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Secondary Somatic Embryogenesis in Hevea brasiliensis *(Müll. Arg.): An Alternative Process for Long-term Somatic Embryogenesis*

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A procedure for long-term somatic embryogenesis called Maintained Somatic Embryogenesis (MSE) was previously described in Hevea brasiliensis *to obtain embryogenic callus lines directly from integument explants. However, the frequency with which embryogenic friable callus lines are directly established from the inner integument of immature fruit is low and restricted to the clones PB 260 and RRIM 703. In this study, the ability of somatic embryos to develop friable callus lines with proliferating and embryogenic capacities was highlighted. Histological analyses revealed dedifferentiation of the epidermal and perivascular cells, giving rise to the formation of friable embryogenic callus on the periphery of somatic embryo explants. Cell proliferation induced by subsequent subcultures of calli on maintenance medium resulted in the establishment of embryogenic callus lines. This new procedure is called indirect Secondary Somatic Embryogenesis (SSE). The potential of embryo-derived embryogenic callus lines was assessed in comparison with the integument-derived embryogenic callus line produced using the MSE procedure. Embryogenic callus lines were established with the clones PB 260 and RRIM 703 for the new process with a higher frequency than those obtained with the MSE procedure. In addition, the number of subcultures needed to establish callus lines was shortened. Secondary somatic embryogenesis also enabled the production of friable callus lines for the PB 217 clone, which was recalcitrant to the previous process. This alternative procedure opens the way for applications of long-term embryogenesis to many other clones for which primary somatic embryogenesis is successful.*

Keywords: Rubber tree; somatic embryogenesis; somatic embryo; micropropagation; *in vitro* culture

Abbreviations:
CIRAD: Centre de Coopération Internationale en Recherche Agronomique pour le Développement; MSE: Maintained Somatic Embryogenesis; SSE: indirect Secondary Somatic Embryogenesis; MH1: Callogenesis medium for primary somatic embryogenesis; MH: basal medium for Hevea *culture; 3,4-D: 3,4-dichlorophenoxyacetic acid; KIN: kinetin; ABA: abscisic acid; MH2: embryo expression medium for primary somatic embryogenesis; MH3: embryo development medium for primary somatic embryogenesis; BA: N 6-benzyladenine; INF: INduction medium for Friable callus obtained in long-term somatic embryogenesis; EXP: embryo EXPression medium for long-term somatic embryogenesis; MM: callus Maintenance Medium for long-term somatic embryogenesis; PAS: Periodic Acid-Schiff; NBB: naphthol blue-black*

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Hevea brasiliensis is the main industrial source of natural rubber. Rubber plantations are traditionally established with selected clones budded onto unselected seedlings. Clone ageing, genetic heterogeneity of rootstocks, and graft union hamper growth and plantation uniformity. Propagation of self-rooting clones by somatic embryogenesis was attempted to improve both growth and natural rubber yield[1]. This potential was recently confirmed[2]. Rejuvenation and self-rooting have respectively led to a gain in both vigour and uniformity in such new planting material.

Several teams in the world are working to develop an *in vitro* technique for mass propagation of rubber trees[1,3,4,5]. At CIRAD, two somatic embryogenesis procedures have been developed using the inner integument of immature seeds: i) primary somatic embryogenesis, a short-term process, and ii) maintained somatic embryogenesis, a long-term process. The inner integument is a maternal tissue, which is rapidly reactive on culture medium to produce callus. Callus structure depends on the calcium concentration in the callogenesis medium[6]. The primary somatic embryogenesis process (PSE) consists of a first phase of callogenesis, generally producing a compact callus, a second phase of differentiation of undifferentiated cells into embryogenic cells, and then a third phase of embryo development at the expense of callus proliferation[1]. Today, more than 22 *Hevea* clones have been regenerated by this technique worldwide[7–13] (*Table 1*). Several maintained embryogenic callus lines were obtained for clone PB 260 from 1993 to 2005. It took 10 to 18 months to establish embryogenic friable callus lines with a frequency of less than 1‰ per initial explant. Maintained somatic embryogenesis (MSE) is based on long-term maintenance of both the proliferation and regeneration of friable calli[12] over several months through successive subcultures. Early cryopreservation of embryogenic lines allowed long-term maintenance over several years[14]. Both the duration and the low frequency of this process remain a constraint for application of the technique to other clones. Indeed, the attempts carried out on clones PB 217, PB 314, RRIM 600, RRIM 703, PB 254, BPM 24 and PR 107 were mainly unsuccessful, generating only one line for each clone RRIM 703 and PR 107 with a poor regenerating embryogenic capacity.

