Tree Physiology 29, 291–298
doi:10.1093/treephys/tpn027

Influences of aging and cloning methods on the capacity for somatic embryogenesis of a mature *Hevea brasiliensis* genotype

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Received February 4, 2008; accepted September 30, 2008; published online December 23, 2008

Summary We compared embryogenic capacities of integument explants excised from three sources of the *Hevea brasiliensis* (Müll. Arg.) mature genotype PB 260. The three sources were 17-year-old (BT 86) and 7-year-old (BT 96) budded trees and 7-year-old emblings (EM 96). The highest proportions of embryogenic calluses obtained from the total number of integument explants initially used were from trees of EM 96 origin, followed by BT 96 trees, with explants from BT 86 trees producing the lowest number of embryogenic calluses. Further initiation of embryogenic callus lines from the primary somatic embryos derived from the three sources was successful only for EM 96. Somatic embryo cultures from BT 86 and BT 96 sources produced only friable calluses that could not be further amplified. Overall, somatic embryo explants derived from EM 96 responded over a wider range of 3,4-dichlorophenoxyacetic acid and kinetin concentrations than the somatic embryo explants from BT 86 and BT 96 origins. The effects of chronologic, ontogenetic and physiologic aging on explant capacity for somatic embryogenesis and on the overall efficiency of the process in *H. brasiliensis* are discussed.

Keywords: callogenesis, maturation, ontogenesis, phase change.

Introduction

Somatic embryogenesis of *Hevea brasiliensis* (Müll. Arg.) has potential as a method for mass clonal propagation of mature selected genotypes (Carron et al. 1995), and for genetic engineering (Montoro et al. 2000). We recently reported a new process for obtaining embryogenic callus lines quickly based on secondary somatic embryogenesis; however, success rates were low ($< 0.1\%$ of initial explants of clone PB 260) and unpredictable (Lardet et al. 2008).

Previous studies have shown that physiologic aging negatively affects the micropropagation capacity of *H. brasiliensis* from microcuttings (Lardet et al. 1990, Perrin et al. 1994). This phase change phenomenon has been observed in many studies and is associated with a noticeable decline in the potential for micropropagation or somatic embryogenesis of most arborescent species (e.g., Bonga 1982, Hackett 1985, Monteuuis 1989, von Aderkas and Bonga 2000). Explants with a high ability for somatic embryogenesis generally originate from reproductive organs (e.g., anthers, inner integument of the seed and zygotic embryos), young seedlings (Dunstan et al. 1995) and somatic-embryogenesis-derived plantlets (hereafter referred to as emblings). In *Picea abies* (L.) Karst., for instance, in-vitro-cultured somatic embryos showed higher ability for somatic embryogenesis than their similarly aged or older zygotic counterparts (Ruaud et al. 1992). In *Larix decidua* Mill., somatic embryogenesis from cotyledons and needles has been successful only for tissues obtained from emblings or from immature and mature zygotic embryos (Lelu et al. 1994).

In addition to explant origin, the in vitro culture conditions have a major effect on the success of somatic embryogenesis from mature tree tissues. Growth regulators have a determining influence on the capacity for somatic embryogenesis of tree species (Dunstan et al. 1995, Klimaszewska et al. 2001). The regulation of plant aging by phytohormones has been recognized for a long time (Haffner et al. 1991*a*), and endogenous cytokinins appear to play a major role in the aging process in *H. brasiliensis* (Perrin et al. 1997). In this species, the success of microcutting cultures derived from shoot apices was highly dependent on the concentrations of growth regulators in the culture medium, and these concentrations changed with the developmental stage of the shoot apex at the time the explants were collected (Lardet et al. 1990, Haffner et al. 1991*b*).

These observations prompted us to assess possible ways of improving the efficiency of the secondary somatic embryogenesis technology developed for *H. brasiliensis* (Lardet et al. 2008). We adopted a two-step procedure for evaluation: (1) we analyzed the effects of physiologic aging and cloning methods (budding versus somatic embryogenesis) on explant capacity for primary somatic embryogenesis and (2) we assessed the effects of various combinations of exogenous growth regulators on the capacity for secondary somatic embryogenesis of explants derived by primary somatic embryogenesis.

