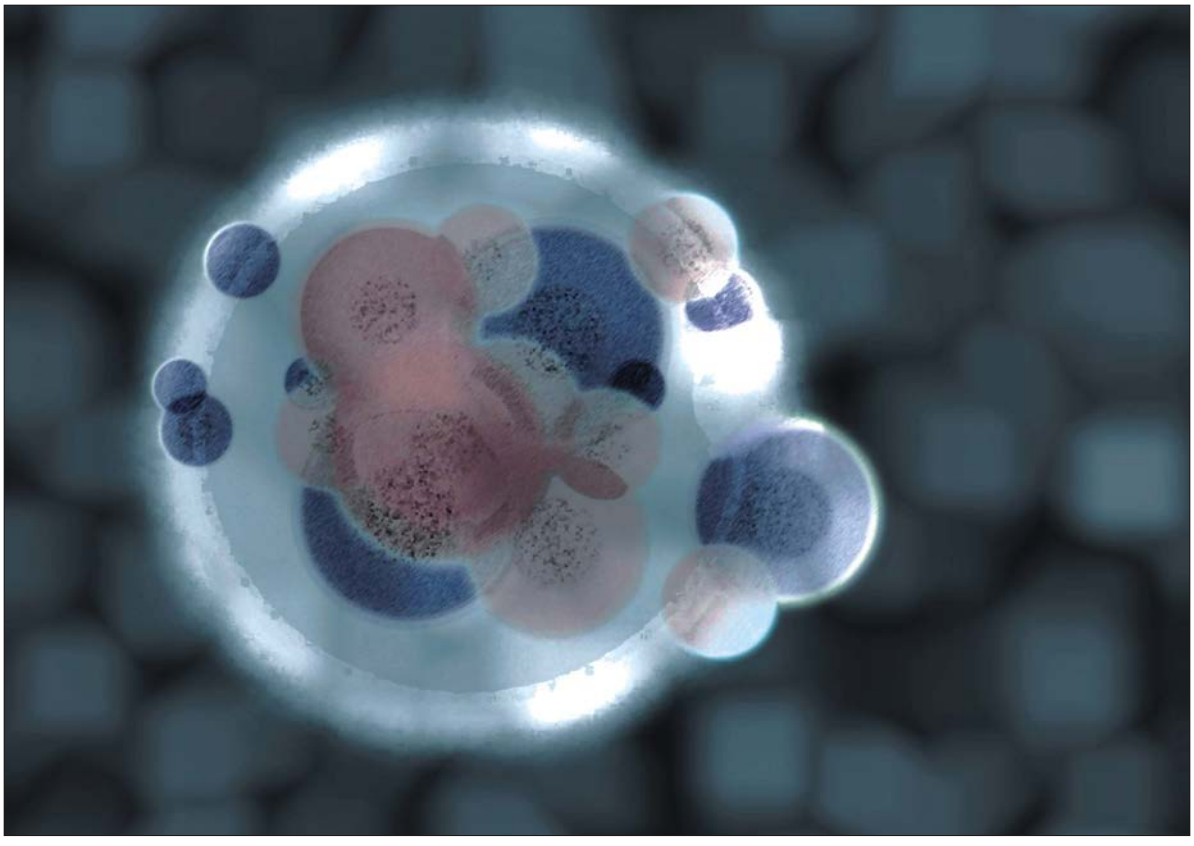

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Effect of Culture Media with Changes in Phenols Content and Soluble Peroxidases Activities During Somatic Embryogenesis in *Baillonella toxisperma* Pierre (Sapotaceae)

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Abstract: *Baillonella toxisperma* is a vulnerable multipurpose species threatened of extinction. The study is conducted in view of its domestication via somatic embryogenesis. Calli were induced from leaf explants in half-strength Murashige and Skoog or in half-strength Driver and Kuniyuki media, containing 2,4-dichlorophenoxyacetic acid (2,4-D) and Benzylaminopurine (BAP) combinations. The highest percentage of embryogenic calli formation (94%) was obtained in half-strength Driver and Kuniyuki medium with 0.5 mg L^{-1} 2,4-dichlorophenoxyacetic acid and 0.5 mg L^{-1} benzylaminopurine. Globular somatic embryos were induced from embryogenic calli in the same basal media supplemented with various 2,4-D concentrations. Maximum differentiation rate (100%) and maximum number per callus (50) of globular embryos were obtained in half-strength Driver and Kuniyuki medium supplemented with 2 mg L^{-1} 2,4-dichlorophenoxyacetic acid. After subculture of globular embryos in 2,4-dichlorophenoxyacetic acid and abscisic acid combinations in the same basal media, maximum differentiation rate (34%) and maximum number per callus (13.6) of bipolar somatic embryos occurred in half-strength Murashige and Skoog medium containing 0.5 mg L^{-1} 2,4-dichlorophenoxyacetic acid and 0.5 mg L^{-1} abscisic acid. For the best results recorded in both media, phenols content increased in globular embryos stage with high concentration of 2,4-dichlorophenoxyacetic acid and dropped in bipolar embryos stage with low concentration of 2,4-dichlorophenoxyacetic acid combined to 0.5 mg L^{-1} abscisic acid. At this late stage, peroxidase activities increased in half-strength Murashige and Skoog medium and decreased in half-strength Driver and Kuniyuki medium. Three peroxidases as highest number were detected in bipolar stage. Phenols and peroxidases may be playing key role in bipolar somatic embryos differentiation.

