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Effect of antioxidants on the callus induction and the development of somatic embryogenesis of cocoa [*Theobroma cacao* (L.)]

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Abstract

The browning of plant tissue and organs is a major constraint in tissue culture of cocoa (*Theobroma cacao* L.). This study aims to evaluate callogenic and embryogenic potentialities of three genotypes cocoa on culture media supplemented with different types and concentrations of antioxidants. Polyvinylpyrrolidone (PVP), ascorbic acid, silver nitrate and cysteine were used as antioxidant agents. The explants staminode and petal were excised from immature buds of the flowers of genotypes cocoa C1, C8 and C14. The results showed that the induction rate of callus derived from the petal and the staminode varied with the genotypes tested. Except 21 mg/l of silver nitrate, the others antioxidants used namely ascorbic acid, PVP and cysteine have enhanced the callus induction of the genotype C8. In comparison with the control, the callus browning of the three genotypes was reduced two to three times when the culture media were supplemented with various concentrations of the silver nitrate. After 72 days of culture, somatic embryos were induced on callus derived from the petal explants with all the studied genotypes. Addition of PVP (300 mg/l) in the induction medium improved the rate of somatic embryos of the genotypes C1 and C14. The improvement of responses to somatic embryogenesis in reducing the rate of browning of cultures will allow a mass production of the high-yield cocoa genotypes.

Keywords: *Theobroma cacao*, Somatic embryogenesis, antioxidants, phenolic compounds, staminode, petal.
Abbreviation: PVP_ Polyvinylpyrrolidone; PCG_Primary callus growth medium; SCG_secondary callus growth medium; ED_ embryo development medium; MS_Murashige and Skoog (1962) medium.

Introduction

Cocoa (*Theobroma cacao* L.) is the subject of major interest worldwide. It is grown mainly for its beans used as raw materials in the manufacture of food, pharmaceutical and cosmetics (Dillinger et al., 2000). Cocoa product contains phenols, flavonoids and its antioxidant activity is higher than black tea, green tea or red wine (Subhashini et al., 2010). In the last decade, its consumption has increased due to the functional properties conferred by these antioxidant polyphenols. The beneficial effects of cocoa on cancer, diabetes control, cardiovascular diseases and the prevention of hepatitis have been reported by several authors (Rusconi and Conti, 2010; Sarmadi et al., 2011, 2012). Cocoa has an important economic role as a source of foreign exchange in numerous tropical countries including Côte d'Ivoire and thereby, crop provides a substantial income to the smallholders in the tropics (Alemanno et al., 2003). Even though global cocoa demand has increased significantly, its production is not yet sufficient and there is a world deficit in cocoa supply in recent years (International Cocoa Organization, 2014). Global climate change, pest and disease infestation and reduce plant productivity because of aging have caused world cocoa production instability (Ajijah et al., 2016). Cocoa is well known for its genetic variability due to its natural propagation system (allogamous), which generates a high degree of yield variation (Maximova et al., 2002). Seeds are one of the main sources of genetic variation in cocoa because they are, in most cases, the result of cross linking between two genotypes. To minimize the negative effects of seed propagation, traditionally the crops are propagated by asexual propagation, such as rootstocks or grafting. However, these techniques have been described as inefficient and costly (Figueira and Janick, 1995). A potential solution to these problems could be found using selected tissue culture techniques. In this context, somatic embryogenesis is the most frequently adopted regeneration method, which has been used not only for plant propagation but also for genetic engineering (Loyola and Vasquez, 2006), virus eradication (Quainoo and Dwomon, 2012a) and germplasm preservation (Fang et al., 2004; Juarez, 2012). Although somatic embryogenesis has been reported for several cocoa varieties (López-Baez et al., 2001; Maximova et al., 2002), a low conversion rate of somatic embryos into normal plants has remained a problem, limiting the utilization of embryogenic cells for genetic transformation and plant