Materials and methods

Plant material

The study material comprised three origins (17-year-old (BT 86) and 7-year-old (BT 96) budded trees and 7-year-old emblings (EM 96)) of clonal offspring of the *H. brasiliensis* genotype PB 260, which is widely planted on a commercial scale. The BT 86 plants were obtained by budding on 10-month-old seedling rootstocks. The BT 86 plants were 17 years old and 23–25 m in height on average when the experiment started. The BT 96 plants were obtained by budding on 10-month-old seedling rootstocks. The BT 96 trees were 7 years old with a mean height of 10–12 m when the experiment started. The EM 96 trees or emblings were derived by somatic embryogenesis and had been cultivated in the nursery for 15 months before being planted outdoors. The EM 96 trees were 7 years old and had a mean height of 10–11 m on average when the experiment started. The BT 86, BT 96 and EM 96 trees have been grown outdoors at Osse River, Nigeria under the same environmental conditions since they were budded (BT 86 and BT 96) or following the 15-month period of cultivation in a nursery (EM 96).

Obtaining primary somatic embryogenesis from integument explants

The protocol for primary somatic embryogenesis has been described in detail by Carron et al. (1995). The primary explant was a thin slice of inner integument of immature seeds collected from fruits 9–12 weeks after anthesis. Integument explants were induced to embryogenesis by a 25-day culture on MH1 medium consisting of the basal medium for *Hevea* culture (MH) as described by Carron et al. (1989) supplemented with 4.44 μM 3,4-dichlorophenoxyacetic acid (3,4-D), 4.44 μM kinetin (KIN), 30 μM $AgNO_3$ and 234 mM sucrose. Expression of embryogenesis was obtained following a second 25-day subculture on MH2 medium of the same basic composition as MH1 but with 1.35 μM 3,4-D and KIN, 50 μM spermidine, 30 μM $AgNO_3$, 234 mM sucrose and 5×10^{-3} μM abscisic acid. Development of embryos was achieved at the end of a third 30-day culture on MH3 medium having the same basic composition as MH1 but with 1.8 μM 3,4-D, 0.9 μM N^6-benzyladenine in place of KIN and 234 mM sucrose. Media were solidified with 2.3 g l^{-1} phytagel. All cultures were maintained in darkness at 27 °C.

There were 1144 integument explants of BT 86 originating from 14 fruits (30–267 explants per fruit), 1819 integument explants of BT 96 originating from 33 fruits (36–102 explants per fruit) and 537 integument explants of EM 96 originating from four fruits (108–135 explants per fruit).

The differences in explant number per fruit reflect the varying size of the seeds produced per fruit.

Obtaining embryogenic callus lines from primary somatic embryo explants (secondary somatic embryogenesis)

We followed the protocol for secondary somatic embryogenesis described by Lardet et al. (2008). Well-formed mature primary embryos, displaying well-developed embryo axis and cotyledons, were collected at the end of the 30-day culture on MH3 medium (Lardet et al. 1999). The primary somatic embryos were cut longitudinally into two or three sections, each of 2–3 mm in length, and each section was placed in contact with friable callus induction medium (INF) enriched with growth regulators and calcium (Montoro et al. 1993). Embryo explants were maintained in these conditions until friable callus clusters formed. When the friable callus fragments reached a fresh mass ranging between 30 and 50 mg, they were transferred to maintenance culture medium (MM) and then subcultured every 2 weeks until they gave rise to embryogenic lines characterized by a stabilized callus proliferation rate of two to three per subculture, as described by Carron et al. (1998).

