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Full Paper

Induction of somatic embryos of *Coffea arabica* cv. Catimor CIFC 7963 using spirulina extract

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Abstract: The influence of spirulina extract (SPE) in the process of somatic embryo (SE) induction of *Coffea arabica* cv. Catimor CIFC 7963 was studied. The explants of the second expanded leaves (2nd leaves) were cultured on solid Murashige and Skoog (MS) medium supplemented with 1 μM 2,4-dichlorophenoxyacetic acid (2,4-D) + 5 μM 6-benzlyaminopurine (BA). The SPE was varied in the following order: 0%, 3%, 4% and 5% (v/v). To determine the germination of SE, 6-month-old cotyledonary embryos were cultured on MS medium without plant growth regulators (PGRs). The results showed that cells of the 2nd leaves developed callus within 1 month. Some of the obtained calli developed into SEs on the same medium within 3 months. The maximum percentage of SE induction (61%) was obtained from the leaves cultured on MS medium supplemented with 3% SPE + 1 μM 2,4-D + 5 μM BA. The somatic embryos germinated to shoots and some germinated to secondary somatic embryos on PGR-free MS medium. After 7 months, approximately 26-28% of the somatic embryos had developed into normal plantlets. This protocol would be useful for SE induction and somaclonal variation reduction in hybrid coffee.

Keywords: somatic embryo, explants, spirulina extract, leaves, hybrid coffee, *Coffea arabica*

INTRODUCTION

Arabica coffee (*Coffea arabica* L.), an allotetraploid (2n=4x=44) and self-fertilising plant [1, 2], is one of the main agricultural export goods of Thailand. Currently, *C. arabica* accounts for about 65% of coffee production in the world, while *C. canephora* (Robusta) provides most of the remaining 35% of production [3]. Moreover, coffee is the world's largest traded commodity after oil production [4]. In 2018, the economic importance of green and roast beans was evident through the exports from

Thailand, valued at THB 137 million. It is an extremely important perennial agricultural crop in Thailand with annual production exceeding 633,000 kilograms of green and roast beans [5].

At present, *C. arabica* cv. Catimor CIFC 7963 has been reported with the characteristics of leaf rust resistance in Thailand [6]. This variety is the offspring of the cross between *C. arabica* cv. Caturra rojo and a hybrid from Timor (a natural cross of *C. arabica* x *C. canephora*). It provides a low-caffeine coffee with fine aroma underlying its well-known, superior quality. Although a resistant coffee hybrid has been announced, there are other problems to consider, such as the low rate of propagation [7]. Seed propagation results in uncontrolled genetic variation in heterozygous cultivars, a slow rate of multiplication and a short span of seed viability [8]. Consequently, seed is not recommended for hybrid coffee propagation because of the segregation in the F2 generation. There is a greater genetic uniformity in coffee propagation via vegetative grafting or rooting of cuttings, but these are labour-intensive and time-consuming for large-scale production [9].

Tissue culture via somatic embryogenesis and organogenesis provides a better chance for the production of true-to-type hybrid plantlets on a large scale within a short period [6, 10]. Organogenesis of *C. arabica* has low efficiency owing to technical difficulties such as explant sterilisation, high concentration of phenolic compounds, apical dominancy, and a low rate of shoot multiplication [11]. However, somatic embryogenesis might be the most effective technique because of its potential for producing the highest rate of multiplication with a lower production cost [4, 12, 13]. Somatic embryogenesis of coffee was first reported by Staritsky [14], when it was induced from internode sections of *C. canephora* P. ex Fr, followed by leaf sections which increased the frequency of somatic embryos [15, 16].

The somatic embryogenesis of coffee can be achieved via direct somatic embryogenesis (DSE), indirect somatic embryogenesis (ISE) or secondary somatic embryogenesis (SSE) [17]. DSE is characterised by the induction of somatic embryos from pre-embryogenic cells of vegetative tissues, creating an identical clone. Somatic embryos are developed from friable embryogenic callus in ISE [18, 19], while SSE is the process of somatic embryos which are formed from primary embryos [20]. Electron microscopy studies have shown that embryogenic calli are yellowish, friable callus which has a spherical shape and brown callus which is elongated [21]. ISE facilitates the large-scale production of somatic embryos for selected coffee clones and hybrids [22]. DSE can avoid the problem of somaclonal variation. However, the number of somaclones produced through DSE is usually limited and not uniform [10]. Therefore, ISE for crop applications must be improved in order to decrease the production of somaclones from genetic variation at the callus stage by increasing the efficiency of callus development.