The degree of embryogenic response is strongly linked to the nature of the explant. According to several authors, the younger the tissues are the better the initiation of embryogenesis culture is[15–17]. Reproductive organs, such as zygotic embryos, are the preferential explants for embryogenic culture induction in *Picea Abies*[18], hybrid larch[19], *Pinus taeda*[20] or for cereals[21], and cotyledons for cassava[22] or mango[23]. For the rubber tree, somatic embryogenesis was achieved early in the 1980s from the anther[24] and inner integument of immature seeds[25], and more recently from zygotic embryos[13]. Embryogenic callus was induced from floral staminodes in *Theobroma cacao*. However, secondary somatic embryogenesis induced from cotyledons led to an increase in embryogenic capacity in cocoa[26]. Interestingly, Noriega and Söndahl[27] showed that primary somatic embryos were an excellent source for obtaining embryogenic friable calli in a hybrid rose cultivar. Hence, zygotic and somatic embryos have therefore proved to be choice explants to generate embryogenic tissues.

In this study, we investigated the ability of somatic embryos derived from inner integument callus to generate a proliferating friable callus at the origin of embryogenic callus lines. Callus initiation was characterised at the morphological and cytological levels in order to specify the nature of the reactivated tissues. A comparison of both embryo-

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derived callus and integument-derived callus procedures was carried out in terms of callus induction, the time taken to establish callus lines, and culture conditions. The new procedure involving secondary embryogenesis is proposed for developing embryogenic lines from newly selected rubber tree clones.

MATERIALS AND METHODS

Plant Material

The study was carried out with cultivated clones PB 260, PB 217 and RRIM 703. Primary somatic embryogenesis and maintained somatic embryogenesis (MSE) were initiated from a thin slice of the inner integument of immature seeds, which is a maternal diploid tissue[1].

Primary Somatic Embryogenesis (PSE)

The PSE process was previously described by Carron *et al*.[1]. Embryogenesis induction was obtained by culturing integument explants for 25 days on MH1 medium containing a basal medium for *Hevea* culture (MH) described by Carron *et al*.[25], 4.44 µM 3,4-dichlorophenoxyacetic acid (3,4-D), 4.44 µM kinetin (KIN), 30 µM $AgNO_3$ and 234 mM sucrose. Embryogenesis expression was obtained by a second 25-day subculture on MH2 medium having the same basic composition but supplemented with 1.35 µM for both 3,4-D and BA, 50 µM spermidine, 30 µM $AgNO_3$, 234 mM sucrose and 5 nM abscisic acid (ABA). Embryo development was achieved after a third 30-day culture on MH3 medium having the same basic composition but supplemented with 1.8 µM 3,4-D, 0.9 µM N 6-benzyladenin (BA) in place of KIN and 234 mM sucrose. The media were semi-solidified with 2.3 gL^{-1} phytagel and calli were cultured in the dark at 27ºC.

Maintained Somatic Embryogenesis (MSE)

The establishment of embryogenic callus lines was first described by Carron *et al*.[12]. Fragments of inner integument were cultured on a friable callus induction medium (INF) corresponding to the MH1 medium supplemented with 9 mM of $CaCl_2$. Four to eight fortnight-long subcultures were usually required to initiate friable callus formation. The friable callus aggregates were isolated on maintenance medium (MM) containing the basic MH medium also supplemented with 234 mM of sucrose, 30 µM $AgNO_3$, 1.34 µM BA, 1.34 µM 3,4-D, and 6 mM of $CaCl_2$. They were maintained on the MM medium with 2 to 3-week-long subcultures till they gave rise to friable callus lines characterised by a stabilised callus proliferation rate of 2 per subculture, as described by Carron *et al*.[12]. Each callus line originated from a single callus aggregate. Once stabilised, calli were subcultured regularly every two weeks on fresh medium. The media were semi-solidified with 2.3 gL^{-1} phytagel and calli were cultured in the dark at 27ºC.

