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Influence of plant growth regulators on somatic embryogenesis induction from inner teguments of rubber (*Hevea brasiliensis*) seeds

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Generating somatic embryos from the inner teguments of hevea seeds is difficult. Like other ligneous plants, the rubber-tree is generally considered to be recalcitrant with regard to somatic embryogenesis. In this study, the ability of callus from inner integument explants to develop embryogenic callus lines was highlighted. Combination of 2,4-dichlorophenoxyacetic acid (2,4-D/KT) (9 µM/3.375 µM) revealed the positive effect of the 2,4-D on callogenesis and somatic embryogenesis from the inner integument of the seed of immature fruit. The rate of embryogenic calli of about 50% obtained, suggested that 2,4-D has a similar effect as 3,4-dichlorophenoxy acetic acid (3,4-D). So, although 2,4-D is rarely used as a hormone in biotechnology of rubber, its positive influence on callus induction and somatic embryo development shows that it is an alternative to 3,4-D which is commonly used. Optimal combinations of 2,4-D/thidiazuron (TDZ) (9 µM/34.2 nM) produced abnormal embryos at lower rates (approximately 5%) than the optimal combination of 2,4-D/KT.

Key words: Callus, culture medium, *Hevea brasiliensis*, hormones, rubber-tree, somatic embryogenesis.

INTRODUCTION

Rubber tree (*Hevea brasiliensis* Müll. Arg.) is the South American tropical tree of the spurge family (Euphorbiaceae). Cultivated on plantations in the tropics and subtropics, especially in Southeast Asia and western Africa, it replaced the rubber plant in the early 20th century as the chief source of natural rubber. It has soft wood; high, branching limbs and a large area of bark. The milky liquid (latex) that oozes from any wound to the tree bark contains about 30% rubber, which can be coagulated and processed into solid products, such as

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Abbreviations: 3,4-D, 3,4-Dichlorophenoxy acetic acid; **BA,** benzyladenine; **KT,** kinetin.

tires. Latex can also be concentrated for producing dipped goods, such as surgical gloves.

Nowadays, rubber plantations are established with grafted clones. In the same plantation, half of grafted trees provide 70 to 80% of production (Langlois, 1965). A part of this difference is attributed to the interaction between the rootstock and the scion (Carron et al., 1989). To face these various constraints that are linked to clonal heterogeneity, vigor decline and production related to grafting, somatic embryogenesis was suggested. Since it was used with the inner tegument of rubber's immature seed, somatic embryogenesis recorded some progress regarding the use of growth regulators. The efficiency of various somatic embryogenesis protocols described in rubber depends on the cultivars, as some of them are recalcitrant to *in vitro* culture (El Hadrani et al., 1991). One of the major bottlenecks in somatic embryogenesis procedures is the production of primary calli. Exogenous auxins and cytokinins are main plant growth regulators (PGRs) involved in the control of cell division and differentiation (Féher et al., 2003). The role of these PGRs in the regeneration performance of several plants has been previously described (Zouine et al., 2005; Sané et al., 2006; Zouzou et al., 2008; Ashrafi et al., 2010; Yapo et al., 2011). Different combinations of growth regulators were experimented to obtain more embryogenic calli less subject to browning. In this regard, 9 µM of 2,4-dichlorophenoxyacetic acid (2,4-D) and a combination with 5.7 µM of indolylacetic acid (AIA) were experimented by Carron and Enjarlic (1985). Later, 3,4-dichlorophenoxy acetic acid (3,4-D) was preferred to 2,4-D (Michaux-Ferrière and Carron, 1989). This auxin was first combined with 9 µM of benzyladenine (BA) during the induction of somatic embryogenic phase. In addition, Kinetin (KT) was then preferred to the BA (Montoro et al., 1992). In spite of growth regulators combination research, this protocol is not under full control yet. 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 Hadrami et al., 1991). However, much research input and further refinement considering different growth regulators key factor for devising efficient protocol with particular reference to somatic embryogenesis pathway of rubber is required.