Key words: *Baillonella toxisperma*, nutrients, somatic embryos, phenols, peroxidases

INTRODUCTION

Baillonella toxisperma Pierre (Sapotaceae) or African pear wood is one of the trees that dominate the rainforests of Central Africa. It lives at the wild state and is scattered from Nigeria to Democratic Republic of Congo (White, 1983). The plant is used for several purposes. The sweet mesocarp of the fruit is edible and the local populations extract; an oil from the seeds for culinary and cosmetic uses (Vivien and Faure, 1985). In traditional medicine, the decoction of peel is used in the treatment of dental and low back pains and the oil is applied on the body to relieve rheumatic pains (Louppe *et al*., 2008). It offers a very resistant and lasting wood which is very appreciated and marketed by forest operators (Schneemann, 1995). The tree also offers several ritual uses (Schneemann, 1995; Louppe *et al*., 2008).

As a consequence of its multiple uses, *B. toxisperma* like number of African plant species undergoes enormous human pressures notably deforestation, barking and systematic hand-pick up of the seeds by local populations (Debroux, 1996). In addition, natural regeneration is submitted to many constraints. The seeds are subjected to various parasitic attacks and are more consumed by the rodents (Schneemann, 1995). The plantlet generally dies because of the bad conditions of brightness in the forest undergrowth (Debroux, 1996) and besides this, the first flowering intervenes very late when the plant is between 50 and 70 years old (Schneemann, 1995; White, 1983). From the preceding reasons, natural and autonomous

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regeneration of African pear wood is strongly limited. The species is classified among vulnerable species and is threatened of extinction in the ecological systems (Newton *et al*., 2003).

Somatic embryogenesis is the process whereby haploid or diploid cells regenerate complete plants through histodifferentiation patterns resembling zygotic embryos (Williams and Maheswaran, 1986). Formation of somatic embryos is now recognized as a useful method of clonal propagation, but somatic embryogenesis can also be used for plant regeneration from transformed cells, artificial seed production and for the study of plant embryogenesis (Von Arnold *et al*., 2002). Culture medium including plant hormones, macro and micronutrients plays a major role in this developmental process (Pinto *et al*., 2008). Like most biological processes it is also controlled by internal biochemical events. Phenols are then known to be essential in the control of induction and differentiation of somatic embryos (Cvikrova and Hrubcova, 1999). They are the main substrate of peroxidase and are potent to modify their activities (Ndoumou *et al*., 1997). Peroxidases promote auxin metabolism involved in cell differentiation and lignification which are related to embryos maturation (Passardi *et al*., 2005).

The aims of this study were; (1) to evaluate the effect of basal media and plant hormones in somatic embryogenesis of *Baillonella toxisperma* and (2) to analyze the role of phenolic compounds and peroxidases activities at different stages of cultures.

MATERIALS AND METHODS

Plant material: The study was conducted from July 2010 to February 2011. Young leaves of about 2 weeks after bud opening were harvested from plantlets obtained from germinating seeds collected in Nsimalen zone, suburb of Yaounde (Cameroon). They were washed with tap water and sterilized by successive soakings in 10% Mercryl Lauryle and 3% sodium hypochlorite solutions for 10 and 15 min, respectively and then rinsed three times with sterile distilled water for 5 min each. Leaf sections of about 1.5 cm^2 containing a median midrib were used as explants.

Culture media preparation and culture conditions: Two media were used: Half strength solid (Murashige and Skoog, 1962) mineral salts (MS/2) containing 4.5% sucrose, 0.6% agar and 1 mL L^{-1} Morel and Wetmore (1951) vitamins; or half strength solid Driver and Kumiyuki (1984) mineral salts (DKW/2) containing 250 mg L^{-1} glutamine, 100 mg L^{-1} myo-inositol, 20 g L^{-1} glucose, 25 µg L^{-1} TDZ, 2 g L^{-1} phytagel and 1 mL L^{-1} of DKW vitamin solution.

Embryogenic calli induction: Leaf explants were cultured for 28 days on MS/2 or on DKW/2 culture media supplemented with 0, 0.5, 1, 2 or 3 mg L^{-1} of 2,4-dichlorophenoxiacetic acid (2,4-D) associated to 0.5 mg L^{-1} benzylaminopurine (BAP). 300 leaf explants were cultured (5 per flask x 20 flasks x 3 repetitions).

