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Callogenesis and Somatic embryogenesis induction in *Hevea brasiliensis*: effects of fruit shelf-life and carbon source

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An experiment was conducted to study the effect of the fruit shelf-life and the concentrations of carbon source on somatic embryogenesis *via* callogenesis of *Hevea brasiliensis*. Fruits harvested were stored at 15°C during 1, 5, 7, 12 and 15 days and the inner integuments obtained from seed were used as explants. The experiments were performed under carbon source treatments with three concentrations for glucose and five concentrations for sucrose. Under these conditions, the percentage of explants forming calli was better during the first week of fruit preservation regardless of the carbon source but at high concentration. However, beyond 7 days of fruits shelf-life, sucrose is best to induce callus unlike glucose but with high concentration. The best rate of embryogenic calli was also obtained with sucrose. The percentages of callogenic explants and embryogenic calli have decreased sharply with the shelf-life of fruit at 15°C. So, to maintain an embryogenic potential of explants in situations of long-term conservation of fruits, sucrose can be used at 234 mM of concentration or default at 111 mM sucrose. These sucrose concentrations are conducive to induce embryogenic calli with explants coming to rubber fruits after a long time of preservation.

Key words: callogenesis, carbon source, fruit, *Hevea brasiliensis,* shelf-life, somatic embryogenesis

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Introduction

Hevea brasiliensis Muell. Arg., the principal source of natural rubber, is an open pollinated crop belonging to the Family Euphorbiaceae. It is intensively cultivated in South East Asia. The main producers of natural rubber in the world are Thailand, Indonesia, Malaysia, India, Vietnam, China, Ivory Coast and Sri Lanka, in descending order of production (Anonymous, 1981; 2007). Côte d'Ivoire has produced 165,000 t of natural rubber in 2005 and is the first African producer of natural rubber and seventh worldwide (Apromac, 2005). The milky liquid (latex) that oozes from any wound to the tree bark contains about 30 percent rubber. The higher strength, low heat build up, better resistance to wear and flex cracking make natural rubber a suitable raw material for the manufacture of heavy duty automobile tyres. Therefore, the global demand for natural rubber is steadily increasing and hence the production of rubber needs to be increased to meet the demand. The genetic base of rubber is very narrow as it originated from about 10 mother trees grown from the 70,000 odd seeds collected by Wickham in 1876 on the banks of the Tapajo in Para, Brazil (Schultes, 1977; Nayanakantha and Seneviratne, 2007). Rubber plantations were originally established with unselected seedlings, which resulted in considerable hetero-geneity. From 1920 onwards, research was carried out along several lines to improve the quality of plant material. Rubber plantations are constituted of grafted clones. Comparisons between these grafted clones and elites seedlings showed that the best seedlings display a superior growth and production than grafted clones. In fact, the average production of a grafted tree is estimated to 7.5 kg a year of dry rubber against 30 kg/tree/year for one seedling (Djikman, 1951). Moreover, a half of grafted trees provide 70 to 80 % of production of a parcel (Langlois, 1965). A part of this difference is attributed to the interaction between the rootstock and the scion (Carron *et al*., 1989). To overcome these various constraints that are the heterogeneity of clonally plantations and the decline of vigor and production bound to the grafting, the somatic embryogenesis way was envisaged. Since it was used with the inner tegument of rubber's immature seed, the somatic embryogenesis has recorded some progress regarding the use of growth regulators. The efficiency of various somatic embryogenesis protocols described for rubber depends on the cultivars, some of them being recalcitrant to in vitro culture (El Hadrani *et al*., 1991; Carron *et al*., 1995). One of the major bottlenecks in somatic embryogenesis procedures is the production of primary calli. In rubber, the carbon source commonly used in somatic embryogenesis induction is sucrose. Paranjothy and Ghandimathi (1975) showed that 292 mM sucrose clearly promoted the calli growth compared to other carbon sources. Carron (1982) indicated that up to 234 mM, the growth of calli was significantly correlated to the increase of sucrose concentration. In addition, sucrose was no longer a limiting factor and its accumulation in the medium did not cause any significant increase in the size of the crop. Montoro *et al*. (1993) found that 351 mM sucrose in combination with appropriate concentrations of growth regulators favored the friability of the callus. Carron *et al*. (1995) reported the fugacity of the embryogenic capacity and the low rate embryos conversion into plantlets. It is therefore of importance to optimize the somatic embryogenesis conditions in rubber which is generally considered to be recalcitrant with regard to somatic embryogenesis (El Hadrani *et al*., 1991). Moreover, much research seems to consider the fruit shelf-life and carbon source as key factors for devising efficient protocol to somatic embryogenesis pathway of rubber (Sushamakumari *et al*., 2000; Kumari *et al*., 2001; Blanc *et al*., 2002). The explants currently used to somatic embryogenesis induction are the inner integument of the immature seed fruit of

eight to ten weeks (Carron and Enjarlic, 1985). However, in tropical countries, rubber fruit – the source of explants – are not available all the year. Moreover, the immature fruit stage lasts only a few days. The availability of the source of explants is often carried out by picking a large amount of fruit followed by their preservation. This raises the issue of the influence of the shelf-life of the harvested fruit but also the interaction between this shelf-life and culture medium on the production of somatic embryos.