breeding (Suarez and Bozhkov, 2008). According to Alemanno et al. (2003), this low conversion rate of embryos resulted from a significant secretion of phenolic compounds by flower explants. Phenolic secretions and other exudates in plants tissue culture systems lessen explant initiation, growth, and development. Many perennial plants, such as cocoa, are notorious for their ability to produce secondary products, many of which may be phenolic in character. Polyphenol presence has also been associated with *in vitro* recalcitrance (Bailey- Serres and Mittler, 2006). Moreover, consistent with Alemanno et al. (2003), somatic embryos that regenerated were free of inner polyphenols, and only small amounts were distributed in the periphery of the embryo epidermal layer, while in the non-regenerating variety, embryogenic calli were compact and such structures contained polyphenols randomly distributed across all tissues. Embryogenic capacity, therefore, seems to be associated with a balanced concentration and distribution of polyphenols (Gallego et al., 2016). High concentrations are associated with the non-regenerating response of somatic embryos (Alemanno et al., 2003). The oxidation of phenolic compounds could be associated with factors such as wound response, sterilization process and specific components of the tissue culture media (e.g., metal cations), among others (Benson, 2000; Lattanzio et al., 2006). The production of phenolic compounds results in a particular phenomenon called "tissue browning" (Khosroushahi et al., 2011). The tissue browning event involves many toxic compounds through the phenolization process eventually resulting in the necrosis of cells (Murata et al., 2001; Wu and Lin, 2002). The use of antioxidants in culture medium could prevent or reduce the oxidation of phenolic compounds. Thus, the frequency of embryogenic callus induction and the rate of somatic embryos from cocoa explants could be increased (Dubois, 2013). The effectiveness of antioxidants on callus induction and somatic embryos development was already shown in *Taxus brevifolia* (L.) (Khosroushahi et al., 2011) and *Mangifera indica* (L.) (Litz et al., 1982). Furthemore, phenolic compounds production in cell suspension cultures of Mango ginger (*Curcuma mangga*) was most effectively controlled by the incorporation of ascorbic and citric acid as antioxidants (Sundram et al., 2012). In cocoa, to our knowledge there is no report regarding the effect of antioxidants application in controlling lethal browning. Therefore, the objective of the reported work was to investigate the effects of antioxidants on the induction of callus and the somatic embryogenesis development.

Results

Callus induction and tissue browning intensity

Two weeks after the culture establishment, callus formation was observed in both explants tested. These calli were vitreous granular-white and brown granular (figure 1). The experimental values for the induction rate of callus were shown in Table 1. Analysis of the results shows that the addition of antioxidants in PCG medium did not influence the rate of callogenic explants expressed with the genotypes C1 and C14. Indeed, the induction rates of these calli (RCI) were statistically similar and the rates varied from 82 to 100 % for C1 and from 88 to 100 % for C14. With the genotype C8, except 21 mg/l of silver nitrate, the concentrations of the other antioxidants have significantly improved the induction rates of callus derived from staminodes and petals.

Table 2 shows the effect of antioxidants on the browning of the calli produced by the tested genotypes. The calli produced on culture media containing no antioxidants exhibited the highest intensity of browning. The reduction of the callus browning varied according to the type and the concentration of antioxidants. Among the antioxidants used, silver nitrate allowed a significant reduction of the browning of callus derived from petal and staminode whatever the cocoa genotype.

Effect of antioxidants on the induction of somatic embryogenesis

30 to 40 days after callus formation, induction of embryogenic structures was observed on the SCG medium followed by a subsequent development of somatic embryos on the ED medium. The induced somatic embryos were characterized by asynchronous developmental stages (figure 1). In Table 3, the rate of embryogenic callus developed by the tested genotypes depending on the type and concentration of the antioxidant were recorded. The results indicated that only the explants petals have exhibited embryogenic callus formation with the three genotypes. Culture media containing silver nitrate did not produce any embryogenic calli. Somatic embryogenesis occurred on calli initiated on culture media supplemented with ascorbic acid, cysteine and PVP. Among these antioxidants, the PVP (300 mg/l) significantly enhanced the induction rate in somatic embryogenesis of the genotypes C1 and C14.