Somatic embryos initiation: Friable calli obtained from 0.5 mg L^{-1} 2,4-D/0.5 mg L^{-1} BAP combinations were transferred for 60 days on the same enriched MS/2 or DKW/2 basal media without BAP and containing therefore only 0, 0.5, 1, 2 or 3 mg L^{-1} of 2,4-D to induce the formation of Globular Somatic Embryos (GSE). 180 embryogenic calli were cultured (5 per flask×12 flasks×3 repetitions).

Somatic embryos maturation: Globular Somatic Embryos of each treatment were subcultured during 97 days on the same previous culture media containing 0.5, 1, 2 or 3 mg L^{-1} 2,4-D supplemented always with 0.5 mg L^{-1} abscisic acid (ABA) to differentiate Bipolar Somatic Embryos (BSE). Total 120 calli with globular embryos were cultured (5 per flask×8 flasks×3 repetitions).

The pH of all culture media was adjusted to 5.6 with 0.1 N HCl or 1 N NaOH before autoclaving at 115°C for 30 min. The cultures were incubated at 25±2°C under the white fluorescent light (40 µmol m^{-2} sec^{-1}) of 16 h photoperiod.

Extraction and analysis of total phenolic compounds: One gram of fresh material (Embryogenic callus, Globular Somatic Embryos, Bipolar Somatic Embryos) was ground at 4°C in 3 mL of 0.1 N HCl. After incubation to 4°C during 20 min, the homogenate was centrifuged at 6000 g_n during 40 min. The supernatant was collected, the pellet re-suspended in 3 mL of 0.1 N HCl and centrifuged as previously. The two supernatant are mixed and constitute the crude extract of the soluble phenol. The quantity of phenol was determined spectrophotometrically using Folin-Ciocalteu reagent (Marigo, 1973). The reaction mixture containing 100 µL of extract, 2 mL distilled water, 200 µL Folin; 0.5 mL of 20 % Na_2CO_3 was incubated at 40°C for 20 min and absorbance read at 725 nm. A standard curve was established using chlorogenic acid. Total phenol content was expressed as mg g^{-1} of fresh matter weight.

Enzyme extraction and peroxidase assay: One gram of fresh material (EC, GSE and BSE) is ground in 2 mL cold Tris-maleate buffer 0.05 M, pH 7 containing mannitol 0.5 M. The homogenate was incubated at 4°C during 20 min then centrifuged at 6000 xg for 40 min. The supernatant was taken off and constitutes soluble fraction of proteins which was kept as aliquots at -4°C.

Peroxidases activities were measured spectrophotometrically at 420 nm according to Thorpe and Gaspar (1978). To the reaction mixture containing 1V of 2% H_2O_2, 5V of 1/15 M phosphate buffer pH 6.1 and 2V of 1% guaiacol as substrate, 10 µL of extract was added. Peroxidases activities were expressed as OD/min/µg FM.

Electrophoresis profiles analysis: Acidic peroxidase isozymes were separated by non-denaturing polyacrylamide gel electrophoresis (PAGE) adapted from McDougall (1991) and the gel was stained in a solution containing 0.06% (v/v) H_2O_2, 0.1% (w/v) benzidine and 0.1% (v/v) acetic acid. The photography of the gel was released immediately after staining.

Data analysis: Data were subjected to ANOVA analysis following Fischer test at $p \leq 0.05$. If F-test was significant, LSD multiple range tests at $p = 0.05$ were used for comparing measured values. These operations were done using "Statgraphics plus" (version 5.0).

RESULTS

Effect of different BAP/2,4-D combinations on calli formation: In half strength MS medium (MS/2), calli were yellowish with crumbly aspect (Fig. 1a). The maximum percentage (86%) of embryogenic callus was obtained with 0.5 mg L^{-1} BAP/0.5 mg L^{-1} 2,4-D combination. In half strength DKW medium (DKW/2), calli were whitish with crumbly aspect (Fig. 1e). The higher percentage (94%) of embryogenic callus was also registered with 0.5 mg L^{-1} BAP/0.5 mg L^{-1} 2,4-D combination. No response was observed without plant hormone (Table 1).

Effect of 2,4-D on globular somatic embryos formation: On half strength MS medium, the higher percentage (92%) of embryogenic calli which produced globular somatic embryos (Fig. 1b) was obtained with 3 mg L^{-1} of 2,4-D (Table 2). This percentage decreased progressively when 2,4-D concentrations dropped. Without plant hormone, embryogenic calli degenerated after 4 weeks of subculture. The average number of