Therefore, the aim of the present study is to determine the influences of the fruit shelf-life and the concentrations of carbon source on the induction somatic embryogenesis *via* callogenesis.

MATERIALS AND METHODS

Plant material and preparation of explants

Plant material was implemented from the inner integuments obtained from seed of immature fruit of rubber. Fruits were obtained after 8 to 10 weeks of anthesis with the PB 260 clones of rubber. Fruits harvested in plantations in the Centre National de Recherche Agronomique (CNRA) of Côte d'Ivoire between May and June. In the laboratory, fruits collected were stored in a refrigerator at 15 °C during 1, 5, 7, 12 and 15 days before any utilization. These fruits were disinfected with sodium hypochlorite (2.45 % of active chlorine) for 30 minutes followed by 3 washings within sterile distilled water. Seeds were then extracted by section of the fruit and their inner integuments were aseptically cut into fragments of about 5 x 3 x 1 mm. Fragments were used as explants and transferred onto Petri dishes containing 30 ml of medium culture.

Embryogenic calli induction

The experiments were performed with a modified Murashige and Skoog basal medium (MBm) containing 30 µM $AgNO_3$ and Fossard vitamins (Fossard, 1976; Kouassi *et al*., 2008) without choline chloride. This medium was supplemented with 9.0 µM of 2,4-dichlorophenoxyacetic acid (2,4-D) and allowed the preparation of eight media varying by the concentrations of carbon source (sucrose or glucose) and 2.25 µM of kinetin for all media, with exception of M5 culture medium wherein the concentration of kinetin was 3.375 µM. Influences of fruit shelf-life and carbon source on callogenesis were evaluated on media containing sucrose and glucose at different concentrations. Sucrose was used at 58.5 mM (M1), 111 mM (M2) and 175 mM (M3) 175 mM (M3) and 234 mM (M4 and M5). In addition, glucose was used at 55.5 mM (M6), 111mM (M7) and 166.5 mM (M8). The use of fruits according to their shelf-life was recorded in Table 1.

Table 1. Fruits shelf-life at 15 °C and carbon source on callus induction of *Hevea brasiliensis*

Culture medium	Sucrose (mM)	Glucose (mM)	Shelf-life of fruit (day)
M1	58.5	-	1 - 12 - 15
M2	111	-	1 - 12 - 15
M3	175	-	1 - 12 - 15
M4	234	-	1 - 12 - 15
M5	234	-	5 - 7 - 12 - 15
M6	-	55.5	5 - 7 - 12 - 15
M7	-	111	1 - 12 - 15
M8	-	166.5	5 - 7 - 12 - 15

All media were solidified with 2 g/L gelrite, subjected to pH 5.8 before autoclaving (120°C). Explants were incubated at 27±2°C under darkness for callus induction. Percentage of callogenic explants (PCE) was assessed after 25 days of culture and calculated as number of calli forming by total number of explants cultured. Responding calli were transferred to embryogenesis expression medium

containing 2,4-D (1.35 µM), benzyladenin (1.35 µM) and abscissic acid (5.10^{-3} µM) for proembryos induction. Calli cultures were incubated at 27±2 °C under darkness during 25 days (first subculture). Calli derived from each explant were maintained separately on embryogenesis expression medium. Thereafter, healthy calli were sub-cultured on medium having a concentration of 1.8 µM 2,4-D and low concentration of BA (0.8 µM) with 25 days of incubation (2nd subculture). Embryogenic calli were then transferred to the embryo development medium during 25 days at 27 ± 2 °C under darkness (3rd subculture). The basal components of this medium were identical to those of embryogenesis expression medium but without growth regulator. Percentage of embryogenic calli (PEC) was evaluated after 3 times of culture with intervals of 25 days and calculated as number of mature embryogenic calli forming by total number of calli cultured.

Experimental design and statistical analysis

The experiments were conducted randomly with ten replications per treatment. Thirty explants were cultured per treatment and data was pooled from independent experiments. Data were subjected to analysis of variance (ANOVA) using XLSTAT 7.5.3 program and significant differences among treatments were compared using Duncan test at 5 %. Fisher Protected LSD test at $P < 0.01$ level of significance was used.