The success of induction through somatic embryogenesis is influenced by the genotype of the plant and the type of medium formulation [10]. Somatic embryogenesis is very sensitive to the culturing conditions for coffee, such as nutrient levels and the growth regulator hormone composition of the medium [22]. Moreover, there is still the obstacle of coffee genotype. To induce the embryogenic cells, the explant cultures are transferred to a differentiation medium containing growth regulator hormones such as 2,4-dicholorophenoxyacetic acid (2,4-D), indole-3-butyric acid and α-naphthaleneacetic acid combined with kinetin (KIN), and then they eventually develop into somatic embryos [23]. Söndhal and Sharp [15] obtained an embryogenic callus on a primary culture medium containing auxin for callus induction, followed by a second culture medium without auxin for embryo induction.

In recent years there have been reports of using blue-green algae as a tissue culture medium supplement. The effects of *Spirulina platensis* extract have been mentioned by many scientists. Amin

et al. [24] studied the exchange of plant growth regulators (PGRs) and vitamins in culture media using an extract of *S. platensis* to propagate *Sisymbrium irio* callus. Moreover, the hexane extract of *Spirulina maxima* has been reported to increase the shoot length and seed germination, but to inhibit the root length slightly in both *Vigna radiata* and *Oryza sativa* var. Japonica [25]. The benefits of algae led the authors to study the extract from *S. maxima* in order to induce somatic embryogenesis via ISE of coffee plant.

The aim of this study is to determine the influence of the extract of *S. maxima* on ISE induction in *Coffea arabica* cv. Catimor CIFC 7963. The morphological characterisation of the embryogenic callus initiated from leaf sections is also studied.

MATERIALS AND METHODS

Plant Materials and Explant Preparation

The second leaves of 25 explants derived from the apices of 1-year-old coffee trees (*Coffea arabica* cv. Catimor CIFC 7963), which is a hybrid coffee tree obtained by crossing *C. arabica* cv. Caturra rojo with a hybrid of Timor (a natural cross of *C. arabica* x *C. canephora*) [26], were used for inducing and culturing the embryogenic callus. All explants from the basal part of the leaf were used to induce ISE. They were washed under running water for 5 min. Surface sterilisation was accomplished by soaking in 20% (v/v) commercial bleach (25% w/v NaOCl) solution with a few drops of Tween 80 for 5 min. with continuous agitation, followed by rinsing three times with sterile distilled water. After surface sterilisation, the margin and the midrib of each leaf were removed and the remaining leaf tissue was cut into 1-cm^2 pieces to be used as explants.

Embryogenic Callus Induction

Freeze dried *Spirulina maxima* cells (3.5 g) were extracted by hexane (150 mL). Cell disruption was done using a homogeniser at 2000 rpm for 30 min. The slurry was shaken at 37°C for 2 hr. and then stored at 4°C for 12 hr. The slurry was then filtered through filter paper to give 150 mL of the aqueous spirulina extract (SPE). All explants were placed on the culture media consisting of Murashige and Skoog (MS) medium [27] supplemented with different concentrations of SPE and combinations of 1 µM 2,4-D and 5 µM 6-benzlyaminopurine (BA). They were grown in Petri dishes with the abaxial side facing the medium. Seven treatments (A-G) were used (numbers in %)−A: 0 2,4-D, 0 BA, 0 SPE; B: 1 2,4-D, 0 BA, 0 SPE; C: 0 2,4-D, 5 BA, 0 SPE; D: 1 2,4-D, 5 BA, 0 SPE; E: 1 2,4-D, 5 BA, 3 SPE; F: 1 2,4-D, 5 BA, 4 SPE and G: 1 2,4-D, 5 BA, 5 SPE. All media were adjusted to pH 5.7-5.8 before autoclaving for 15 min. at 121°C. Petri dishes with 20 mL of culture media containing five explants and five replicates were used. The cultures were incubated in the dark at 25±2°C for 1 month. After that, the percentages of explants with calli and the numbers of calli per explant were recorded.

Indirect Somatic Embryogenesis (ISE)

The explants that produced calli were transferred to a Petri dish with fresh medium every month. Then they were incubated in the dark at 25±2°C. After 3 months of culture, the somatic embryo (SE) induction percentage (= [no. of SEs / total no. of 1-month-old calli]×100) per explant was recorded.