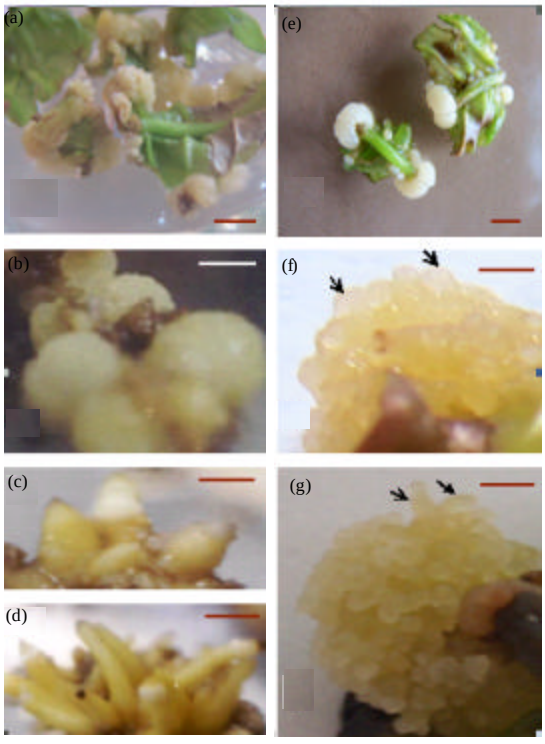

Fig. 1(a-g): Different stages of somatic embryogenesis in *B. toxisperma* in half strength Murashige and Skoog medium (MS/2), (a) Yellowish embryogenic callus, (b) Globular embryos, (c) Short bipolar embryos and (d) Elongated bipolar embryos and in half strength Driver and Kuniyuki medium (DKW/2) (e) Whitish embryogenic callus, (f) Globular embryos and (g) Bipolar embryos, Arrows: Embryos, Bar = 0.5 cm

Table 1: Effect of 2,4-D/BAP ratio on callus formation from leaf explants of *B. toxisperma* after 28 days of culture in MS/2 or DKW/2

Hormones (mg L^{-1})		Callogenesis (%)	
BAP	2,4-D	MS/2	DKW/2
0.5	0	0^{a}	0^{a}
0.5	0.5	86 ± 2^{e}	94 ± 2^{e}
0.5	1	52 ± 3^{d}	18 ± 2^{d}
0.5	2	46 ± 1^{c}	10 ± 3^{c}
0.5	3	24 ± 3^{b}	6 ± 2^{b}

Treatments with the same letter in a column were not significantly different according to LSD multiple range test (p = 0.05), MS/2: Half strength Murashige and Skoog medium, KW/2: Half strength Driver and Kuniyuki medium, BAP: 6-Benzylaminopurine, 2,4-D: 2,4-Dichlorophenoxyacetic acid

Table 2: Effect of 2,4-D concentration on the differentiation of globular embryos in *B. toxisperma* after 60 days subculture of embryogenic calli in MS/2 or DKW/2 media

2,4-D treatments (mg L^{-1})	% of callus with globular embryos in MS/2	Globular embryos per callus in MS/2	% of callus with globular embryos in DKW/2	Globular embryos per callus in DKW/2
0	0.0^{a}	0.0^{a}	0^{a}	0.0^{a}
0.5	4.0 ± 0.1^{b}	23.6 ± 1.5^{b}	100^{b}	11.6 ± 1.1^{b}
1	22.0 ± 2.6^{c}	24.3 ± 1.5^{b}	100^{b}	20.6 ± 1.1^{c}
2	70.0 ± 2.6^{d}	43.3 ± 0.5^{c}	100^{b}	50.0 ± 0.1^{d}
3	92.0 ± 0.4^{e}	45.6 ± 0.5^{c}	100^{b}	49.3 ± 0.5^{d}

Treatments with the same letter in a column were not significantly different according to LSD multiple range test (p = 0.05), MS/2: Half strength Murashige and Skoog medium, DKW/2: Half strength Driver and Kuniyuki medium, 2,4-D: 2,4-Dichlorophenoxyacetic acid

globular embryos formed per callus was also affected by 2,4-D concentration. In general, low 2,4-D concentration differentiated fewer globular embryos (23.6 or 24.3) while, high 2,4-D concentration gave more embryos per callus (43.3 or 45.6) (Table 2). All of embryogenic calli subcultured on half strength DKW medium differentiated into globular embryos. Maximum number of globular somatic embryos (50) was higher than that produced in half strength MS medium (Fig. 1f).

Effect of ABA/2,4-D combinations on the bipolar somatic embryos formation: Globular somatic embryo differentiated basal and distal poles when transferred in MS/2 or DKW/2 supplemented with ABA and 2,4-D. In half strength MS medium, differentiated embryos were more or less elongated (Fig. 1c, d). The maximum differentiation rate of bipolar embryos (34%) was recorded in the culture medium supplemented with 0.5 mg L^{-1} ABA/0.5 mg L^{-1} 2,4-D while, the maximum number of bipolar embryos (13.3 or 13.6) formed per callus was mentioned in culture medium supplemented with 0.5 or 1 mg L^{-1} 2,4-D/0.5 mg L^{-1} ABA. On half strength DKW medium, these embryos were yellowish and smaller than those obtained on MS/2 medium (Fig. 1g). All combinations allowed embryos bipolarization but at relatively low rates compared to half strength MS medium. In DKW/2 medium, a maximum embryos