Embryos Development and Conversion into Plantlets

Embryo induction was achieved by subculturing callus on an expression medium (EXP), which was a modified MM medium supplemented with 9 mM $CaCl_2$, 58.5 mM sucrose, 175.5 mM maltose, 0.44 µM BA, 0.44 µM 3,4-D and 0.5 µM ABA. The development of pro-embryos was carried out in a temporary immersion system (RITA®, CIRAD, France) with 1 min of immersion per day in the liquid development medium (DM), which was a MM medium containing 234 mM sucrose, 3 mM $CaCl_2$, and did not contain any growth regulators. After 8 weeks of culture, well-formed mature embryos displaying a well-developed embryonic axis and cotyledon

were collected[28]. They were transferred into glass tubes containing a germination medium (GER), which consisted of MS macro-elements[29], MH micro-elements and vitamins, 234 mM sucrose and semi-solidified with 7 gL^{-1} Agar (Sigma A1296). Embryos were cultured under a light intensity of 60 µmol m^{-2} s^{-1} with a 12h day/12h dark photoperiod for embryo conversion into plants.

EXPERIMENTAL

Secondary Somatic Embryogenesis

In SSE, embryogenic callus lines were established from somatic embryos derived from inner integument callus. Experiments were conducted with clones PB 260, PB 217 and RRIM 703 (global experimental schema in *Figure 1*).

A first experiment was carried out with clones PB 260 and PB 217. Inner integument explants were cultured under the previously described primary somatic embryogenesis conditions. At the end of the culture on MH3 medium, the somatic embryos were collected and sliced applying an axial longitudinal cut. Each embryo fragment comprised half of the embryonic axis and a more or less developed cotyledon. The embryo explants were placed with the cut side against the friable callus induction medium (INF) and maintained under culture conditions without subculturing until friable callus aggregates formed. Once the friable calli reached sufficient fresh weight (30 to 50 mg), they were isolated on MM maintenance medium and subcultured on fresh medium until the proliferation rate had stabilised.

A second experiment with clone RRIM 703 was used to check the ability of MSE-derived somatic embryos to establish new friable callus lines. The culture conditions for obtaining long-term somatic embryogenesis by the MSE process were already described. The culture conditions for obtaining friable callus lines from somatic embryos were the same as those described above, for clones PB 260 and PB 217.

The efficiency of the two processes was compared according to the following criteria: culture number and culture duration to obtain a proliferating friable callus on INF medium, culture number and culture duration to establish callus lines by subcultures of the friable callus on MM medium, frequencies of callus line achievement. The Chi-square test was used to compare the significance of the frequencies at a confidence level of 95%.

Histological Study

The study was carried out on primary embryo explants of clone PB 260 after 1 to 3 weeks of culturing on INF medium and on calli after 6 weeks' culturing on the same medium. Specimens were fixed in a solution containing 1% glutaraldehyde, 2% paraformaldehyde, 1% caffeine in 0.2 M phosphate buffer, pH 7.2, for 24 h. After progressive dehydration, specimens were embedded in Kulser 7100 resin and cut into 3 µm thick sections. Sections were double-stained with periodic acid-Schiff (PAS) – naphthol blue-black (NBB). PAS specifically stained polysaccharides (walls and starch), while NBB specifically revealed soluble and storage proteins.

RESULTS

Evaluation of the Secondary Somatic Embryogenesis Process for Obtaining Friable Callus Lines

Fragments of inner integument gave rise to embryogenic compact calli under culture conditions for primary somatic embryogenesis