To our knowledge, 2,4-D, KT and thidiazuron (TDZ) combination have never been experimented during the somatic embryogenesis from the inner teguments of rubber. This research is aimed at improving the productions of embryogenic calli. In the present study, the effect of various PGRs, particularly of the auxin, 2,4-D and of the cytokinines, KT and TDZ on the embryogenic calli induction of inner tegument of rubber was explored. The hormonal conditions for the proliferation of calli and the development of somatic embryos were also investigated by combining various concentrations of PGRs.

MATERIALS AND METHODS

Plant material and preparation of explants

Our study was implemented from the inner teguments obtained from seed of immature fruit of rubber. Fruits were harvested after eight to 10 weeks of anthesis in PB 260 clone of rubber. Fruits were harvested in plantations of the Centre National de Recherche Agronomique (CNRA) of Côte d'Ivoire between May and June. Fruits were sterilised with 2.45% aqueous solution of sodium hypochlorite for 30 min and followed by three washings with autoclaved distilled water. Seeds were then extracted by section of the fruit and their inner teguments were aseptically cut into fragments of 5 mm in length. They were then transferred onto Petri dishes containing 30 ml of medium. All the explants were transferred for callus induction with various growth regulators concentrations.

Callus induction

Inner teguments were put in Petri dish containing 30 ml of medium. Mineral basic medium of MB supplemented with 125 mM KH_2PO_4 (MBm) was used. It is a modified Murashige and Skoog (1962) medium that contained 234 mM sucrose, 30 µM $AgNO_3$ and Fossard vitamins (Fossard, 1976) without choline chloride. MBm medium was fortified with different concentrations of 2,4-D (4.5 and 9.0 µM) and KT (1.25, 2.25, 3.375 and 4.5 µM). 2,4-D and KT were used alone or in combination (Table 1). Effect of 2,4-D at 9.0 µM in combination with four concentrations of TDZ (11.40, 22.80, 34.20 and 45.60 nM) were also tested (Table 2). Media were solidified with 2 g/L gelrite (Sigma Chemical Co.), subjected to pH 5.8 before autoclaving (120°C). A total of 13 different hormonal combinations were tested. The effect of the hormonal composition was evaluated by counting the calli obtained after 25 days of culture in the dark at 27 ± 2°C. Percentage of callogenic explants (PCE) were evaluated as follows: PCE = (Number of callus/Total number of explants) x 100.

Somatic embryos induction

The primary calli were chopped with a scalpel and subcultured in embryogenesis induction media for somatic embryos induction. Calli were cultured on 2,4-D and KT combination medium as well as 2,4-D and TDZ combination medium to investigate their effect on somatic embryogenesis. M0 contained 2,4-D (9.0 µM); used as control. Cultures were maintained through subculturing on the same medium condition for three times with intervals of 25 days in the dark at 27 ± 2 °C. At the end of the third subculture (75 days), the percentage of embryogenic calli (PEC) was evaluated [PEC = (Number of embryogenic calli/ Total number of callogenic explants) x100].

Statistical analysis

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. The means are the result of ten replicates (one replicate containing 30 explants).

Table 1. Combination of 2,4-D and kinetin added in medium for callus induction.

Media code	Concentration of plant growth regulator	
	2,4-D (µM)	KT (µM)
M 1	4.5	1.25
M 2	4.5	2.25
M 3	4.5	3.375
M 4	4.5	4.5
M 5	9.0	1.25
M 6	9.0	2.25
M 7	9.0	3.375
M 8	9.0	4.5

2,4-D, 2,4-dichlorophenoxyacetic acid; **KT,** kinetin.

Table 2. Combination of 2,4-D and TDZ added in medium for callus induction

Media code	Concentration of plant growth regulator	
	2,4-D (µM)	TDZ (nM)
M 0	9.0	-
M 9	9.0	11.4
M 10	9.0	22.8
M 11	9.0	34.2
M 12	9.0	45.6

2,4-D, 2,4-dichlorophenoxyacetic acid; **TDZ,** thidiazuron.