Table 3: Effect of 2,4-D/ABA ratio on the differentiation of bipolar embryos after 97 days of culture of globular embryos in MS/2 or DKW/2

Hormones (mg L^{-1})		% of callus with bipolar embryos in	Bipolar embryos per callus in	% of callus with bipolar embryos in	Bipolar embryos per callus in
ABA	2,4-D	MS/2	MS/2	DKW/2	DKW/2
0.5	0.5	34.0 ± 0.1^{d}	13.6 ± 0.2^{b}	12 ± 1^{d}	7.3 ± 1.1^{c}
0.5	1	30.0 ± 0.3^{c}	13.3 ± 0.2^{b}	10 ± 2^{c}	7.6 ± 1.5^{c}
0.5	2	4.0 ± 0.2^{b}	4.3 ± 1.5^{a}	7 ± 2^{b}	5.3 ± 0.5^{b}
0.5	3	2.0 ± 0.0^{a}	4.0 ± 0.1^{a}	3 ± 0^{a}	2.3 ± 0.5^{a}

Treatments with the same letter in a column were not significantly different according to LSD multiple range test (p = 0.05), MS/2: Half strength Murashige and Skoog medium, DKW/2: Half strength Driver and Kuniyuki medium, ABA: Abscisic acid, 2,4-D: 2,4-Dichlorophenoxyacetic acid

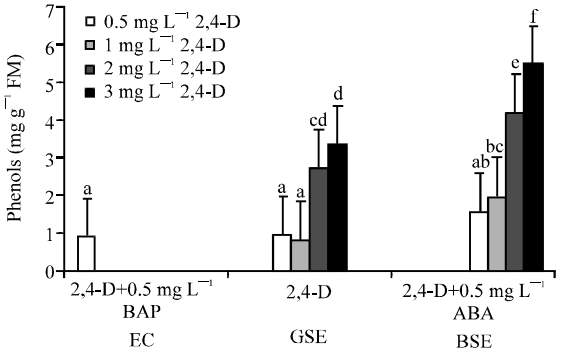

Fig. 2: Variation of phenols content at different stages of somatic embryogenesis in *B toxisperma* in half strength Murashige and Skoog medium (MS/2), Treatments with same letter were not significantly different according to LSD multiple range test (p = 0.05), EC: Embryogenic calli, GSE: Globular somatic embryos, BSE: Bipolar somatic embryos

formation rate (12 %) and a higher average number of embryos per callus (7.6) were obtained with lower 2,4-D concentrations associated to 0.5 mg L^{-1} ABA (Table 3).

Changes in phenol content: Phenol content was measured and compared to somatic embryogenesis process. On MS medium, low quantity of phenols was noted in the embryogenic callus. In globular somatic embryos, phenol levels remain broadly constant for 0.5 and 1 mg L^{-1} 2,4-D while 2 and 3 mg L^{-1} 2,4-D favors its significant increase (Fig. 2). In bipolar embryos stage, phenol levels increased and reached a peak only with 3 mg L^{-1} 2,4-D/0.5 ABA combination. In half-strength DKW medium, the same behavior was noted in all three stages (Fig. 3).

Changes in soluble peroxidases activities: Peroxidases activities were evaluated and compared to somatic embryogenesis process. In MS/2 medium, there was no significant variation from callus to globular embryos stage. Meanwhile in bipolar embryos stage, these

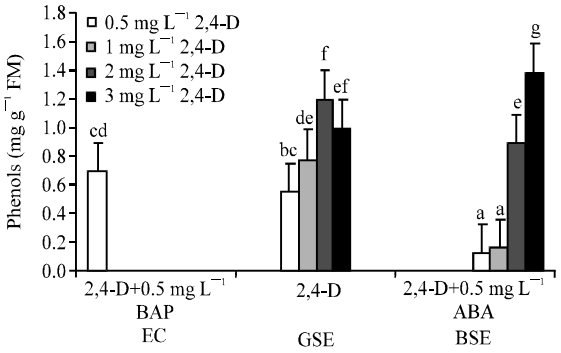

Fig. 3: Variation of phenols content at different stages of somatic embryogenesis in *B. toxisperma* in half strength Driver and Kuniyuki medium (DKW/2). Treatments with same letter were not significantly different according to LSD multiple range test (p = 0.05), EC: Embryogenic calli, GSE: Globular somatic embryos, BSE: Bipolar somatic embryos

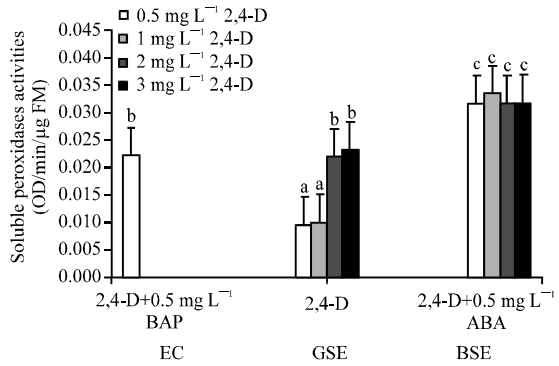

Fig. 4: Soluble peroxidases activities at different stages of somatic embryogenesis in *B toxisperma* in half strength Murashige and Skoog medium (MS/2). Treatments with same letter were not significantly different according to LSD multiple range test (p = 0.05). EC: Embryogenic calli, GSE: Globular somatic embryos, BSE: Bipolar somatic embryos

activities increased significantly for all treatment compared to those of previous stages (Fig. 4). In contrast, peroxidases activities decreased from globular to bipolar embryos stage in DKW/2 medium (Fig. 5).