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Regeneration of SEs

The globular and torpedo stages of SEs obtained in the experiments described above were transferred to the MS medium supplemented with 0.1 µM BA. SEs were cultivated in the dark at 25±2°C in glass bottles closed with polyethylene food wrap**.**

Germination and Conversion of SEs **into Plantlets**

After 6 months 57 cotyledonary-stage embryos were cultured on the MS medium without PGRs. All cultures were maintained for 7 months at 25±2°C under a light intensity of 30 $\mu mol.m^{-2}.s^{-1}$ for a 16-hr photoperiod.

Statistical Analysis

The experiment was arranged in a completely randomised design with five replications. The statistical analysis was performed using a one-way ANOVA and the significant differences among treatment means were determined using Duncan's multiple range test at $p < 0.05$. The SPSS program [28] version 14.0 was used.

RESULTS AND DISCUSSION

Cell Proliferation and Callus Induction

Cells at the incision site of each leaf explant started to proliferate after 2 weeks on the callus induction medium. This stage was characterised by small clumps of spherical and elongated cells (Figures 1A, 1B). The small clumps of spherical cells were embryogenic cells while the elongated cells were non-embryogenic cells. These results were supported by reports that embryogenic calli were composed of different types of cells, i.e. large elongated and smaller spherical cells [26, 29], while the non-embryogenic calli of the same age as the embryogenic calli were formed by loosely arranged large cells, usually elongated, with large vacuoles and thin walls [29]. Then they developed into the green friable and compact calli which were observed on the primary callus induction medium after 1 month (Figures 1C, 1D). The compact calli were characterised as non-embryogenic callus while the friable calli were embryogenic calli. Similarly, the compact non-embryogenic calli developed on the cut edge of the leaf and continuous growth was not observed [10]. The embryogenic calli of coffee were characterised by small clumps of densely cytoplasmic and spherical cells that were very friable [30].

Embryogenic Callus Induction

In treatments D, E, F and G (2,4-D, BA, and SPE), the calli were observed in the leaf sections. There was no callus in treatments A, B and C (control) (Table 1). The calli were observed in the MS medium supplemented with 2,4-D, BA and SPE. These results suggested that 1 µM 2,4-D plus 5 µM BA induced leaf cells of *C. arabica* cv. Catimor CIFC 7963 to develop callus. Gatica et al. [31] reported that MS medium supplemented with 2,4-D and KIN induced embryogenic calli in *C. arabica.* Similarly, *C. arabica* cv. Catimor leaf that had been cultured on MS medium supplemented with 4.52 µM 2,4-D and 18.56 µM KIN produced embryogenic calli [32]. Thus, different combinations and concentrations of PGRs such as 2,4-D plus KIN or 2,4-D plus BA increase the pre-embryogenic calli due to alleviation of cell division to produce a pre-embryogenic mass [10]. Aga and Khillare [33] also reported that the leaf explants of *C. arabica* L. induced more callus on medium supplemented with 2,4-D in combination with BA. Thus, the 2,4-D in combination with BA was most

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useful for greater callus induction and proliferation compared to other auxin/cytokinin combinations in initiating callus formation.

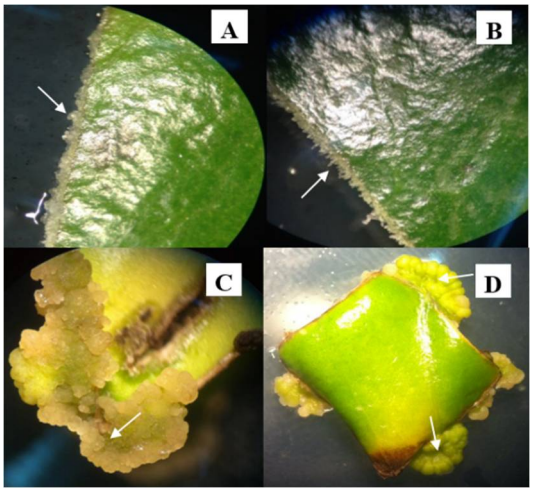

Figure 1. Leaf explants of *Coffea arabica* cv. Catimor CIFC 7963 showing different cell type formations and calli induction: formation of small clumps of spherical cells (arrowed) (A); formation of small clumps of elongated cells (arrowed) (B); leaf with friable callus (arrowed) (C); leaf with compact callus (arrowed) (D)

