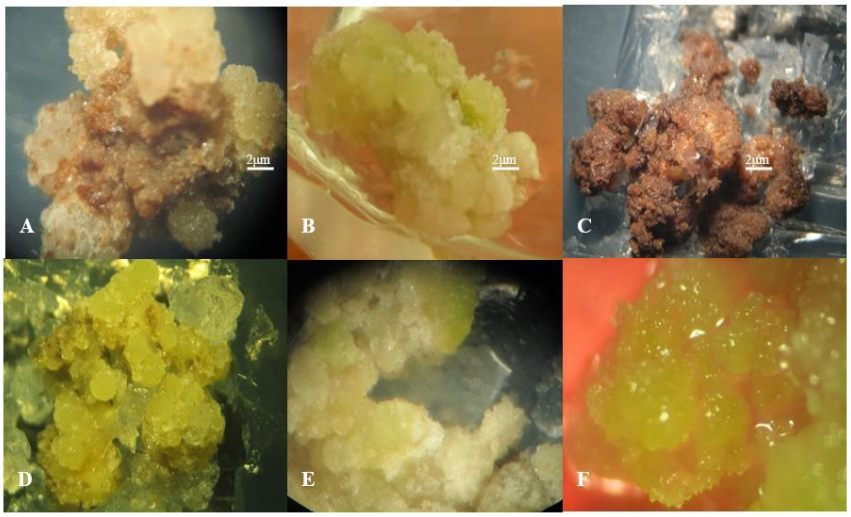

Figure 1. Proliferated pineapple calli on MSB5 medium containing different sources **Of carbon at 30 g/L.**

(A) Galactose: Brownish callus; (B) Saccharose: Greenish callus; (C) Mannitol: Brownish callus;

(D) Glucose: Greenish callus; (E) Maltose: Greenish callus; (F) Fructose: Greenish callus

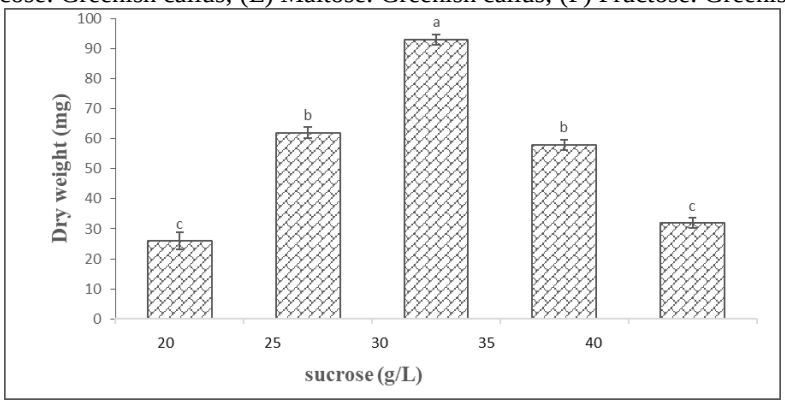

Figure 2. Effect of sucrose concentrations on callus proliferation **Taking into account the dry weight after six weeks of culture.**

The tapes followed by the same letter are not significantly different (Newman Keuls test at 5%).