Soluble peroxidases electrophoresis profiles: Soluble peroxidases patterns in embryogenic structures cultured in MS/2 and DKW/2 media were revealed electrophoretically. In MS/2 medium, four bands (i_1, i_2, i_3, i_4) were detected (Fig. 6). The peroxidase i_3 is present in all

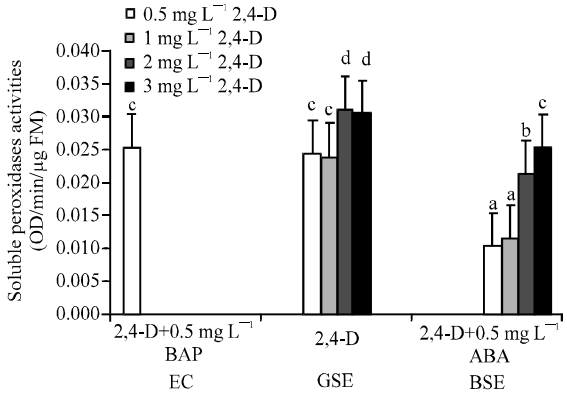

Fig. 5: Soluble peroxidases activities at different stages of somatic embryogenesis in half strength Driver and Kuniyuki medium (DKW/2). Treatments having the same letter were not significantly different according to LSD multiple range test (p = 0.05), EC: Embryogenic calli, GSE: Globular somatic embryos, BSE: Bipolar somatic embryos

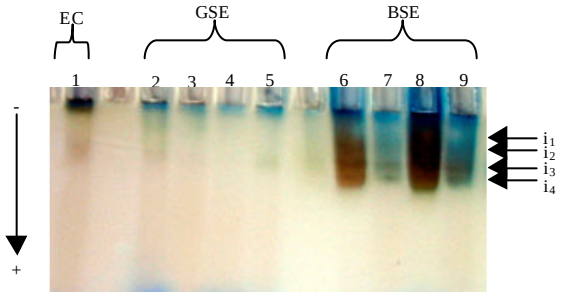

Fig. 6: Soluble peroxidases Profiles in half strength Murashige and Skoog medium (MS/2) at different stages of somatic embryogenesis with variable concentrations of plant hormones in *B. toxisperma*. 1: 0.5 mg L^{-1} 2,4-D/0.5 mg L^{-1} BAP, 2-5: 0.5, 1, 2 or 3 mg L^{-1} 2,4-D, 6-9: 0.5 mg L^{-1} ABA/0.5, 1, 2 or 3 mg L^{-1} 2,4-D, i_1...i_4: Peroxidases 1-4, EC: Embryogenic calli, GSE: Globular somatic embryos, BSE: Bipolar somatic embryos

stages of culture. Isoenzyme i_1 enzyme is specific to globular stages while i_2 and i_4 are specific to bipolar stage. In DKW/2 medium where 3 bands (j_1, j_2, j_3) were detected (Fig. 7). At the embryogenic callus stage, only j_1 and j_2 isoenzymes were expressed. In globular and bipolar embryos stages, all three bands are present.

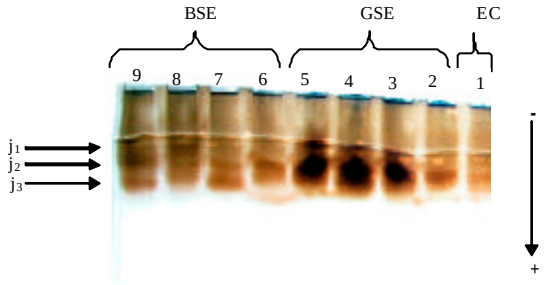

Fig. 7: Soluble peroxidases profile in half strength Driver and Kuniyuki medium (DKW/2) at different stages of somatic embryogenesis with variable concentrations of plant hormones in *B. toxisperma*. 1: 0.5 mg L^{-1} 2,4-D/0.5 mg L^{-1} BAP, 2-5: 0.5, 1, 2 or 3 mg L^{-1} 2,4-D, 6-9: 0.5 mg L^{-1} ABA/0.5, 1, 2 or 3 mg L^{-1} 2,4-D, j_1...j_3: peroxidases 1...3, EC: Embryogenic calli, GSE: Globular somatic embryos, BSE: Bipolar somatic embryos