Evaluation of integument explants' capacity for primary somatic embryogenesis

We recorded data for each integument explant of each fruit. Integument explant capacity for primary somatic embryogenesis was assessed as: (1) mean proportion of callogenic explants expressed as a percentage of the total number of integument explants used per fruit after 25 days of culture on MH1 medium; (2) mean proportion of embryogenic calluses expressed as a percentage of the total number of integument explants used per fruit after 80 days of culture (25 days on MH1, 25 days on MH2, and 30 days on MH3); and (3) mean number of embryos per embryogenic callus after 80 days of culture (25 days on MH1, 25 days on MH2 and 30 days on MH3).

Evaluation of primary somatic embryo-derived explants' capacity for secondary somatic embryogenesis

Capacity for secondary embryogenesis of the three different origins of plant material was assessed in two experiments that differed in the combinations of exogenous growth regulators added to the INF, as detailed below.

Experiment 1 The INF medium was supplemented with 1 of 12 combinations of 3,4-D and KIN (0, 0.44 and

4.4 μM 3,4-D combined with 0.44, 1.34, 4.44 and 8.88 μM KIN). The 4.4 μM 3,4-D and 4.4 μM KIN combination served as the control. The experiment comprised the following numbers of embryo explants of each origin: 414 explants of BT 86 were inoculated on five of the 12 INF combinations (76–86 explants per combination), 1213 explants of BT 96 were inoculated on the 12 INF combinations (90–110 explants per combination) and 770 explants of EM 96 origin were inoculated on the 12 INF combinations (40–80 explants per combination).

Experiment 2 Based on the results of Experiment 1, a second experiment was conducted on somatic embryo explants from BT 96 and EM 96 origins exclusively, exposed to nine 3,4-D and KIN combinations (2.22, 4.44 and 6.66 μM 3,4-D combined with 2.22, 4.44 and 6.66 μM KIN for BT 96 origin and 0.44, 2.22 and 4.44 μM 3,4-D combined with 1.34, 2.22 and 4.44 μM KIN for EM 96 origin). The experiment comprised 680 explants of BT 96 (70–80 explants per combination) and 720 explants of EM 96 (80 explants per combination).

We assessed primary somatic embryo-derived explants' capacity for secondary somatic embryogenesis as: (1) mean proportion of friable calluses, recorded after 3 months of culture on INF medium, expressed as a percentage of the number of primary somatic embryo explants initially inoculated on INF medium and (2) mean proportion of embryogenic lines, recorded after at least three subcultures on the MM, expressed as a percentage of the number of primary somatic embryo explants inoculated on INF medium.

Statistical analyses of the data

Data, which are expressed as means, were analyzed using SAS statistical software (CATMOD and GLM procedures, SAS Institute, Cary, NC) for assessing the effects of the experimental treatments (plant material origins, growth regulators as well as possible interactions between these two factors for Experiments 1 and 2). The general model for one-factor analysis was: $Y_{ij} = \mu + O_i + \epsilon_{ij}$, for one-factor analysis, where Y_{ij} is the jth observation corresponding to the ith level of the factor plant material origin, μ = overall mean, O_i = effect of the factor plant material origin, $1 \leq i \leq 3$; and ϵ_{ij} = residual error. The general model for two-factor analysis was: $Y_{ijk} = \mu + O_i + G_j + (OG)_{ij} + \epsilon_{ijk}$, where Y_{ijk} is kth observation corresponding to the ith level of the factor plant material origin and the jth level of the factor growth regulators; G_j = effect of the factor growth regulator, $1 \leq j \leq 12$; $(OG)_{ij}$ = effect of the interaction between plant material origin and growth regulator; and ϵ_{ijk} = residual error. The χ^2-Pearson's test and the Duncan's multiple comparison test (Sokal and Rohlf 1995) were used to determine significant differences among treatment means. A $P \leq 0.05$ was considered significant for all the statistical analyses.

Results

Integument explants' capacity for primary somatic embryogenesis

The three sources of plant material tested significantly influenced the proportions of callogenic (P = 0.0044) and

Table 1. Characteristics and capacity for primary somatic embryogenesis of integument explants obtained from the BT 86, BT 96 and EM 96 origins derived from the same PB 260 clone of *H. brasiliensis*. Mean proportions were calculated from individual records as the percent of callogenic explants or embryogenic callus obtained from the total number of integument explants per fruit initially used. Abbreviations: SE, standard error; CV, coefficient of variation. Within a row, means followed by different letters are significantly different at P = 0.05 (Duncan's multiple comparison test).