Both compact and friable calli were observed after 1 month on the primary callus induction medium. The highest percentage of explants responding to the medium (92%) was obtained on leaf explants (19.78±10.86 clusters of callus/explant) cultured on MS medium supplemented with 1 µM 2,4-D, 5 µM BA and 3% SPE. Furthermore, 80% of leaf explants (42.20±10.82 clusters of callus/explant) produced significantly more calli when they were cultured on MS medium supplemented only with 1 µM 2,4-D and 5 µM BA. The addition of SPE had significant effects on callus induction, resulting in the reduction of number of callus per explant (Table 1). However, it was observed that treatment D (2,4-D and BA) produced compact rather than friable callus, while the addition of SPE in the MS medium improved friable callus induction (treatments E, F and G) (Table 1), probably due to the presence of various vitamins in SPE, including β-carotene, inositol, riboflavin, thiamine, vitamin E and ascorbic acid [24]. Vitamins play a role as a cofactor involved in cell metabolism. Thus, the SPE extract might have affected the cell structure or cell organisation such as cytoplasmic viscosity and cell wall thickness. In general, embryogenic calli are friable and their cells are small, isodiametric, and arranged in clusters with a dense cytoplasm [34]. This would stimulate the cell to develop into an embryogenic cell and divide into friable callus.

At the end of the second month, the green embryogenic calli which were very friable turned yellowish and had a smooth surface (Figure 2A). Then abundant calli of white-beige colour differentiated into embryos after a little more than 3 months (Figure 2B). Our results corresponded with Ibrahim et al. [10] who reported that the yellow embryogenic calli of *C. arabica* var. Kartika changed to black brownish colour after 2 months. Then the 3-month-old calli appeared brown blackish and white in the pre-embryo stage. However, the compact non-embryogenic calli did not show continuous growth (Figure 2C). Non-responding explants were also observed as necrotic, brown friable calli (Figure 2D).

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Table 1. Callus induction and callus types on MS medium containing different combinations of supplements

Treatment	Supplement			% of explant with calli	No. of calli per explant (1 month)	Callus type
	2,4-D (µM)	BA (µM)	SPE (% v/v)			
A	0	0	0	0	0	-
B	1	0	0	0	0	-
C	0	5	0	0	0	-
D	1	5	0	80	42.20±10.82[a]	compact, friable[1]
E	1	5	3	92	19.78±10.86[b]	compact, friable[2]
F	1	5	4	84	21.00±6.74[b]	compact, friable[2]
G	1	5	5	80	20.90±8.76[b]	compact, friable[2]

Note: a and b superscripts represent significant differences among treatment means within the same column ($p < 0.05$); [1]compact calli > friable calli; [2] friable calli > compact calli.

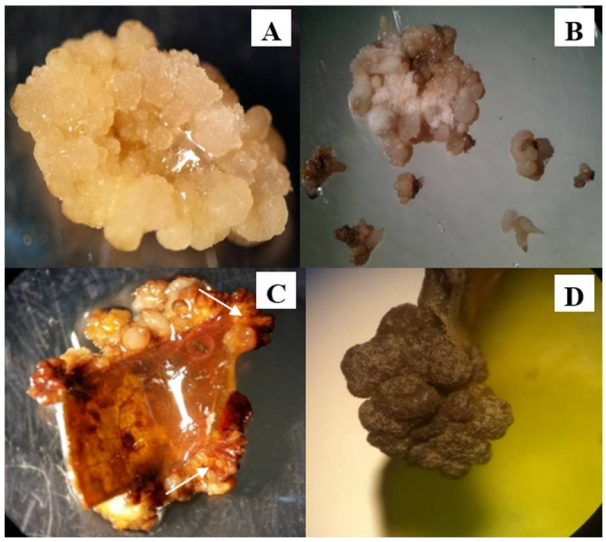

Figure 2. Calli and somatic embryos of *C. arabica* cv. Catimor CIFC 7963 on induction media: clusters of friable embryogenic callus at 2 months (A); embryogenic callus with many somatic embryos at different stages at 3 months (B); compact non-embryogenic callus at 3 months (arrowed) (C); friable non-embryogenic callus at 3 months (D)