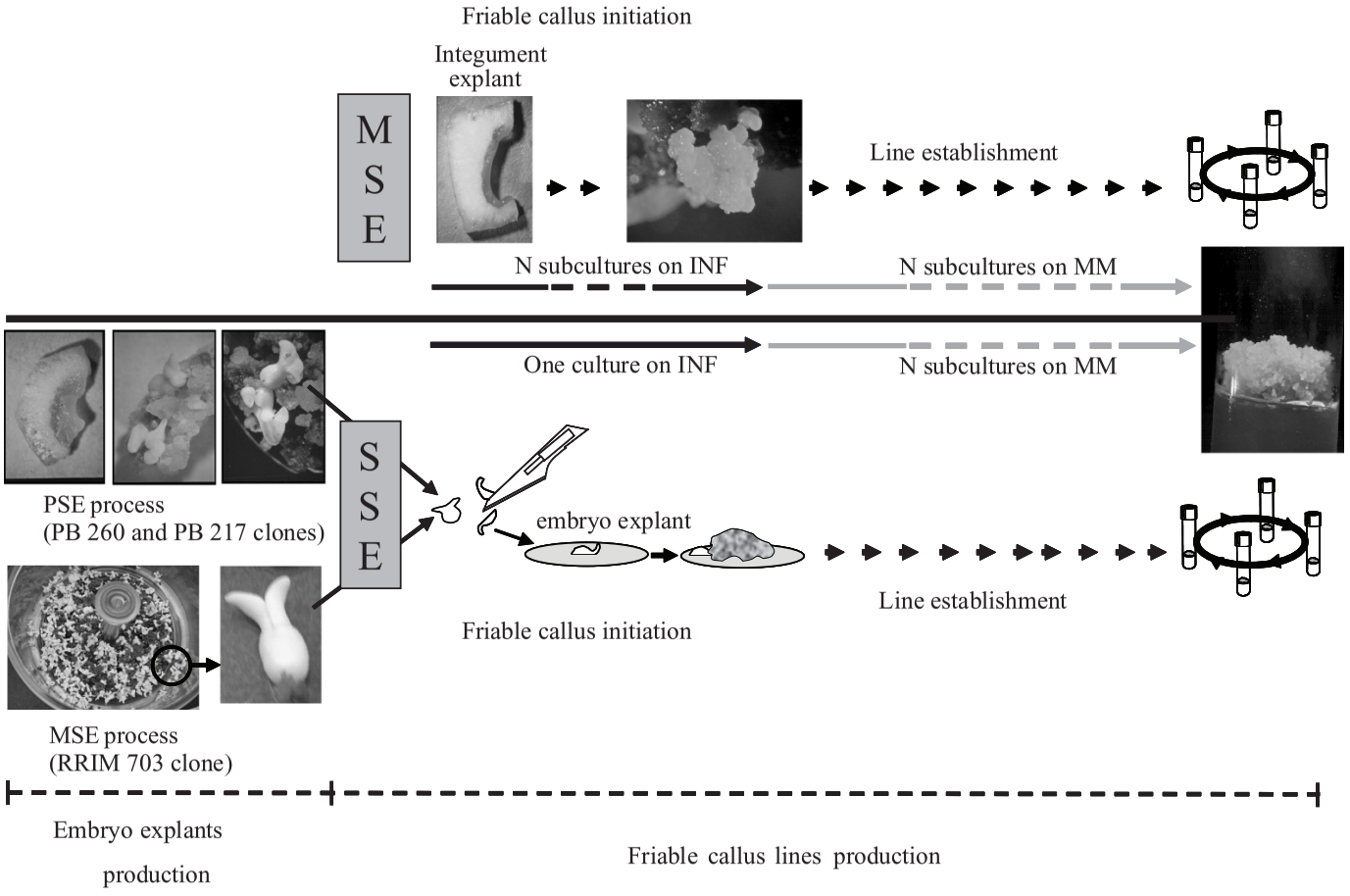

Figure 1. Global experimental schema for SSE compared to MSE implementation for obtaining friable callus lines.

(PSE, *Figure 2A*). By contrast, conditions for MSE induced a yellow proliferating friable callus after at least four subcultures on INF medium. Such an achievement required only one subculture on INF for the SSE process. Although a large number of friable callus aggregates failed to proliferate after transfer to MM medium, friable callus lines were established for clone PB 260 with a frequency of 0.72‰ and 1.09‰ for the MSE and the SSE processes respectively (*Table 2*). This result confirmed the ability of somatic embryo fragments to produce friable callus lines. Interestingly, the time taken to establish callus lines was substantially shorter for the SSE process (7 to 10 months as in *Table 2*) than for the MSE process (10 to 18 months). This advantage was acquired during the line establishment phase, which was greatly shortened for SSE (4 to 7 subcultures) compared to MSE, requiring 6 to 15 subcultures. Better callus proliferation was found from embryo fragments, leading to rapid establishment of friable callus lines as soon as those calli were isolated on MM medium.