DISCUSSION

Calli obtained after 28 days of culture were all crumbly with yellowish and whitish color in MS/2 and DKW/2 media, respectively. Unlike compact callus which is generally more favorable to organogenesis (Emons and Kieft, 1991), friable calli are embryogenic and able to produce somatic embryos. In some species, however, these two aspects of callus can be embryogenic (Chaudhury and Qu, 2000). Percentages of calli formation vary with plant hormones combinations used in both culture media. The highest percentages of calli formation were respectively 86 and 92% in MS/2 and DKW/2 media using 0.5 mg L^{-1} BAP/0.5 mg L^{-1} 2,4-D combination. In general, the presence of auxins and auxin-active substances is required for the induction and proliferation of cells that later differentiate into somatic embryos (Bobak *et al*., 2004). Meanwhile, high doses of auxin seem to inhibit the callogenesis. These results are in accordance with those of Vengadesan and Pijut (2009) on *in vitro* callogenesis from immature cotyledons of *Quercus rubra*. There is no callus formation without growth regulators. Similar results were obtained in *Arnebia euchroma* (Manjkhola *et al*., 2005). Indeed, the exogenous growth regulators are essential for initiation of somatic embryogenesis process in plants (Ammirato, 1983). When BAP was also used alone, no callus differentiation was observed. This indicates a synergistic effect and/or complementary relationship between BAP (cytokinin) and 2,4-D (auxin) in the development process in plants. The synergetic effect of auxin/cytokinin was observed in several studies of somatic embryogenesis studies in woody species such as *Catharanthus roseus* (Junaid *et al*., 2006) and *Vitis vinifera* (Olah *et al*., 2009).

Transfer of embryogenic calli in MS/2 or DKW/2 media supplemented with various 2,4-D concentrations promote development of globular embryos. When they were cultured without plant hormones, necroses appeared. However, in *Quercus rubra* (Vengadesan and Pijut, 2009) and in *Papaver nudicaule* (Yang *et al*., 2010) globular somatic embryos have been obtained on MS medium without growth regulators. On MS/2 medium, the percentage of embryogenic callus on which globular somatic embryos are differentiated increased with the concentration of 2,4-D (4 to 92%). 2,4-Dichlorophenoxiacetic acid would have a stimulatory effect on the formation of globular somatic embryos and this has been observed in *Santalum album* (Rao *et al*., 1996). In some species, the formation of globular embryos required the combination auxin/cytokinin as recently noted in *Guizotia abyssinica* (Naik and Murthy, 2010). On DKW/2 medium, all embryogenic calli subcultured in the presence of 2,4-D were able to produce embryonic cells. This permitted to note that 2,4-D is not the only factor that governs the production of globular embryos. Indeed, DKW/2 medium was supplemented with other specific compounds such as glutamine and glucose that may influence plants development. It is generally observed for both media that the number of globular somatic embryos increases with the concentration of 2,4-D (11.5 to 49.1 on DKW/2 medium and 23.9 to 45.4 on MS/2 medium). This trend was observed during somatic embryogenesis in *Acacia arabica* (Nanda and Rout, 2003).

After 97 days of culture, some globular embryos differentiated into bipolar structures with apical and basal poles. Such structures were considered as the final stage of somatic embryos and including the heart, torpedo or cotyledonary embryos (Akula *et al*., 2000; Solis-Ramos *et al*., 2010). The percentages of bipolar embryo formation decreased when 2,4-D rate associated to ABA increased. This result matches with that of Rao *et al*. (1996) in *Santalum alba* in which the they concluded that if the differentiated pro-embryonnaire masses on embryogenic calli are positively sensitive to 2,4-D for their transformation into globular embryos they are negatively sensitive to their transformation into bipolar embryos. At this stage, the role of ABA was determinative, compared to the previous stage where that growth regulator was not used. In some species, especially in conifer, treatment with ABA is necessary for the differentiation of bipolar embryos (Von Arnold *et al*., 2002). Therefore, Dodeman *et al*.

(1997) specified that the synthesis and the accumulation of storage proteins during this phase of embryos development depend generally on the ABA gene expression. ABA is known as osmotic agent needed for embryos desiccation that accompanies their maturation (Maruyama *et al*., 2002).

The best percentages of embryogenic calli formation and globular embryos differentiation were obtained using DKW/2 medium while MS/2 medium was more suitable to bipolar embryos formation. This indicates that the processes of in vitro tissues culture vary with types of media (Jimenez, 2005). The MS medium is richer in nitrogen with (39.4 mM of NO_3^- and 20.6 mM of NH_4^+) than DKW medium (30 mM of total nitrogen), which is rather rich in sulphate and in calcium (Pinto *et al*., 2008). An adequate report of NO_3^-/NH_4^+ would stimulate morphogenesis and embryogenesis (Ramage and Williams, 2002). However, the influence of culture medium in somatic embryogenesis process is not only relative to the concentration of the different mineral elements of the culture media but their similarity with mineral elements of cultured plant tissues (Pinto *et al*., 2008).