Types of Non-embryogenic Callus

The non-embryogenic callus of *C. arabica* cv. Catimor is characterised as friable with rough surface (Figure 3A) or creamy compact callus (Figure 3B), both being clearly distinguished. Friable, non-embryogenic callus with rough surface was also reported by Silva et al. [26], who observed that the callus was organised by isodiametric cells in a compact form which then developed into embryos, while the friable callus with rough surface was observed to be necrotic with brown characteristics. In addition, creamy compact calli involved in the DSE were observed when they were cultured on a medium containing triacontanol [17]. On the contrary, our results showed that all compact calli also

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had necrotic callus without continuous growth after 3 months of culture. These results suggest that the compact non-embryogenic calli which were cultured on the MS medium supplemented with 1 µM 2,4-D, 5 µM BA and SPE were involved in the ISE. Our result corresponds with that of Ibrahim et al. [10], who reported that the compact nodular non-embryogenic calli of *C. arabica* var. Kartika did not show continuous growth.

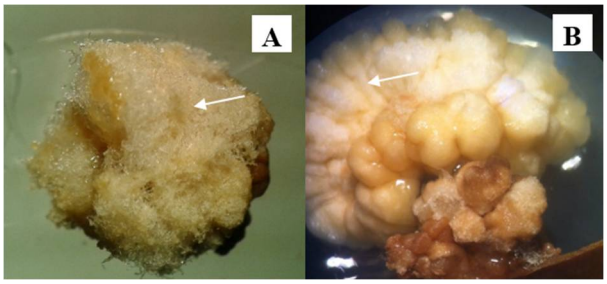

Figure 3. Non-embryogenic calli of *C. arabica* cv. Catimor CIFC 7963 on induction media at 3 months: friable callus with rough surface (arrowed) (A); creamy compact callus (arrowed) (B)

SE Induction

The number of SEs (22.86±14.23 embryos) per explant was highest when the callus was cultured by treatment D, followed by treatment E (12±4.18 embryos), F (11±3.65 embryos) and G (9±2.15 embryos). The presence of SPE significantly reduced the average number of SEs per explant. However, the highest percentage of SE induction (61%) was recorded in treatment E (1 µM 2,4-D, 5 µM BA, and 3% SPE) (Table 2). This experiment suggests that the interaction between SPE and 2,4-D plus BA has an effect on SE induction. Although fewer embryos were obtained, they induced the 1-month-old calli to convert into SEs more effectively and the 3% SPE concentration caused almost all calli to form SEs. Treatment D without SPE produced a greater percentage of 3-month-old callus than treatment E (Table 2) and also produced a higher number of 3-month-old calli (18.78±0.7 calli/explant) than the other treatments.

When tested to establish its effect on somatic embryogenesis, the SPE showed no significant difference among the different concentrations but did have a significant effect on the number of 3-month-old calli per explant (Table 2). These calli of *C. arabica* entailed some risk of somaclonal variations via somatic embryogenesis. Thus, the 3-month-old calli might increase the frequency of variants due to the long callus culture period inducing aneuploidy (monosomy) during cell culture ageing [35]. The reduction of the culture duration may contribute to minimising the occurrence of somaclonal variants [36].

The culture medium composition has a considerable influence on the induction of cells of coffee to become a SE [37]. Auxin plays a role in the embryogenesis of coffee tissues via acidification of the cytoplasm and cell wall [38]. Thus, SPE containing, in particular, abundant indole acetic acid and various concentrations of gibrellin, benzyl adenine, abscisic acid, jasmonic acid and methyl jasmonate [24] might influence the embryogenesis of coffee tissue. Consequently, treatment E (MS medium supplemented with 1 µM 2,4-D, 5 µM BA and 3% SPE) might induce most embryogenic calli to develop into SEs.

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Table 2. SE induction and number of 3-month-old calli on MS medium containing different combinations of supplements

Treatment	Supplement			No. of calli per explant (1 month)	No. of SEs per explant (3 months)	No. of calli per explant (3 months)	% SE induction
	2,4-D (µM)	BA (µM)	SPE (% v/v)				
D	1	5	0	42.20±10.82[a]	22.86±14.23[a]	18.78±0.70[a]	54
E	1	5	3	19.78±10.86[b]	12±4.18[b]	7.60±0.53[d]	61
F	1	5	4	21.00±6.74[b]	11±3.65[b]	9.78±0.76[c]	52
G	1	5	5	20.90±8.76[b]	9±2.15[b]	11.63±0.55[b]	43

Note: a, b, c and d superscripts represent significant differences among treatment means within the same column ($p < 0.05$).