RESULTS AND DISCUSSION

Effect of fruit shelf-life and carbon source on callogenesis

Fruit shelf-life and carbon source have explained about 97 % of the variability observed during callus formation (data not show). These variables influenced significantly ($P < 0.0001$) the percentage of explants forming calli as well as their interaction when sucrose (58.50 mM) and glucose (55.50 mM) are used with almost similar concentration. Thus, the percentage of callogenic explants decreased with the fruit shelf-life during explants incubation on medium supplemented with sucrose. So, the percentage of explants forming calli after one day (88.12 %) was more important than those obtained after 12 days (42.24 %). On contrary, the percentage of explants forming calli after 15 days of fruit shelf-life is the lowest statistically (Table 2). Regarding with medium containing glucose, the evolution of results is similar. Indeed, the percentage of explants forming calli which is 76.11 % for 5 days of fruit shelf-life is higher but statistically identical to the 7 days of fruits preservation (71.66 %).

Table 2. Effect of shelf-life fruits of *Hevea brasiliensis* on callogenesis from the inner integuments

Carbon source	Shelf-life of fruits at 15 °C (days)	Explant forming callus (%)
Sucrose	1	88.12 ± 1.68 a
	12	42.24 ± 7.03 b
58.5 mM	15	16.67 ± 4.27 c
Glucose	5	76.11 ± 2.74 a
	7	71.66 ± 3.55 a
55.5 mM	12	20.61 ± 3.30 b
	15	12.17 ± 3.53 c

Means followed by a different letter are significantly different at P=0.05 (Duncan's test);
± s.d: standard deviation; each value represents the mean of three replicates.

In other hand, for 12 days of fruit shelf-life, only 20.61 % of explants induced calli and 12.17 % for explants from fruit preserved during 15 days at 15 °C. With carbon sources provided at the same concentrations, the first week (1 - 7 days) of fruit preservation is not fatal and best rates of callogenic explants were obtained (71.66 - 88.12 %). All explants used give statistically identical percentage callus induction.

However, after 12 days of fruits shelf-life, the explants which are collected have a potential for callus induction significantly lower and that increases when the shelf-life of fruits reached 15 days. The percentage of callogenesis was decreased also proportionally to the shelf-life of fruits, which means that there is a negative correlation between shelf-life of fruit and calli induction. The decrease of percentage of explants forming calli observed from 12 days of fruit preservation could be explained by the change of physiological state of the explants. It is assumed that after a relatively long shelf-life, the explants were no longer able to assimilate as much sugar as they did during the first days of fruit preservation. These results are in agreement with those of Zouzou *et al*. (1997) and Koné *et al*. (2004) which showed that the ability of explants forming callus in cotton decreases with cultured explants age. Thus, when the shelf-life of fruits is low, i.e. youth explants, rubber callogenesis is important. Otherwise, when the sugar content is not fixed there is a variation in the rate of callus formation depending on the concentration as well as of the carbon source type (Table 3). This would mean that the percentage of explants forming calli varied with the quality and quantity of the carbon source as already reported by Zouzou *et al*. (2008) and Sié *et al*. (2010) in cotton and hibiscus, respectively.

Table 3. Effect of carbon source (sucrose and glucose) concentration and fruit storage time on callogenesis from the inner integuments of *Hevea brasiliensis*

	Explants forming callus (%)				
	Shelf life at 15 °C (days)				
	1	5	7	12	15
Sucrose (mM)					
58.5 (M1)	82.81±4.21 ab	-	-	70.00±2.62 c	34.17±1.54 d
111 (M2)	87.04±3.98 a	-	-	46.67±1.45 d	01.17±0.12 f
175 (M3)	89.45±4.29 a	-	-	39.17±1.33 d	20.00±1.87 e
234 (M4)	91.67±6.74 a	-	-	04.17±0.31 f	00.00±0.00 f
234 (M5)	89.60±3.68 a	-	-	51.20±2.26 d	28.00±1.63 e
Glucose (mM)					
55.50 (M6)	-	70.00±5.04 a	73.33±4.86 a	24.00±1.52 c	13.33±1.30 d
111 (M7)	-	-	-	20.83±1.64 ce	11.17±0.26 d
166.50 (M8)	-	82.22±4.77 b	70.00±3.34 a	17.00±2.11 e	12.00±1.33 e

Means followed by a different letter are significantly different at P=0.05 on a line and column (Duncan's test); ± s.d: standard deviation. Each value represents the mean of three replicates.