Phenolic compounds concentrations varied according to culture media and culture stages. On MS/2 medium containing higher 2,4-D concentrations, phenols level in the embryogenic callus increased in the globular embryos and can be associated to best percentages and numbers of globular embryos formed. These results are in accordance with those obtained in some woody plants species like *Feijoa sellowiana* (Cangahuala-Inocente *et al*., 2004) and *Ceratonia siliqua* (Canhoto *et al*., 2006) in which that early stage of somatic embryos differentiation was associated with phenols-rich cells. In bipolar embryos stage, phenols levels increased proportionally with 2,4-D concentrations combined to 0.5 mg L^{-1} of ABA. This variation seems to have a negative effect on the differentiation of bipolar embryos since percentages and numbers of embryos per callus decrease significantly as noted in *Theobroma cacao* (Ndoumou *et al*., 1997). Phenolic compounds are known to have deleterious effects *in vitro* since their exudation and their oxidation can cause explants browning and necrosis especially for woody plants (Martin and Madassery, 2005). In DKW/2 medium, the same behavior as in MS/2 was observed despite phenols high quantities in embryogenic callus. Generally the concentrations of phenolic compounds were higher on MS/2 medium (2.7 to 16.32 mg g^{-1} FM) than they were on DKW/2 medium (0.55 to 1.38 mg g^{-1} FM). Although low phenols levels might account for higher callus and globular embryos formation recorded on DKW/2 medium and optimal synthesis of phenolic compounds seems to be essential to allow effective development of bipolar embryos on MS/2 medium.

Soluble peroxidase activities were analyzed in different somatic embryogenesis stages. Differences in callus formation rates were not closely related to enzymatic activities in embryogenic callus, since these values were not different statistically. In globular embryo soluble peroxidases activities increased with 2,4-D rates and were reflected by the increases in rates and numbers of globular embryos formation. Bonfill *et al*. (2003) associated also plants peroxidases activities with endogenous levels of auxins. In this stage, peroxidases activities were higher on DKW/2 medium than on MS/2 medium. This result shows that some media promoted more peroxidases activities (Zur *et al*., 2009). In bipolar embryos steps, there was a significant increase in soluble peroxidase activity on MS/2 medium than in the previous stages. This behavior may be attributed to ABA supply. Indeed, Wei (2001) mentioned that ABA promotes peroxidases synthesis, which in turn catalyze H_2O_2 reduction and protects embryos from oxidative damage. In general, highlighting our best results at different stages of culture observed, soluble peroxidases activities on DKW/2 medium increased in globular embryos stage (with of 2 or 3 mg L^{-1} 2,4-D) and then decreased during bipolar embryos stage (with of 0.5 or 1 mg L^{-1} 2,4-D/0.5 mg L^{-1} of ABA). A similar observation was done in *Cicer arietinum* (Kiran *et al*., 2009). In MS/2 medium, soluble peroxidases activities remained relatively stable between embryogenic callus stage and globular embryos stage for best rate and number of embryos and then increased significantly at bipolar embryos stage. These results corroborate those of Malabadi and Nataraja (2007) in *Pinus roxburghii* in which peroxidase activities rose during maturation stages of somatic embryos. Electrophoresis profiles of soluble peroxidases performed at different stages of cultures showed differences. According to Gaspar *et al*. (1982), peroxidases exist in several isoforms regulated with stages of development and are highly sensitive to external stimuli. On MS/2 medium, there is only one isoform i_3 in embryogenic callus step which appeared to play a leading role in initiating and maintaining the process of somatic embryogenesis since it existed at all others stages. At globular embryo stage, i_1 isoform appeared only for low concentrations of 2,4-D and was negatively correlated to globular embryos formation. Obtaining bipolar embryos may be associated with the synthesis of two specific peroxidases i_2 and i_4 and confirmed the spectrophotometric observations which indicated at this

stage that soluble peroxidases activities are higher. In DKW/2 medium, embryogenic calli expressed two forms of peroxidases j_1 and j_2 which persisted in the other two stages and whose role could be devolved to the i_3 isoperoxidases expressed on MS/2 medium. Stages of bipolar and globular embryos were marked by the expression of peroxidase j_3 which occurred in the transformation of embryogenic cells in bipolar and globular structures.

CONCLUSION

This study revealed that for callus formation and globular embryos differentiation, DKW/2 medium is more propitious respectively in the presence of 0.5 mg L^{-1} 2,4-D/ 0.5 mg L^{-1} BAP combination and 2 or 3 mg L^{-1} of 2,4-D. For bipolar embryos differentiation, MS/2 medium was more favorable with the presence of 0.5 mg L^{-1} 2,4-D/ 0.5 mg L^{-1} ABA combination. For the best results registered, phenols content in both media seems to affect positively globular somatic embryos differentiation but negatively late bipolar embryos formation. However, peroxidase activities increased from callus to somatic embryos stages. Soluble peroxidases profiles showed more enzymes in bipolar somatic embryos stages than others.

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