Discussion

The study revealed that callogenesis in pineapple is significantly influenced by the carbohydrate nature. Since the *in vitro* photosynthesis is low or near zero (Pierik, 1987), sugars are very important ingredients. Their addition to the culture medium enables to bring the necessary energy for tissues development. According to Swankar *et al*. (1986), sugars influence cell proliferation and differentiation. However, all sugars have not the same effects on callogenesis. The results of this study showed that the fresh weight of calli is greater with glucose followed by mannitol and sucrose. These results confirm those of Kouakou (1996) and Zouzou *et al.* (1997), which showed that glucose in cotton produces friable, voluminous calli as compared to other sugars. Also, Najiba *et al*. (2008) reported that sucrose and mannitol in the olive tree produced the most developed nodular and compact calli. On the other hand, dry weight analysis revealed that sucrose induced the highest dry weight followed by galactose and glucose. These results could be explained by the fact that the glucose, which enabled us to obtain the highest fresh callus matter, would cause a strong entry of water into the cell while the sucrose would favor an increase of the medium osmotic pressure, therefore an entry of necessary minerals for the growth of calli. The results also showed that maltose and galactose have little beneficial effect on callus proliferation. These two sugars could not be assimilated by plant cells. However, the hydrolysis of maltose, in two molecules of glucose, should supply the plant cells with assimilable sugars. According to N'cho (2006), the unfavorable effect of maltose on cotton callus proliferation seems to indicate an absence or low amount of degradation enzymes, maltases, in pineapple calli. The positive effect of sucrose on pineapple calli dry weight is due to the fact that sucrose is composed of two monomers; fructose and glucose. Indeed, in an acid medium (pH 5.5), the sucrose would hydrolyze to, glucose and fructose to be assimilable. Therefore, there would be a complementary relation between these two sugars, which would together provide the necessary energy for the growth of calli. Moreover, this hydrolysis leads to an accumulation of glucose and fructose and consequently, to an increase in osmolarity that would induce an accumulation of reserves and mineral elements in the calli, which reflects the good development of these calli. In the culture medium, the osmotic role of sucrose has been reported in several studies (Tremblay and Tremblay, 1995; Taber *et al*., 1998; Find *et al*., 1998). According to Rugini (1995), the sucrose content of the culture medium affects the callus growth and subsequently the somatic embryogenesis through its interaction with growth regulators. Indeed, among the various carbon sources involved in the differentiation and osmotic adjustment, sucrose is the most frequently used in *in vitro* culture. In addition to its degradation for the synthesis of ATP and NADH via glycolysis and the Krebs cycle, sucrose is required for the biosynthesis of primary metabolites

important for tissue growth and development (Sturm, 1999). Sucrose also participates in the synthesis of reserve substances such as starch and polypeptides (Sturm, 1999; Fernie *et al*., 2002). Moreover, the work of Zouzou *et al*. (1997) have also shown that glucose gives voluminous calli as compared to other sugars in cotton. These results corroborate those of the present study when only fresh calli weight is taken into account. However, glucose allowed us to obtain a much lower dry weight compared to sucrose. This means that glucose produces a small amount of matter. Thus, this work has shown that the calli proliferation in pineapples is highly correlated with the carbon source. These differences in effects observed by carbon source in this study would reflect specific affinities for a given type of sugar (Najiba *et al*., 2008). In addition, the content of the carbon source could influence the calli proliferation in pineapple. Indeed, the results of this study revealed that the sucrose content of 30 g/L gave the most voluminous calli. These results were observed by Najiba *et al*. (2008) on the olive tree. Several authors have reported that the sugar concentration generally used for the induction and development of somatic embryos in several species is 20-30 g/L (Han and Xi, 1989; Rout *et al*., 1991). However, other authors have reported that high concentrations of sucrose may have adverse effects on embryo formation. This is the case of *Geranium* where concentrations 6; 9 or 12% inhibit the formation of somatic embryos (Gill *et al*., 1993). In other species, the induction and development of somatic embryos require a high rate of sucrose, like in the *Asparagus* (5%) (Komura *et al*., 1990) and *Chrysanthemum* (12-18 %) (May and Trigiano, 1991). Indeed, according to the works of Buffard-Morel (1968) and those of Rabechault *et al*. (1974) high levels of sugars would promote the absorption of the mineral elements of the culture medium and consequently, lead to cell growth. These results show that the carbohydrate concentration effect depends on the plant species in the presence. In this study, sucrose at 30 g/L was the most successful in the proliferation of calli. The dose effect of sucrose therefore has a great influence on the development of calli in pineapples.

Conclusion

The results of this study based on the influence of carbohydrates on the proliferation of calli in pineapple made it possible to identify sucrose as the preferred carbon source for callus proliferation enable us to obtain a large quantity of dry matter. On the contrary, glucose gives a much higher fresh weight. Comparison of sucrose levels showed that the 30 g/L concentration improves callus proliferation in pineapple. Commonly used in *in vitro* culture, the concentration of 30 g/L remains optimal for the proliferation of calli in pineapple. In view of these results, we can say that the proliferation of calli in pineapple (*Ananas comosus* var. Smooth Cayenne) depends on the nature and

content of the carbon source. Galactose and maltose were less favorable to proliferation.

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