Discussion

In this work, all treatments induced callus formation, in both staminodes and petals. Addition of antioxidants in the induction media has influenced the rate of callogenic explants in genotype C8 but not in C1 and C14. Thus, there was a variability of responses to callogenesis induction in respect to the genotype, the type and the concentration of the antioxidants. This also suggests that phenolic compounds are more synthesized in genotype C8 in comparison to the other two genotypes. The beneficial effect of phenolic compounds during the callogenesis with genotype C8 could be explained by the fact that these antioxidants have acted synergistically with growth regulators in the culture medium to induce callus formation on the surface of explants. This synergistic action seems specific to genotype C8. With this genotype, in addition to their role as antioxidants, they would be directly involved in cell division and cell elongation (Sujatha and Mukta, 1996). Phenolic secretions and other exudates in the plant tissues culture systems diminish the initiation, growth and development of the callus on the surface of explants. The antioxidants were tested in the present investigation to minimize lethal browning of the cocoa callus during the development of somatic embryos. The results showed that most of the explants on medium without antioxidants were brown. The browning of callus often correlates with excessive accumulation of phenolic compounds (Dubravina et al., 2005). Thus, the accumulation of phenolic compounds in the culture medium adversely affects the growth and survival of explants under in vitro conditions. Roussos and Pontikis (2001) and Arnaldos et al. (2001) showed that the accumulation of phenolic compounds results in browning and possibly to the death of organs and tissue grown in vitro. Low intensities of the callus browning were observed when the silver nitrate has been used as an antioxidant in culture medium. This product would have prevented the accumulation of toxic substances resulting from the oxidation of phenolic compounds or reduces the explants exudation in sectioned portions.

Table 1. Effect of different types and concentrations of antioxidants on callus induction from petals and staminodes in cocoa genotypes coded C1, C8 and C14.

Genotypes	Antioxidants	Concentrations (mg/l)	Rates of callus induction (%)	
			Staminodes	Petals
C1	Control	0	96 ± 1.63 ^{ab}	100 ^{a}
	Ascorbic acid	20	100 ^{a}	100 ^{a}
		100	96.55 ± 4.44 ^{ab}	94.17 ± 0.15 ^{ab}
		180	97.14 ± 2.85 ^{ab}	97.66 ± 1.33 ^{ab}
	Silver nitrate	7	87.39 ± 6.80 ^{abc}	95.13 ± 3.69 ^{ab}
		14	84.44 ± 15.55 ^{abc}	98.61 ± 2.38 ^{ab}
		21	80.57 ± 6.14 ^{bc}	96.9 ± 1.67 ^{ab}
	Cysteine	8	96.71 ± 4.28 ^{ab}	96.33 ± 11.43 ^{ab}
		16	95.22 ± 4.30 ^{ab}	95.46 ± 3.91 ^{ab}
		24	97.66 ± 1.33 ^{ab}	98,.57 ± 1.42 ^{ab}
	PVP	150	90.57 ± 4.20 ^{ab}	100 ^{a}
		300	96.45 ± 4.54 ^{ab}	95.65 ± 3.45 ^{ab}
		450	100 ^{a}	100 ^{a}
C8	Control	0	53.22 ± 15.55 ^{e}	75.05 ± 12.95 ^{cd}
	Ascorbic acid	20	97.33 ± 2.66 ^{ab}	98.57 ± 1.42 ^{ab}
		100	95.23 ± 3.76 ^{abc}	91.05 ± 3.49 ^{abc}
		180	96 ± 4.00 ^{ab}	97.33 ± 2.66 ^{ab}
	Silver nitrate	7	73.96 ± 9.86 ^{bcd}	89.95 ± 4.89 ^{abc}
		14	86.58 ± 9.41 ^{abc}	88.16 ± 7.95 ^{abc}
		21	68.58 ± 10.68 ^{cde}	80.58 ± 9.57 ^{bcd}
	Cysteine	8	96.04 ± 2.88 ^{ab}	95.02 ± 1.84 ^{abc}
		16	100 ^{a}	93.33 ± 3.84 ^{abc}
		24	100 ^{a}	92.92 ± 3.46 ^{abc}
	PVP	150	96.66 ± 1.92 ^{ab}	98.33 ±1.66 ^{ab}
		300	100 ^{a}	100 ^{a}
		450	100 ^{a}	100 ^{a}
C14	Control	0	100 ^{a}	91,23 ± 8.09 ^{abc}
	Ascorbic acid	20	100 ^{a}	96.55 ± 4.44 ^{abc}
		100	93,20 ± 7.79 ^{abc}	100 ^{a}
		180	100 ^{a}	90.09 ± 10.90 ^{abc}
	Silver nitrate	7	100 ^{a}	97.5 ± 2.41 ^{abc}
		14	94.63 ± 4.03 ^{abc}	94.33 ± 6.66 ^{abc}
		21	90 ± 8.00 ^{abc}	100 ^{a}
	Cysteine	8	98.90 ± 1.09 ^{ab}	100 ^{a}
		16	100 ^{a}	98.97 ± 1.02 ^{ab}
		24	100 ^{a}	96.61 ± 2.38 ^{abc}
	PVP	150	100 ^{a}	100 ^{a}
		300	100 ^{a}	100 ^{a}
		450	98.33 ± 1.66 ^{abc}	98.33 ± 1.66 ^{ab}