RESULTS

After 25 days of culture, explants were covered with compact yellow calli. After transfer in embryogenic media, the colour of these calli turned to brown at the end of 75 days of incubation with appearance of somatic embryos (Figure 1).

Combination of 2,4-D and KT

Callus were produced in all media containing 2,4-D/KT combination. Percentage of callogenic explants was influenced by the balance of growth regulator. Variables 2,4-D, KT and their interaction explained 91% of the variability observed during callogenesis (data not show). Moreover, they influenced significantly ($P < 0.0001$) the callogenesis. Taken individually, the percentage of callogenic explants increased when the 2,4-D concentration also increased from 4.5 to 9.0 µM.

The difference between the effects of these two concentrations on callogenesis was significant ($P < 0.0001$). The Duncan's test allowed the division of the eight induction media into two different classes on the basis of 2,4-D effect on callogenesis. The first class was constituted by the most callogenic media with a mean of 81.11% callogenic explants (data not show). These media contained 9.0 µM 2,4-D (M5, M6, M7 and M8). The second class was defined by the least callogenic media with an average of 50.56% callogenic explants (data not show). These media contained 4.5 µM of 2,4-D (M1, M2, M3 and M4). Thus, 9.0 µM 2,4-D was more callogenic than 4.5 µM (Table 3). The influence of the kinetin concentration on callogenesis was significant ($P = 0.001$). The percentage of callogenic explants increased when the concentrations of KT were superior to the weakest concentration (1.125 µM) (Table 4). The combined effect of the various levels of the factors 2,4-D and KT on callogenesis revealed three classes. The class of the most callogenic media included media combining 9.0 µM 2,4-D with various concentrations of KT (3.375; 2.25; 4.5 and 1.125 µM). The moderately callogenic media was formed by 4.5 µM 2,4-D combined with KT varying from 2.25 to 4.5 µM. The media combining 4.5 µM 2,4-D to 1.125 KT µM were the least callogenic. 2,4-D, KT and their interaction expressed 87% of the variability observed during somatic embryogenesis induction (data not show). In addition, they have, in general, significantly influenced ($P = 0.0001$) the induction of embryogenic calli. All combinations of 2,4-D/KT were proved to be embryogenic, except M1 medium consisting of 4.5 µM 2,4-D combined with 1.125 µM KT. The most embryogenic

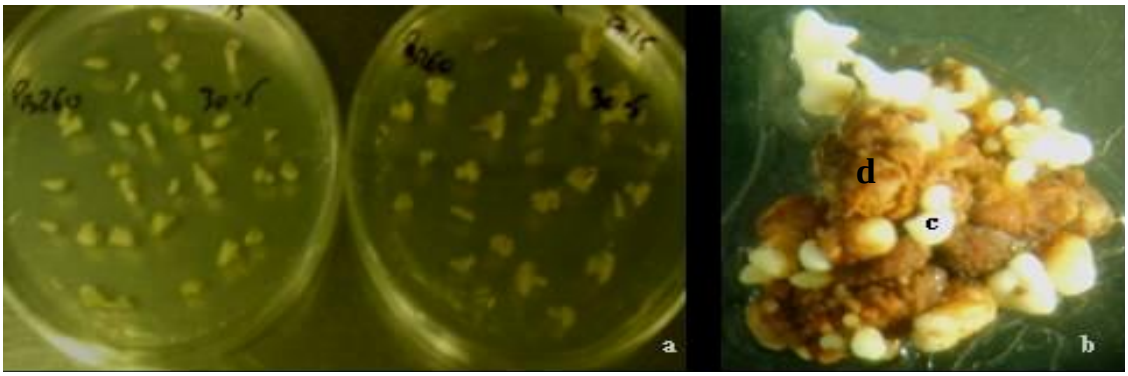

Figure 1. Embryogenic callus induction from inner tegument of *Hevea brasiliensis* fruit in clone PB 260. **a)** Callogenic explants (Gx0.26); **b)** embryogenic callus (Gx12.5) ; **c)** embryogenic cell; **d)** non-embryogenic cell.