	Explant origin		
	BT 86	BT 96	EM 96
Sampling			
Number of trees collected	6	14	2
Number of fruits	14	33	4
Number of fruits per tree	1–4	1–4	1 and 3
Number of explants inoculated	1144	1819	537
Number of explants per fruit	30–267	36–102	108–135
Primary somatic embryogenesis			
Number of callogenic explants obtained after 25-day culture	690	1323	386
Number of embryogenic calluses obtained after 80-day culture	133	633	209
Mean proportion of callogenic explants obtained per fruit (% ± SE)	62.7 ± 11.9 a	90.6 ± 3.0 b	72.5 ± 4.1 ab
CV (%)	65.4	18.9	11.4
Mean proportion of embryogenic calluses obtained per fruit (% ± SE)	12.6 ± 3.2 a	35.3 ± 2.8 b	55.0 ± 5.3 c
CV (%)	96.3	46.2	19.4
Grand mean of somatic embryos obtained per embryogenic callus (±SE)	1.33 ± 0.18 a	2.24 ± 0.13 b	2.37 ± 0.19 b
CV (%)	51.6	34.0	15.9

embryogenic explants ($P < 0.0001$) obtained per fruit after the 25- and 80-day cultures, respectively (Table 1). An origin effect was also found for mean number of somatic embryos obtained per embryogenic callus ($P = 0.0009$) (Table 1). For these three criteria, the BT 86 planted in 1986 gave rise to the lowest scores with noticeable between- and within-tree variation, as reflected by large standard errors (SE) and coefficients of variation (CV) (Table 1). The proportions of callogenic explants obtained varied from 0% (0/54) to 59.5% (50/84) between fruits from the same BT 86 tree ($P < 0.001$), whereas variations in the proportions of callogenic explants were much less between and among fruits and even between and within trees of BT 96 and EM 96 origins, as illustrated by the lower SE and CV compared with the corresponding values for the BT 96 origin. The 7-year-old trees of BT 96 and EM 96 origins produced higher proportions of callogenic explants than 17-year-old trees of BT 86 origin. Integument explants from the EM 96 source had a greater capacity to produce embryogenic calluses than integument explants from the BT 96 source, and a much greater capacity than integument explants from the BT 86 source. The capacity of these embryogenic explants to further develop somatic embryos was also significantly less for the BT 86 source than for the BT 96 and EM 96 sources.

Capacity of primary somatic embryo-derived explants for secondary somatic embryogenesis

Figure 1A shows the proportions of friable calluses obtained from the various primary somatic embryo sources and growth regulator combinations tested in Experiment 1.