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 PVP: polyvinylpirrolidone; Observations were made after 28 days of culture.

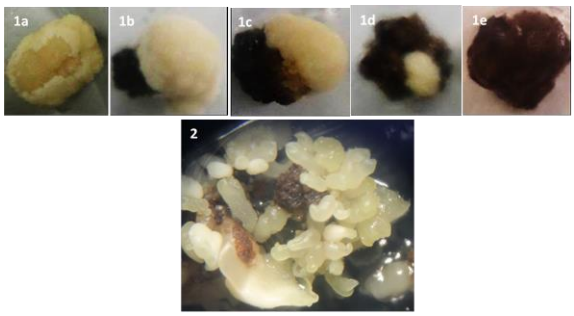

Fig 1. Callogenic explants and embryogenic calli of cocoa. Callogenic explant displaying criteria for browning intensity assessment. Without browning (1a) and with different browning intensities: 1-25 % (1b); 26-50 % (1c); 51-75 % (1d); 76-100 % (1e).
(2) Embryogenic calli induced from petal of cocoa genotype C1 on medium supplemented with 300 mg/l PVP.

Table 2. Effect of different antioxidants types and concentrations on browning of callogenic explants in cocoa genotypes coded C1, C8 and C14.

Genotypes	Antioxidants	Concentrations (mg/l)	Callus browning rating*	
			Staminodes	Petals
C1	Control	0	2	3
	Ascorbic acid	20	2	3
		100	1	2
		180	2	3
	Silver nitrate	7	0	1
		14	1	2
		21	0	1
	Cysteine	8	1	2
		16	2	3
		24	1	3
	PVP	150	1	3
		300	1	2
		450	1	1
C8	Control	0	3	4
	Ascorbic acid	20	4	3
		100	3	3
		180	1	2
	Silver nitrate	7	0	1
		14	1	1
		21	1	1
	Cysteine	8	2	3
		16	0	3
		24	3	3
	PVP	150	2	2
		300	1	3
		450	1	3
C14	Control	0	2	3
	Ascorbic acid	20	2	4
		100	2	3
		180	1	2
	Silver nitrate	7	0	1
		14	0	1
		21	0	1
	Cysteine	8	1	2
		16	1	3
		24	1	3
	PVP	150	1	2
		300	1	3
		450	0	3

*In columns four and five, 0 corresponds to no browning of the callus, 1 corresponds to the callus with 1-25 % of browning, 2 corresponds to the callus with 26-50 % of browning, 3 corresponds to the callus with 51-75 % of browning, 4 corresponds to the callus with 76-100 % of browning. PVP: polyvinylpirrolidone; Observations were made after 28 days of culture.

Table 3. Rate of embryogenic calli in cocoa genotypes coded C1, C8 and C14 from cultures on induction media supplemented with different types and concentrations of antioxidants.