For clone PB 217, two friable callus lines were established by the SSE process, whilst the MSE process did not generate any, even using a larger number of explants at culture initiation (11001 compared to 6682 fragments of inner integument) and a large number of subcultures on induction medium (*Table 2*). This was the first evidence of friable callus lines obtained with clone PB 217.

For clone RRIM 703, 3 friable callus lines were obtained with a frequency of 0.69‰ using the MSE process (*Table 3*). Somatic embryos derived from those 3 lines were then cultivated according to the SSE process (First cycle = C1). After a reduced number of cultures (one on INF medium and 2 to 5 on MM medium), friable calli gave rise to new embryogenic callus lines with significantly higher frequencies ($P < 0.0001$) ranging from 25.6 to 39.2‰. As a consequence of the reduced number of subcultures, the total culture duration to establish the new lines was brought down to 4 to 5 months whilst 9 months were required for the MSE process. A second SSE cycle (C2) using somatic embryos from SSE line A02 from cycle 1 was attempted. The short initial duration was maintained and that frequency remained at the same level (26.3‰). The high frequency observed with clone RRIM 703 line establishment revealed the high responsiveness of somatic embryos derived from long-term somatic embryogenesis compared to that of primary somatic embryos.

With the three clones assessed in the SSE process, proliferating friable calli were obtained after a single culture on INF medium, and friable callus lines were also established with a reduced number of subcultures on MM medium compared to MSE process. Moreover, for clone RRIM 703, friable callus lines were established at a significantly higher frequency than that allowed by the MSE process.

Histological Study

The purpose of this study was to identify the reactive tissues giving rise to calli and the cellular characteristics of the calli. The responsive embryo explants, which were cultured on INF medium, displayed cellular activity after 1 to 2 weeks of culturing revealed by surface yellowing of the explant (*Figure 2B*). The first friable callus clusters were observed from the third week of culture (*Figure 2C*). They grew rapidly to form a callus with a fresh weight of 30 to 50 mg after 2 to 3 weeks of culture (*Figure 2D, 2E*). Friable calli must be subcultured on MM medium otherwise they tend to become compact (*Figure 2F*).

TABLE 1. THE DIFFERENT CLONES REGENERATED BY PRIMARY AND LONG-TERM SOMATIC EMBRYOGENESIS PROCESSES.

Process type	Explants used	Clones regenerated	References
Primary somatic embryogenesis			
	Immature seed integument	PB 260, PR 107, RRIM 600, PB 235, IRCA 109*, PB 254*, PB 310*, PB 311*, BPM 24*, RRII 105*	Carron *et al.*[1]
	Anther (mononuclear stage)	Haiken 1, Haiken 2, GL1 SCATC 43 Dafeng 95*, Reyan 7-33-97*, Wenchang 217*, Yun Yan 77-2*	Chen *et al.*[7] Yuan *et al.*[8]
	Anther (immature floral buds)	PR 300	Sumarmadji and Darmono[9]
	Anther (immature floral buds) - Inflorescence	RRII 105	Thulaseedharan *et al.*[10]
	Cotyledons of zygotic embryos	Non selected clones	Huang Tiandai *et al.*[11]
Long-term somatic embryogenesis			
	Immature seed integument	PB 260, RRIM 703*	Carron *et al.*[12]
	Anther (immature floral buds) - Inflorescence	RRII 105	Kumari Jayasree and Thulaseedharan[13]

Note: * Unpublished results until now

TABLE 2. COMPARISON OF THE CULTURE CONDITIONS, CULTURE DURATION AND FREQUENCIES FOR ESTABLISHMENT OF FRIABLE CALLUS LINES BETWEEN THE MAINTAINED SOMATIC EMBRYOGENESIS (MSE) PROCESS AND THE SECONDARY SOMATIC EMBRYOGENESIS (SSE) PROCESS USING EXPLANTS DERIVED FROM PRIMARY EMBRYOS FOR CLONES PB 260 AND PB 217.