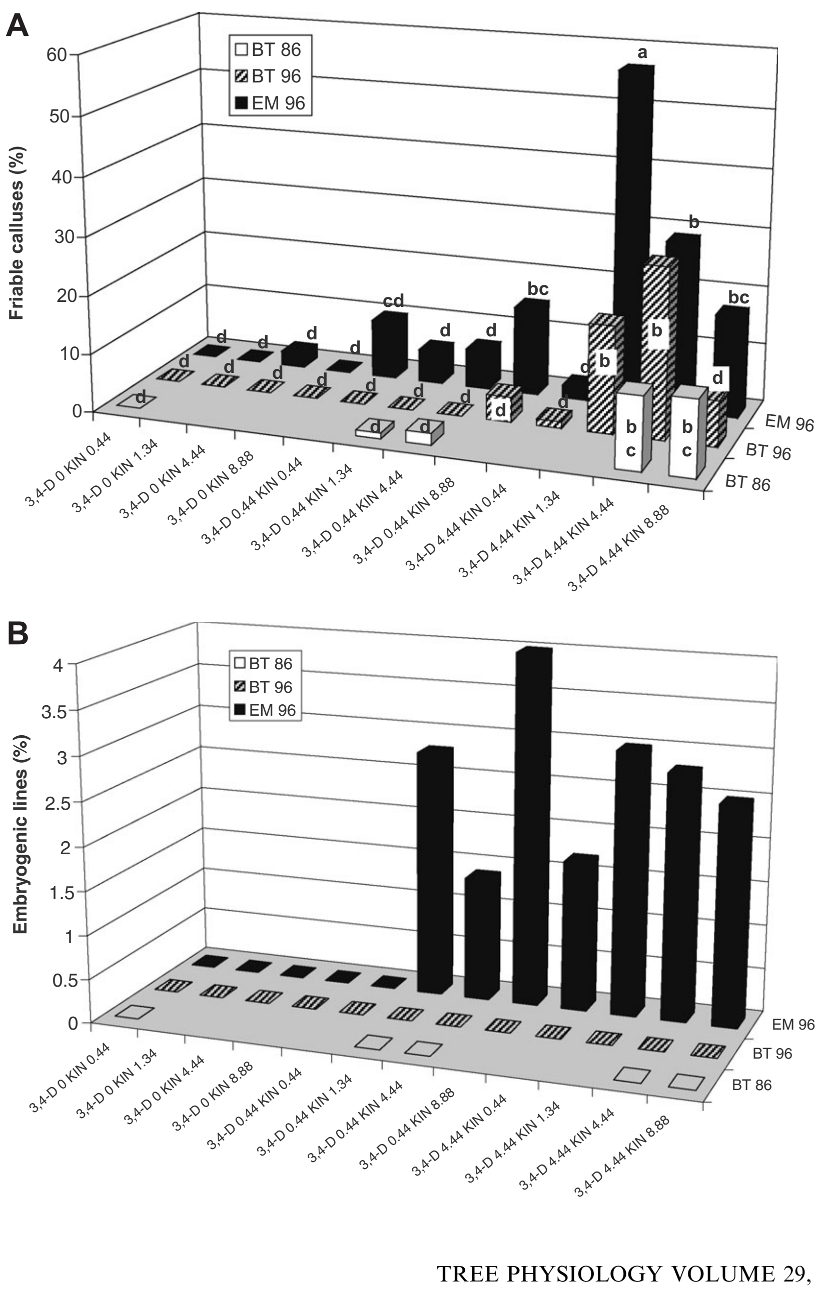

Figure 1. Secondary somatic embryogenesis of mature primary embryos in Experiment 1. (A) Proportions of friable calluses obtained for each of the three plant material origins derived from the same PB 260 *H. brasiliensis* genotype, and tested on various growth regulator combinations added to the INF culture medium. Different letters distinguish values that differ significantly at $P = 0.05$ (χ^2-Pearson's test). (B) Proportions of embryogenic lines obtained for each of the three plant material origins derived from the same PB 260 *H. brasiliensis* genotype, and tested on various growth regulator combinations added to the INF culture medium. There were no significant differences between the values at $P = 0.05$ (χ^2-Pearson's test).

Considering only plant material origin across all growth regulator treatments, the EM 96 source gave rise to higher proportions of friable calluses (92/770 = 11.9%) than the BT 96 (61/1213 = 5%) and BT 86 (34/414 = 8.21%) sources in all growth regulator treatments tested. Considering only growth regulators across the three plant origins, friable callus formation was noticeably stimulated by 4.44 μM 3,4-D combined with 1.34, 4.4 or 8.88 μM KIN, with scores of 33.5% (56/167), 22.4% (57/254) or 13.6% (36/264), respectively. The highest proportion of friable calluses (56.7% (34/60)) was obtained for EM 96 explants exposed to 4.44 μM 3, 4-D and 1.34 μM KIN. However, neither plant material origin nor growth regulator treatment had a significant influence on the proportions of friable calluses recorded. Only EM 96 explants gave rise subsequently to embryogenic lines under the experimental conditions tested (Figure 1B), but the yield was low (14/660 = 2.1%, across all growth regulator treatments), and there were no significant differences between growth regulator treatments.