Table 3. Effect of 2,4-D and kinetin concentration on callogenesis induction from inner tegument of *Hevea brasiliensis* fruit in clone PB 260.

Hormone	Explants forming callus (%)
2,4-D (µM)	
4.5	50.56 ± 3.30^{b}
9	81.11 ± 2.41^{a}
Kinetin (µM)	
1.125	55.56 ± 9.60^{b}
2.25	65.00 ± 7.32^{c}
3.375	75.00 ± 6.34^{d}
4.5	67.78 ± 5.95^{cd}

Values for each parameter followed by a different letter within each column are significantly different by Duncan test (P< 0.05). Each value represents the mean of ten replicates; ± SD, standard deviation; 2,4-D, 2,4-dichlorophenoxyacetic acid.

medium was M7 (9 µM 2,4-D/3.375 µM KT).

However, it is interesting to reveal that the concentrations 2.25 and 3.375 µM KT promoted somatic embryogenesis induction regardless of the concentration of 2,4-D (Table 4).

Combination of 2,4-D/TDZ

The influence of 2,4-D/TDZ combination was consigned in Table 5. Compact callus were observed in media fortified with different concentrations of TDZ (11.4, 22.8, 34.2 and 45.6 nM) in combination with 2,4-D (9.0 µM) alone. The induction media had significantly influenced (P = 0.0001) the percentage of callogenesis because they explain 83% of total variability (data not show). M11 medium (34.2 nM TDZ) was the most callogenic whereas M9 medium (11.4 nM TDZ) was the less callogenic. M10 (22.8 nM) and M11 (34.2 nM) media had callogenesis rates significantly more elevated than those of the controls. Thus, 2,4-D use alone promotes callogenesis but combination with TDZ improved callus induction. The percentage of embryogenic calli was not significantly different in the four media containing TDZ (0 to 4.96%). Only 21% of the variability observed as regard the percentage of embryogenesis was explained by the induction media. Embryogenic calli were observed on M10 (3.57%) and M11 (4.96%) media.

DISCUSSION

In the present study, 2,4-D alone favored the induction of callogenesis; the emphasis was put on the search for the influence of its association with two cytokinins, KT and TDZ. A factorial influence in the case of the association of 2,4-D/KT showed that the percentage of callogenic explants increased in proportion with the concentration of

Table 4. Effect of 2,4-D and Kinetin combination on callogenesis and somatic embryogenesis from inner tegument of *Hevea brasiliensis* fruit in clone PB 260.

Hormone			Explants forming callus (%)	Embryogenic callus (%)
2,4-D (µM)	Kinetin (µM)	Code media		
4.5	1.25	M1	34.44 ± 3.93^{c}	0^{d}
4.5	2.25	M3	50.00 ± 4.04^{b}	21.67 ± 5.00^{e}
4.5	3.375	M4	61.11 ± 2.00^{b}	22.22 ± 5.07^{e}
4.5	4.5	M5	56.67 ± 2.03^{b}	05.33 ± 1.33^{a}
9.0	1.25	M6	76.67 ± 2.03^{a}	35.19 ± 7.76^{c}
9.0	2.25	M7	80.00 ± 5.13^{a}	16.45 ± 3.67^{ei}
9.0	3.375	M8	88.89 ± 1.00^{a}	46.74 ± 3.65^{b}
9.0	4.5	M9	78.89 ± 7.22^{a}	07.58 ± 2.09^{f}

Values for each parameter followed by a different letter within each column are significantly different by Duncan test (P< 0.05). Each value represents the mean of ten replicates.