Clone	Process designation	Integument explants		Culture conditions to obtain friable callus lines						Friable callus lines	
		Mean per replicate	Total (N)	Primary embryogenesis Duration (day)	Friable callus initiation (INF medium) Subcultures Number	Friable callus initiation (INF medium) Duration (day)	Line establishment (MM medium) Subcultures Number	Line establishment (MM medium) Duration (month)	Total duration (month)	Number (L)	Frequency (L/N) (‰)
PB 260	MSE process[1] (11 replicates)	1523	16754	-	4 to 8	60 to 135	6 to 15	8 to 13	10 to 18	12	0.72[a]
	SSE process[2] (1 replicate)	1819	1819	80	1	72	4 and 7	2 and 5	7 and 10	2	1.09[a]
PB 217	MSE process[3] (12 replicates)	916	11001	-	4 to 7	75 to 104	No friable proliferant callus obtained			0	0[a]
	SSE process[4] (1 replicate)	6682	6682	80	1	58 and 88	7 and 10	7 and 9	13 and 15	2	0.3[a]

Values with same letters are not significantly different according to the Chi-square test ($P > 0.05$).

An analysis of yellowish explants revealed reactivation of the epidermal and perivascular cells towards an undifferentiated state (*Figure 2G, 2H*). Those cells were characterised by a highly fragmented vacuole, reserve proteins, as indicated by the intense blue staining of the cytoplasm, and the presence of starch grains (*Figure 2I*). There was also substantial thickening of the middle lamella of the pink stained cells (pectin compounds) indicating impending dislocation of the tissues to form a friable callus. When the callus was forming, the histological analysis showed the formation of embryogenic nodules on the periphery of the explant (*Figure 2J*). By isolating themselves, those nodules gave rise to embryogenic friable calli (*Figure 2K*) containing clusters of typical embryogenic cells (high nucleus: plasma ratio, clearly visible nucleolus, reserve proteins, and presence of starch displaying major mitotic activity (*Figure 2L*).

That process of dedifferentiation has already been described during the formation of embryogenic friable callus from zygotic embryos of rice[30] from cotyledon cuts of immature zygotic embryos of mango[23] and during the secondary embryogenesis process in grapevine[31]. In cocoa, the epidermal cells of primary embryos also give rise to secondary embryos[26].

Embryos and Plantlets from SSE Lines

The embryogenic and regeneration capacities of SSE lines were assessed from 10 g of callus cultured on EXP medium. With PB 260 clone, the total number of embryos was 310 and 2900 for each line. Out of them, 90 and 270 were well-formed and transferred to germination medium, regenerating respectively 0 and 10 plantlets. By contrast, no embryo

TABLE 3. COMPARISON OF FREQUENCIES AND TOTAL CULTURE DURATION FOR THE ESTABLISHMENT OF EMBRYOGENIC FRIABLE CALLUS LINES BETWEEN THE MAINTAINED SOMATIC EMBRYOGENESIS PROCESS AND THE SECONDARY SOMATIC EMBRYOGENESIS PROCESS USING EXPLANTS DERIVED FROM EMBRYOS FROM MAINTAINED SOMATIC EMBRYOGENESIS FOR CLONE RRIM 703. CYCLE 1: FIRST SSE PROCESS APPLIED ON SOMATIC EMBRYOS REGENERATED FROM MSE LINES 1, 2 AND 3;
CYCLE 2: SECOND SSE PROCESS APPLIED ON SOMATIC EMBRYOS REGENERATED FROM SSE LINE A02*, ISSUED FROM MSE LINE 1.

Process designation	Explants (N) Integument (MSE) or embryo (SSE)	Somatic embryo origin	Culture duration for line establishment (month)	Embryogenic lines obtained Number (L)	Frequency (L/N) (‰)
MSE process	4307		9	3	0.69[b]
SSE process (Cycle 1)	39	MSE line 1	4	1*	25.6[a]
	408	MSE line 2	4.5 to 5	16	39.2[a]
	465	MSE line 3	5	17	36.5[a]
SSE process (Cycle 2)	1028	SSE line A02*	3 to 4	27	26.3[a]

Values with different letters are significantly different according to the Chi-square test ($P < 0.05$).