SE Development

In our study, embryos were produced by ISE from brown/beige calli after being grown on a regeneration medium for 3 months. All treatments produced compact, white embryos with a smooth surface comprised of individual or groups of SEs (Figure 4A). Moreover, a few roots were found to develop from calli in all treatments (Figure 4B). Our results showed that after 3-6 months of culturing SEs could be distinguished at different stages of development (globular, torpedo and cotyledonary stages) (Figures 4A-D). In coffee somatic embryogenesis usually takes several months (from leaf explant to SE)−7-8 months for *C. canephora* and 9-10 months for *C. arabica* [4]. Interestingly, SEs of *C. arabica* cv. Catimor CIFC 7963 could be developed within 3 months in a single medium and showed a normal pattern of somatic embryogenesis following the report by Zimmerman [39].

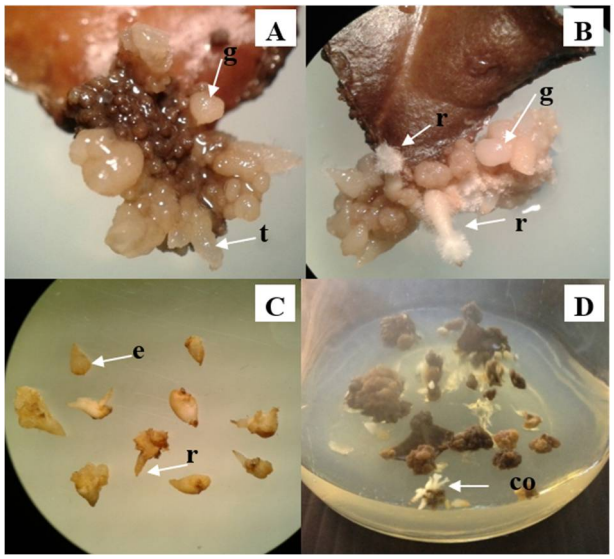

Figure 4. ISE of *C. arabica* cv. Catimor CIFC 7963: white SEs at different stages: globular (g) and torpedo (t) at 3 months (A); root (r) formation via organogenesis (B); dissected embryo (e) and root (r) at 3 months (C); cotyledonary embryo at 6 months (co) (D)

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Secondary Somatic Embryogenesis (SSE)

Cotyledonary-stage embryos were observed on the MS medium supplemented with 0.1 µM BA after 6 months (Figure 4D). The MS medium solely supplemented with 0.1 µM BA induced SEs into a cotyledonary stage because auxins were required for the proliferation of proembryogenic masses but the SE development was inhibited by auxin [37]. Then we transferred them to PGR-free MS medium. We found that some SEs germinated to produce translucent, white, secondary SEs at both the globular and torpedo stages which lacked a primordial root (Figures 5A-C). In this experiment, some SEs were able to germinate (Figure 5D). In *C. arabica* cv. Catimor, the secondary SEs develop directly by cell division at the epidermal and subepidermal levels in the hypocotyl of the primary SEs [26], while in *Myrtus communis* [40] and *Solanum tuberosum* [41], the secondary embryos can differentiate in different zones of the primary embryo, such as the base of the primary embryo hypocotyl and also in zones close to the root pole. This result suggests that the process of SSE in *C. arabica* cv. Catimor CIFC 7963 occurs directly without callus formation. Similarly, the development of secondary SEs from the torpedo phase in Arabica coffee that occurred directly without callus induction produced some SEs on the media with 9.08 µM thidiazuron [42]. Thus, the SSE is a useful process for regeneration of embryo cells under certain transformation methods as in the primary SE.