Table 5. Effect of 2,4-D (9.0 µM) and TDZ combination on callogenesis and somatic embryogenesis from inner tegument of *Hevea brasiliensis* fruit in clone PB 260

Hormone			Explants forming callus (%)	Embryogenic callus (%)
2,4-D (µM)	TDZ (nM)	Code media		
9.0	**0**	M0	42.71 ± 5.24^{c}	0^{a}
9.0	11.4	M9	56 ± 8.3^{bc}	0^{a}
9.0	22.8	M10	70 ± 6.12^{ab}	3.57 ± 1.57^{d}
9.0	34.2	M11	76.67±6.01^{a}	4.96 ± 2.37^{d}

Values for each parameter followed by a different letter within each column are significantly different by Duncan test (P< 0.05). Each value represents the mean of ten replicates; ± SD (standard deviation); 2,4-D (2,4-dichlorophenoxyacetic acid), TDZ (thidiazuron).

2,4-D (from 4.5 to 9.0 µM). These results dealing with 2,4-D were similar with those of Carron (1982) which indicated that the level of callus proliferation stemming from epicotyl fragments of *Hevea* increased in the same sense that the concentration of 2,4-D until 9.0 µM and beyond the threshold of toxicity was reached. Kumari et al. (1999) showed from the immature anthers of *Hevea* that the percentage of callogenic explants increased when the concentration of 2,4-D increased from 2.0 m to 3.0 mg/l and decreased when this concentration was 1.0 mg/l. Growth regulators play a key role by intervening in the reactions that lead to a reorientation of the program of gene expression. This expression can lead either to an unorganized growth of the cells (callus) without embryogenesis or to a polarized growth leading to a somatic embryogenesis (Dudits et al., 1995). In the present study, the embryogenesis was induced when the 2,4-D with 4,5 or 9.0 µM was associated to KT (2.25 or 3.375 µM). Montoro et al. (1993) reported with clone PB 260 that percentages values of embryogenic calli varied from 32 to 48% when the concentrations of 3,4-D or KT varied from 2.25 to 9.0 µM. This study registered 47% of embryogenic calli on the induction medium containing 9.0 µM 2,4-D/3.375 µM KT. This rate is comparable to those obtained in other studies using 3,4-D and KT to induce the embryogenesis from the inner tegument of the seed. The present results evidenced that an inappropriate concentration of 2,4-D combined with an inadequate or a suboptimum concentration of cytokinin may consider the 2,4-D as not very suitable for somatic embryogenesis from inner tegument of rubber's fruit. Actually, 2,4-D is considered as a growth regulator conducive to somatic embryogenesis from the inner tegument of the immature rubber's fruit. It could therefore be used as an alternate solution to 3,4-D.

TDZ has never been used as cytokinin for rubber's callogenesis and embryogenesis. The combination of 2,4-D/TDZ used in this study helped obtain compact calli with a percentage of callogenic induction of more than 70%. Embryogenic calli were induced from 4 to 5% on medium containing 9.0 µM of 2,4-D/34.2 nM of TDZ and 9 µM of 2,4-D/22.80 nM of TDZ. These rates were weaker than those one obtained with the combination of 9 µM of 2,4-D/3.375 µM of KT. Indeed, an optimum association of 2,4-D/cytokinin favorably influences the rate of somatic embryogenesis induction. In addition, all the somatic embryos obtained with TDZ were abnormal. This suggests that the optimal concentrations of this

cytokinin associated with 2,4-D (9.0 µM) were not deepened in the present study. Thereby, embryogenic potentialities of TDZ can be improved. With concentrations (being associated with other growth regulators or not) different from these ones used in this study, it could be another alternate to the traditional growth regulators used in rubber's somatic embryogenesis. Moreover, a protocol of secondary somatic embryogenesis from abnormal embryos observed could be envisaged. With regard to many other species, TDZ alone or associated with another growth regulator was used to produce somatic embryos or regenerate shoots with leaves. In case of the cocoa tree, the association of 9.0 µM of 2,4-D/22.70 nM of TDZ proved to be optimum for the induction of somatic embryos (Li et al., 1998). As for lentil, TDZ alone used with 0.25 mg/l regenerated, in eight weeks, young shoots from cotyledons (Kwawar et al., 2003).

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