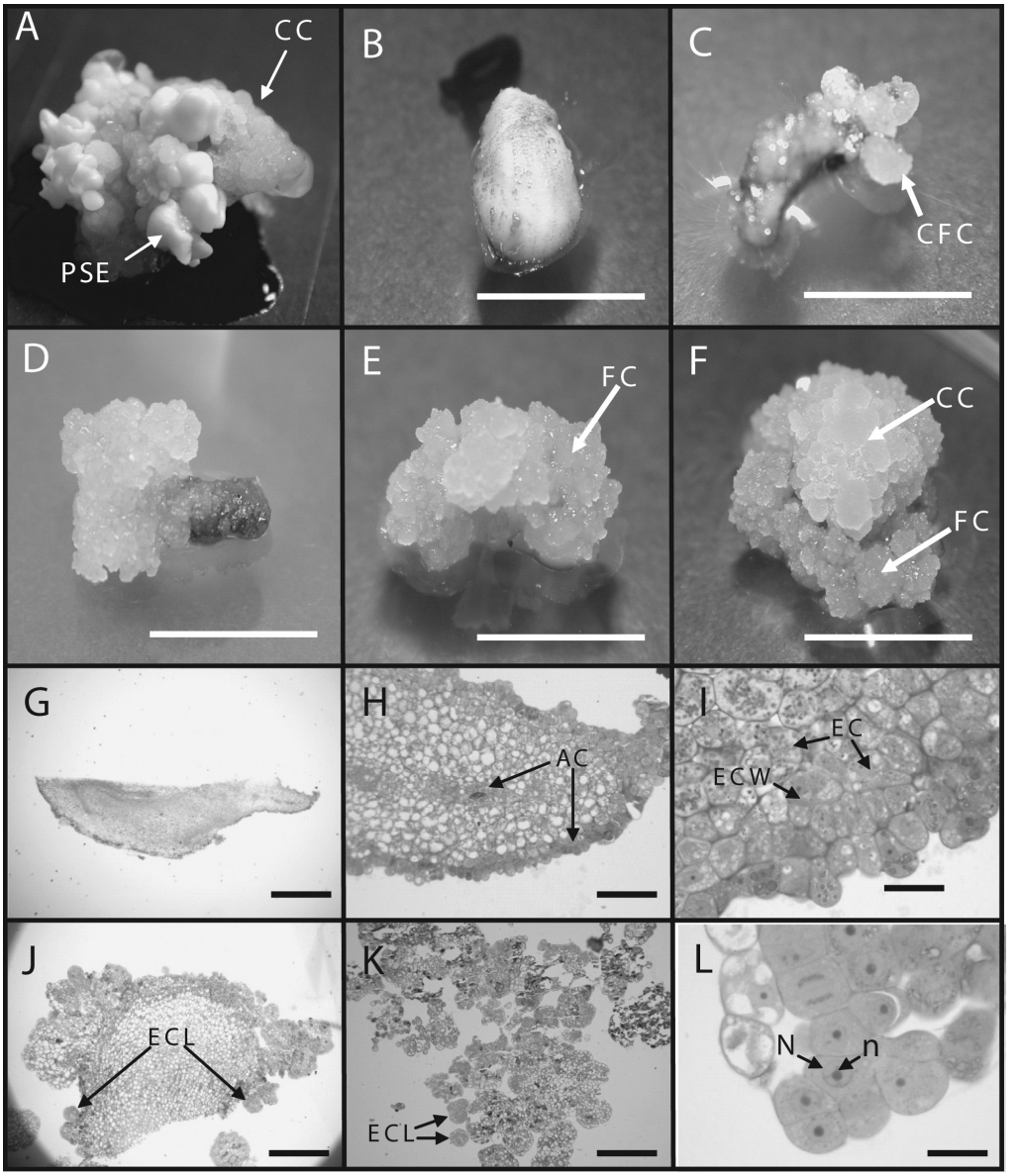

Figure 2. A to F: Morphological study of friable callus development
A: Compact callus (CC) bearing primary somatic embryos (PSE) ; B: Somatic embryo explant after 1 week of culture; C: Somatic embryo explant after 3 weeks of culture, CFC: clumps of friable callus; D and E: Friable callus (FC) developed on somatic embryo explant after respectively 2 and 3 months of culture; F: Friable and compact callus developed on somatic embryo explant after 2.5 months of culture FC: friable callus, CC: compact callus
G to L: Histological study of friable callus development
G: Longitudinal section of the somatic embryo explant after 1 week of culture; H: Active cells (AC) at the epidermis and perivascular level; I: Embryogenic cells (EC) in the sub-epidermal cell layers, ECW: enlarged cell wall; J: Longitudinal section of the somatic embryo explant after 3 weeks of culture, ECL: embryogenic cluster; K: Friable embryogenic callus derived from somatic embryo explant after 6 weeks of culture; L: Detail of active embryogenic cells (N: nucleus, n: nucleolus)