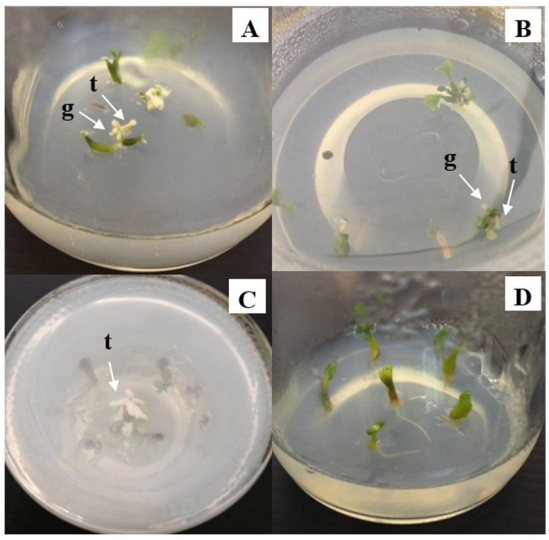

Figure 5. Secondary SEs of *C. arabica* cv. Catimor CIFC 7963 on MS medium: secondary SE (white) in globular (g) and torpedo (t) stages (A-C); germinated embryos (D)

Germination of SE and Conversion into Plantlet

The white/beige, 6-month-old cotyledonary SEs changed to a green colour within 1 month (Figure 6A), produced shoots after 2 months (Figure 6B) and rooted within 3 months (Figure 6C). We found that 100% of the cotyledonary SEs of *C. arabica* cv. Catimor CIFC 7963 germinated. They developed successfully into plantlets on the PGR-free MS medium used for embryo germination and converted into plantlets within 7 months (Figure 6D). The somatic embryogenesis of coffee plantlets usually takes several months–9 months for DSE and 12-13 months for ISE [43, 44]. In addition, the time taken to propagate plantlets via somatic embryogenesis (from leaf explant to SE) was 9-10

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months in *C. arabica* [4, 45]. Similarly, the SEs of *C. arabica* cv. Catimor CIFC 7963 developed into plantlets within 7 months, whereas cultured leaves of *C. canephora* accomplished this in 7-8 months [4]. We observed that approximately 26-28% of SEs developed into normal plantlets after 7 months. About two-thirds of the SEs lacked roots. It can be suggested that in the ISE process the origin of SE is unicellular or multicellular [46]. The results showed that some SEs which originated from the multicellular process lacked roots or shoots. Similar problems were reported to occur in *C. arabica* cultivars, Rubi, Catuaí Vermelho 81 and IAPAR 59, which showed one-third of SEs without roots or shoots [47]. Further research is needed to solve this problem.

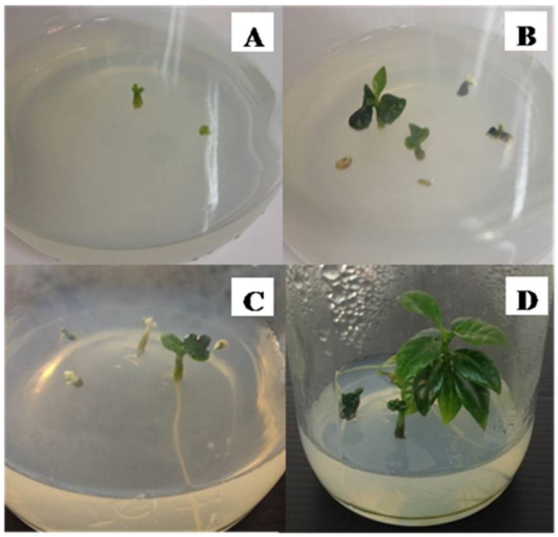

Figure 6. Germination and SE conversion into plantlets of *C. arabica* cv. Catimor CIFC 7963 on MS medium: green cotyledonary embryo at 1 month (A); germinated embryo at 2 months (B); germinated embryo with root at 3 months (C); regenerated plantlet at 7 months (D)

CONCLUSIONS

Leaf explants of *C. arabica* cv. Catimor CIFC 7963 are able to successfully produce SEs via ISE in a single culture medium, responding favourably to the SPE. The ratio and concentration of auxin (2,4-D) and cytokinin (BA) are critical for successful callus formation. In ISE the best result is obtained from solid MS medium supplemented with 1 µM 2,4-D + 5 µM BA + 3% (v/v) SPE. Clumps of these embryos germinate into plantlets and some embryos developed to secondary SEs on MS medium without PGRs. Using the extract of *S. maxima* appears to enhance SE induction for multiplication of *C. arabica* cv. Catimor CIFC 7963. This protocol is simple and reproducible for the mass micropropagation of coffee hybrids and might be useful for further development of the propagation of *C. arabica* hybrids using leaf explants. In addition to the ISE study, we have also established that SSE would be the most appropriate method for coffee improvement programs using genetic engineering.

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