The results of Experiment 2 indicated that somatic-embryo-derived explants of EM 96 had a higher capacity to produce friable calluses than somatic-embryo-derived explants of BT 96 (114/720 (15.8%) versus 80/670 (11.9%); $P < 0.0001$ across all growth regulator treatments tested). The various growth regulators significantly influenced the capacity of the somatic-embryo-derived explants to produce friable calluses ($P < 0.0001$), and there was a significant interaction ($P = 0.0002$) between plant material origin and the growth regulators tested. The 4.44 μM 3,4-D and 4.44 μM KIN treatment combination resulted in an overall mean value of 20% (32/160) across the BT 96 and EM 96 origins. Somatic-embryo-derived explants of EM 96 produced the most friable calluses (33.8% (27/80)) among the plant origins and growth

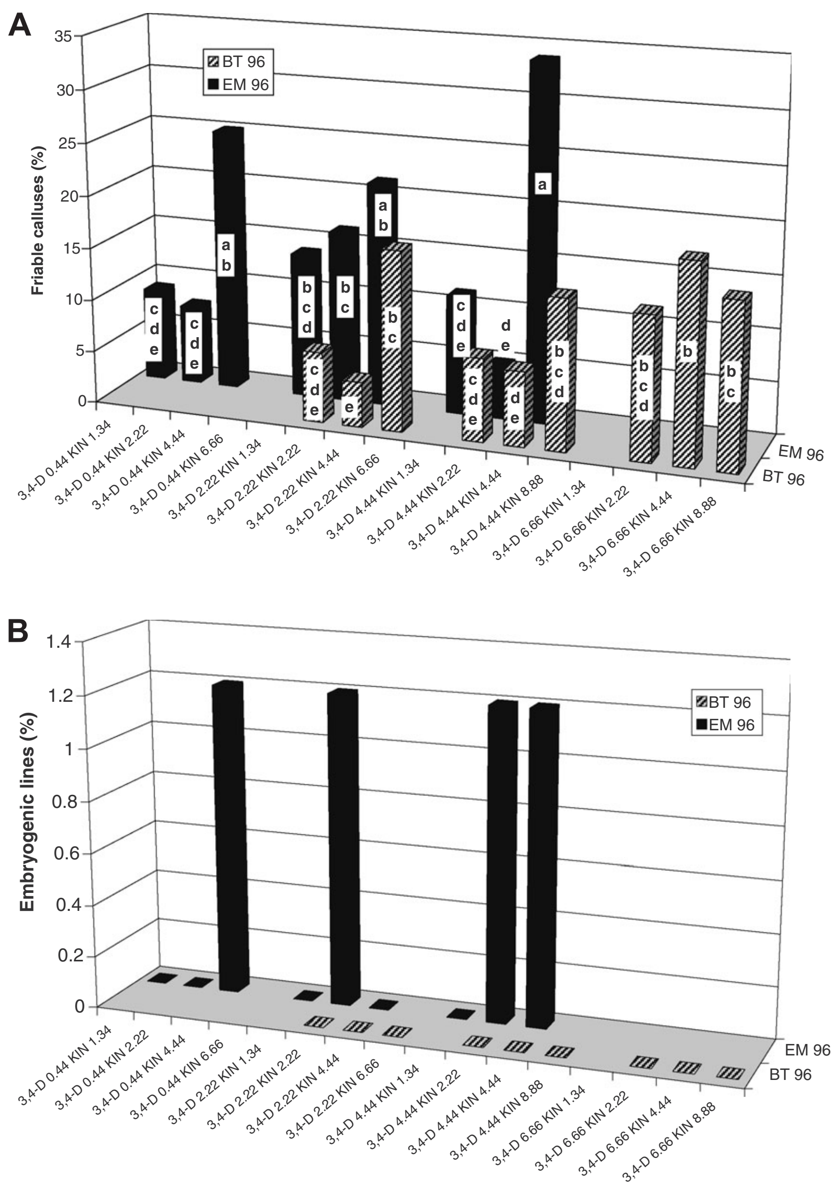

Figure 2. Secondary somatic embryogenesis of mature primary embryos in Experiment 2. (A) Proportions of friable calluses obtained for each of the two plant material origins (BT 96 and EM 96) derived from the same PB 260 *H. brasiliensis* genotype, and tested on various growth regulator combinations added to the INF culture medium. Different letters distinguish values that differ significantly at $P = 0.05$ (χ^2-Pearson's test). (B) Proportions of embryogenic lines obtained for each of the two plant material origins derived from the same PB 260 *H. brasiliensis* genotype, and tested on various growth regulator combinations added to the INF culture medium. There were no significant differences between the values at $P = 0.05$ (χ^2-Pearson's test).

Table 2. Summary of the combined data from Experiments 1 and 2 showing the capacity for secondary somatic embryogenesis of primary somatic embryos obtained from integument explants excised from the BT 86, BT 96 and EM 96 origins derived from the same PB 260 clone of *H. brasiliensis*. Abbreviation: SE, standard error. Within a row, means followed by different letters are significantly different at $P = 0.05$ (χ^2-Pearson's test).

	Explant origin		
	BT 86	BT 96	EM 96
Sampling			
Number of integument explants inoculated for primary embryogenesis	644	1035	537
Number of primary somatic-embryo-derived explants inoculated for secondary embryogenesis	414	1893	1490