Genotypes	Antioxidants	Concentration (mg/l)	Rates of embryogenic calli (%)	
			Staminodes	Petals
C1	Control	0	0.00	10.4 ± 2.3^{b}
	Ascorbic acid	20	0.00	0^{c}
		100	0.00	0^{c}
		180	0.00	6 ± 1.40^{bc}
	Silver nitrate	7	0.00	0^{c}
		14	0.00	0^{c}
		21	0.00	0^{c}
	Cysteine	8	0.00	5.67 ± 1.64^{bc}
		16	0.00	6.11 ± 1.89^{bc}
		24	0.00	5.89 ± 3.20^{bc}
	PVP	150	0.00	5.17 ± 3.16^{bc}
		300	0.00	20.16 ± 3.59^{a}
		450	0.00	0^{c}
C8	Control	0	0.00	2.20 ± 0.60^{a}
	Ascorbic acid	20	0.00	0^{c}
		100	0.00	0^{c}
		180	0.00	0^{c}

	Silver nitrate	7	0.00	0[c]
		14	0.00	0[c]
		21	0.00	0[c]
	Cysteine	8	0.00	1.67 ± 0.34 [b]
		16	0.00	0.10 ± 0.03 [bc]
		24	0.00	0.97 ± 0. 02 [bc]
	PVP	250	0.00	0.12 ± 0.16 [bc]
		500	0.00	2.15 ± 0.09 [a]
		1000	0.00	0 [c]
C14	Control	0	0.00	4.97 ± 2.44 [bc]
	Ascorbic acid	20	0.00	0[c]
		100	0.00	4.42 ± 1.42 [bc]
		180	0.00	0[c]
	Silver nitrate	7	0.00	0[c]
		14	0.00	0[c]
		21	0.00	0[c]
	Cysteine	8	0.00	0[c]
		16	0.00	0[c]
		24	0.00	0[c]
	PVP	150	0.00	5.17 ± 3.16 [bc]
		300	0.00	10.53 ± 2.59 [a]
		450	0.00	0 [c]

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 PVP: polyvinylpirrolidone; Observations were made after 72 days of culture.

The beneficial effect of silver nitrate on reducing the browning was also observed during the callus induction in tomato (Shah et al., 2014). Necrosis observed in rice callus and corn crops was reduced by adding silver nitrate into the culture medium (Ptak et al., 2010).

Induction of somatic embryogenesis was observed on callus derived from petal explants in genotypes C1, C8 and C14 of cocoa at variable rates. The response to somatic embryogenesis depends on the genotype, the explant and the callus induction medium. Genotypes C1 and C14 were more favorable to the somatic embryogenesis when the explants petal were placed onto ED medium. The influence of genotype in response to somatic embryogenesis of cocoa has also been reported (Issali et al., 2008b; Quainoo and Dwomon, 2012b). The embryogenic potential observed with the petal is in agreement with the results reported by Issali et al. (2008a). In contrary to our observations, many studies have rather reported an embryogenic potential higher with the staminode explant in cocoa (Li *et al*., 1998; Tan and Furtek, 2003).

Although silver nitrate has significantly reduced the intensity of callus browning, this antioxidant has not favored the induction of somatic embryos. There should therefore be a compromise between the level of callus browning and their ability to induce somatic embryos. Indeed, on their development medium (ED), embryos are usually induced at the expense of calli on browning phase. For all tested antioxidants, only PVP (300 mg/l) has improved the frequency of embryogenic calli in genotpes C1 and C14. This concentration is optimum for induction of somatic embryos of cacao with PVP. The use of the antioxidant PVP would avoid the accumulation of toxic phenolic compounds for the induction of somatic embryos.

Materials and Methods

Plant material

Flowers buds about 4 to 5 mm long were collected (early in the morning before 9.00 am) from trees of three elite genotypes coded C1, C8 and C14 from Centre National de Recherche Agronomique (CNRA) experimental farm in Divo (Côte d'Ivoire). Genotypes C1 and C14 are originated from Côte d'Ivoire while C8 is from Trinidad.