However, all the explants derived from the fruits of one day old storage at 15 °C gave callus rate largest regardless of the sucrose concentration used. In addition, with glucose, the percentage of explants forming calli is higher when explants are 5 or 7 days of age. The highest concentration (166.50 mM glucose) gave the percentage of callus induction most significant. Moreover, when the fruits were preserved for 12 or 15 days, only the highest concentrations of sucrose gave the best percentages of calli formation. With glucose results evolve in the same direction, but the percentage of callus induction was statistically lower compared to those obtained with sucrose. So, in a

situation of long time preservation of fruit (12 to 15 days), the percentage of explants forming calli was relatively high with sucrose compared to glucose. Therefore, the effect of glucose was not significant in long time storage situation of fruits. These results show a clear influence of the rate and the type of carbon source used and the fruit shelf-life on callus induction in rubber. Thus, during the first week of storage fruit, the type of carbon source has no influence on the percentage of callus induction. However, it would be best to induce callus with high concentration of carbon source. In the other hand, beyond 7 days of fruits shelf-life, sucrose seems to be best to induce rubber callus with low concentration. Sucrose containing glucose and fructose would therefore provide more energy and carbon (12 carbons) than glucose which contains only 5 carbons. The difference between M4 and M5 media shows the positive influence of high concentrations of kinetin on callus induction Zouzou *et al*., 1999; Sié *et al*., 2010; Yapo *et al*., 2011). It is worth noting that despite everything, almost all concentrations of glucose and sucrose could induce callus. This justifies the fact that these two carbon sources were described by several authors as sugars which are beneficial induction of callus (Chua, 1966; El Maataoui *et a*l., 1998; Zouzou *et al*., 2000).

Influence of fruit shelf-life and carbon source on embryogenesis

Fruit shelf-life and carbon source explained 78 % of the variability observed during embryogenic calli induction (data not show). These two variables have significantly effect (P = 0.008) on embryogenic calli formation. This influence has led to a drop of the percentage of embryogenic calli (from 46.95 to 15.28 %; in M5 medium) between 5 and 15 days of fruit preservation. The decrease of percentage of embryogenic calli induction could be explained by the change of explants physiological state. It is assumed that after a relatively long shelf-life of fruit, the explants were no longer able to assimilate as much sugar as they did during the first days of fruit preservation. This should result in low sugar assimilation and thus a low callogenesis; the same effect should cause consequently a low rate of embryogenic calli formation. As regards the influence of the carbon source on embryogenesis, with the exception of medium containing 58.5 mM sucrose (M1), all media tested were produced embryogenic calli. The medium containing 111 mM glucose (M7) gave 6.25 % of embryogenic calli whereas with medium supplemented of 111 mM sucrose (M2) the percentage of embryogenic calli was 42.93 % at one day of fruit shelf-life. Regarding to these result (Table 4), we can conclude that the best rate of embryogenic calli recorded was 46.74 % on medium containing 234 mM sucrose (M5). This is explained by the facts that explants which have 12 days old have the same embryogenic capacities as explants derived from fruit stored for one day unlike in other culture media. The results confirm the best embryogenic potential of sucrose compared to glucose as reported by Blanc *et al*. (2002). However, it is worth noting that Montoro *et al*. (1993) have mentioned the effect of sucrose on somatic embryogenesis from the inner integument. They reported that 117 mM sucrose gave 2.60 % of embryogenic calli and 234 mM sucrose gave 36.2 % of embryogenic calli.

Moreover, Kouassi et al. (2008) using unpollinated ovules as explants obtained 19.23 and 32.78 % of embryogenic calli in medium containing 111 mM and 234 mM sucrose respectively. These results are below those that of this study; this indicates that M2 and M5 media are conducive to the induction of embryogenic calli with older explants (Kumari and Thulaseedharan, 2004). Sucrose has been shown to be highly embryogenic carbon source (Millam and Davidson, 1993; El Maataoui *et al*., 1998; Thomas, 2006; Zouzou *et al*., 2008).

Table 4. Effect of interaction between carbon source and shelf-life fruits of *Hevea brasiliensis* on embryogenic calli induction

Shelf-life at 15 °C (days)	Embryogenic callus (%)		
	Sucrose (mM)		Glucose (mM)
	111 (M2)	234 (M5)	111 (M7)
1	42.93 ± 2.14 a	-	6.25 ± 0.24 b
5	-	46.95 ± 1.78 a	-
7	-	46.80 ± 2.15 a	-
12	38.33 ± 1.47 c	46.74 ± 1.73 a	2.31 ± 0.34 d
15	20.60 ± 1.12 d	15.28 ± 1.80 d	1.95 ± 0.11 a

Means followed by a different letter are significantly different at P = 0.05 on a line and column (Duncan's test); ± s.d: standard deviation; Each value represents the mean of three replicates.

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