Secondary somatic embryogenesis (Experiments 1 and 2 combined)			
Number of friable calluses obtained	34	142	210
Number of embryogenic lines obtained	0	0	16
Mean proportion of friable calluses obtained from the number of explants initially inoculated for secondary embryogenesis (% ± SE)	8.21 ± 0.07 a	7.50 ± 0.01 a	14.09 ± 0.02 b
Mean proportion of embryogenic lines obtained from the number of explants initially inoculated for secondary embryogenesis (% ± SE)	0 a	0 a	1.07 ± 0.01 b
Mean proportion of embryogenic lines obtained from the number of integument explants initially inoculated for primary embryogenesis (% ± SE)	0 a	0 a	2.98 ± 0.03 b

regulator treatments tested (Figure 2A); however, only 1.33% (4/320) of the friable calluses from EM 96 origin subsequently produced embryogenic lines (Figure 2B).

To gain a broader perspective of the respective capacities of the sources of plant material studied for secondary embryogenesis, data from Experiments 1 and 2 were compiled and are summarized in Table 2. The results demonstrate that, compared with the BT 86 and BT 96 sources, primary somatic embryos derived from integument explants of EM 96 have a higher capacity to produce friable calluses and subsequent embryogenic lines.

Discussion

Hevea brasiliensis is one of a few arborescent species for which somatic embryos can be obtained from mature individuals via integument explants from seed (Carron et al. 1995). The nature of the initial explant has been recognized for a long time as a determining factor for successful plant tissue culture (Bonga 1982), particularly for somatic embryogenesis which is strongly influenced by the age of the donor plants (Bonga and von Aderkas 1992). Somatic tissues of flowers of several species are reported to have a higher potential for vegetative reproduction than tissues from other plant parts, presumably because of their proximity to rejuvenating sexual cells (Bonga 1982, Bonga and von Aderkas 1992). However, our study has shown that, despite using primary explants obtained from the same integument seed tissues, collected at the same time and under the same conditions from donor plants of the same PB 260 genotype, significant differences in capacity for somatic embryogenesis were observed in the same culture conditions based on the origins and age of these donor plants.