Preparation of explants

The flower buds were surface-sterilized by using 1 % (w/v) calcium hypochlorite for 20 min. They were then rinsed 4 times in sterile distilled water and afterwards sliced perpendicular to their longitudinal axis by using a sterile scalpel blade. The staminodes and petals were extracted with a sharp sterile forceps and these were used as explants.

Basal media

The basal culture media used were those for the induction and the development of somatic embryogenesis in cocoa by Driver and Kuniyuki (1984). These are the primary callus growth medium (PCG), the secondary callus growth medium (SCG) and the embryo development medium (ED). For this study, no changes were made to SCG and ED media.

Composition of induction media

Different concentrations of antioxidants namely, cysteine (8, 16 and 24 mg/l), polyvinylpirrolidone (PVP) (150, 300 and 450 mg/l), acid ascorbic (20, 100 and 180 mg/l) and silver nitrate (7, 14 and 21 mg/l) were added separately to medium PCG (Li et al., 1998). The medium used as control did not contain any antioxidant.

Culture conditions

The pH was adjusted to 5.8 into PCG medium or to 5.7 into SCG and ED media using solutions of 0.1 N NaOH or HCl. Culture media were solidified with Phytagel (2 g/l) and then autoclaved for 20 min at 121 ° C under a pressure of 1 bar. After sterilization, the culture media were poured in sterile Petri dishes under a laminar flow hood. Incubations were carried out in a culture room in continuous darkness with a temperature of 24 ± 1 °C and a relative humidity of 70 %. The Petri dishes were arranged out on the racks according to a completely randomized device.

Culture steps

The methodology for obtaining somatic embryos is that established by Li et al. (1998). Fifteen (15) staminodes and 15 petals used as explants were placed on induction medium called PCG. After two weeks of culture, explants were transferred onto SCG medium for a further growth. Twenty-eight (28) days after cultures induction, induced callus were transferred onto ED medium and subcultured every 28 days.

Evaluation of callus browning before transfer on ED medium

After 28 days of culture, the protocol of Coyne et al. (2012) was adapted and enabled to affect a rating according to the browning intensity. Rating 0 corresponded to callus without any browning while 1; 2; 3 and 4 corresponded respectively to 1-25 %, 26-50 %, 51-75 % and 76-100 % of browning (Fig. 2). The average value of browning rate was determined by: Average rating of browning = (Sum of browning rating / Total number of calli) x 100

Evaluation of callus and somatic embryogenesis induction

Callus induction was recorded on SCG medium after 28 days of culture whereas somatic embryogenesis was estimated on ED medium after 72 days of culture. The rate of callus induction (RCI) and the rate of embryogenic calli (REC) were calculated following the formulas:

- RCI = (Number of explants that induced calli / Total number of explants cultured) x 100
- REC = (Number of callogenic explants that induced somatic embryos / Total number of callogenic explants) x 100

Statistical analysis of data

The results were submitted to analysis of variance (ANOVA) with the Statistica 7.1 software. For unequal numbers, analysis of variance through the generalized linear model (GLM) was adopted. When a significant difference was observed between treatments, the multiple range test of Newman-Keuls at 5% threshold was used to separate the averages. For the rate assessment, a transformation Arc sin (p = proportion) was realized before performing the ANOVA tests.

Conclusion

Callus browning is reduced in the presence of antioxidants with varying intensities. The best results to control browning of cacao genotypes were obtained with all concentrations of silver nitrate. Addition of different antioxidants in the culture medium did not influence the responses to callus induction of the genotypes C1 and C14. However, these antioxidants have improved the callus induction rate with genotype C8. Induction of somatic embryogenesis was observed on calli derived from explants petals with the three genotypes of cocoa tested. Among the antioxidants tested, only PVP at 300 mg/l has improved the rate of somatic embryogenesis induction in the genotypes C1 and C14. The enhanced responses to somatic embryogenesis by reducing the rate of browning of cultures will allow the mass production of cocoa elite genotypes and genetic transformation of these important genotypes.

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