was regenerated from clone PB 217, revealing no embryogenic capacity of those callus lines. Regeneration tests with the eleven RRIM 703 lines gave well-formed embryos ranged from 2 to 450 (mean 70) and plantlets ranged from 2 to 80 (mean 18) respectively. Despite the high variability of regeneration intensity and low rate of the embryo conversion, these results indicated that friable callus lines of PB 260 and RRIM 703 derived from the SSE process are embryogenic.

DISCUSSION

Characterisation of the SSE Process

For several species, secondary somatic embryogenesis has proved to be an efficient way to achieve long-term somatic embryogenesis. According to the definition of Raemakers *et al*.[32], secondary embryogenesis is a process for inducing new embryos from pre-existing embryos. However, the secondary embryogenesis phenomenon takes on several forms depending on the species considered. In cocoa, the formation of secondary embryos is direct, without passing through a callogenesis phase[26]. In banana, clusters of secondary embryos are formed on the primary embryo[33]. In pepper, secondary embryos are formed from a small clump of embryogenic tissues at the base of the root pole of primary embryos[34]. In grapevine or some species of roses, the formation of secondary embryos involves the formation of an embryogenic callus where proliferation and regeneration are concomitant[27,31,35]. The technique developed in this paper on the rubber tree corresponds to indirect secondary somatic embryogenesis in the sense mentioned above, where proliferation and regeneration are dissociated. The somatic embryo fragments develop an embryogenic friable callus capable of proliferating or regenerating embryos on maintenance medium or embryogenesis expression medium respectively.

SSE Process with Regard to Growth Regulator Exposure

Friable callus initiation was achieved by subculturing explants on a high 3,4-D and KIN enriched medium (4.4 µM of each). With the MSE process, this phase required several subcultures, whereas it was carried out with a single culture with the SSE process. Moreover, the number of subcultures on MM medium required for line establishment was reduced with the SSE process compared to the MSE process. It has been shown that somaclonal variation increases with the number of multiplication cycles or with the length of time spent in culture[36–39]. Abnormalities in canopy architecture, branching, leaf shape and colour on some trees derived from the MSE process have been observed in the rubber tree[2]. The finding of variants on some loci in microsatellite tests has provided some evidence for mutations[2]. The SSE process is therefore of particular interest since it reduces the time cultures are exposed to high concentrations of growth regulators, hence a reduced risk of somaclonal variation occurring.

Clonal Diversification of the Long-term Somatic Procedure using the SSE Process

To our knowledge, friable callus lines were obtained in this study for the first time with clone PB 217 using the SSE process. Although lines were not embryogenic for this clone, a first step was achieved. Several other clones of economic interest have been regenerated by several teams in the world (*Table 1*). Most of those clones are only responsive to the primary somatic embryogenesis process which does not allow mass propagation in the absence of embryogenic tissues proliferation. The indirect secondary somatic embryogenesis process developed in this study should lead to long-term somatic embryogenesis with clones that respond even weakly in primary somatic embryogenesis. It may help in diversifying the

clones established in the form of embryogenic lines, with a view to developing a process for mass propagation.

CONCLUSION

This is the first study showing the feasibility to obtain callus lines, somatic embryos and plantlets through an indirect secondary somatic embryogenesis process in rubber trees. Besides usual ways to obtain somatic embryogenesis from anther or inner integument of immature seed, somatic embryos through indirect secondary somatic embryogenesis provide an alternative way to achieve long-term somatic embryogenesis in rubber trees. Moreover, reducing the culture duration on INF and MM medium for callus lines establishment significantly reduces the growth regulator exposure then the risk of somaclonal variation. It would be necessary to carry on with the experiment to validate this way on many selected clones.

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