Although of the same age, from the same clone and at a similar developmental stage, the EM 96 trees were produced by somatic embryogenesis, whereas the BT 96 trees were derived from budding. This basic difference between EM 96 and BT 96 had a major effect on the somatic embryogenic capacities of the integument explants excised from immature seeds of these trees. Somatic embryogenesis represents the only experimental method of achieving true ontogenetic rejuvenation (Fortanier and Jonkers 1976). Because all vegetative shoot tissues of a tree, or of its clonal offspring, derive from the initial single shoot primary meristem at the apical pole of the zygotic embryos (Monteuuis 1989), the greater the shoot system development, the higher the number of mitotic divisions required within the shoot apical meristems for the acropetal expansion of the shoots, and hence the more advanced the ontogenetic aging of these meristems (Fortanier and Jonkers 1976). Ontogenetic aging can therefore increase considerably with the number of successive generations of clonal offspring vegetatively propagated from the pre-existing meristems, for instance, by grafting or by cuttings. In contrast, de novo organogenesis or somatic embryogenesis is assumed to reset the process of ontogenetic aging to zero (Fortanier and Jonkers 1976, Greenwood 1984, Lawson and Poethig 1995). Thus, the EM 96 were ontogenetically younger than the grafted BT 96 trees, although of the same chronologic age (Fortanier and Jonkers 1976), and they were chronologically and ontogenetically younger than the grafted BT 86 trees, that were in turn chronologically and ontogenetically older than the BT 96 trees. In fact the BT 86 had 10 years more than the BT 96 to develop ontogenetically. As trees age ontogenetically and chronologically, the shoot apical meristems become more distant from the root system, which serves as the source of many nutrients and hormones (Hackett 1985, Monteuuis 1989), resulting in the shoot apical meristems becoming more prone to physiologic aging (Borchert 1976, Monteuuis 2004). Thus, physiologic aging can be closely associated with the ontogenetic aging that occurs in the shoot apical meristems during tree development and which results entirely from the organogenic activity of these meristems. This physiologic aging affects all the tissues produced by the meristems, including those from maternal origin of the reproductive organs (Borchert 1976, Monteuuis 1989, Baurens et al. 2004).

The results of these cited studies imply that, first, the variation in somatic embryogenesis capacity among fruits obtained from the same donor plant could be associated with the location of the fruits and hence the physiologic age of the integument tissues used as explants. This physiologic age varies according to the physiologic zonation existing within each tree in relation to its development and the proximity of its root system (Borchert 1976, Hackett 1985, Monteuuis 1989). Second, because of their respective ontogenetic origins, EM 96 seed integuments should be physiologically younger than BT 96 seed integuments, which, in turn should be younger than BT 86 seed integuments, a ranking that is consistent with the differing capacities for primary somatic embryogenesis of the explants from the origins studied. The finding that these differences persisted during the subsequent steps of secondary embryogenesis further demonstrates the overriding influence of donor plant age, especially physiologic age, on somatic embryogenesis success rates (Bonga and von Aderkas 1992, Dunstan et al. 1995). Higher endogenous concentrations of zeatin riboside in EM 96 tissues than in tissues from grafted plants of the same PB 260 genotype lends further support to our conclusion that the EM 96 trees are physiologically younger than the grafted trees we studied from the same clone (Perrin et al. 1997).

Secondary embryogenesis has been developed in *H. brasiliensis* trees for improving the efficiency of our former somatic embryogenesis technology, which was based on the use of budded-tree integuments as primary explants, mainly by reducing subculture number and duration (Lardet et al. 2008). However, despite these improvements, the success rate of the secondary embryogenesis technology based on explants from budded-tree integuments remained unexpectedly low ($< 0.1\%$), unpredictable and highly dependent on the composition of the medium (Lardet et al. 2008). The possibility of obtaining $> 1\%$ of embryogenic lines from embling-derived integuments as primary explants represents a major advance in *H. brasiliensis* secondary somatic embryogenesis compared with the scores of $< 0.1\%$ obtained from explants of integument tissue obtained from budded trees. A higher organogenic responsiveness on a wider range of culture media for physiologically juvenile tissues compared to mature ones was observed in other species (Monteuuis 1987, 2004), indicating that it may be a general phenomenon.

Acknowledgments

This work was carried out under a research agreement between CIRAD and Michelin Company on in vitro mass propagation of *H. brasiliensis*. The authors are indebted to Michelin Company, and more particularly to Dr. Josiane Julien and Dr. Françoise Granet, Heads of the Cell Biology Laboratory, for supplying the rubber tree fruits used in this study.

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