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Development of an in vitro regeneration system for *Theobroma cacao* from mature tissues

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Abstract

Induction of somatic embryogenesis from tissues in unopened flower buds of cocoa was studied with respect to physiological age, type of floral explant, genotype, and medium composition and phytohormones. Two-to-three-week-old staminodes were found to be the best explants for embryogenesis. Embryogenesis was affected by genotype and sugars. Two main types of abnormalities of the embryos were observed: fusion of the hypocotyls and multiple cotyledons. These embryos have a lower rate of conversion into plantlets. Cytological analyses of somatic embryo-regenerated plants revealed a somatic chromosome number of $2n = 2x = 20$, similar to seed-derived plants.

Keywords: Cocoa tissue vulture: Floral explants: Somatic embryogenesis: Wood) species; Cytological analyses

1. Introduction

Somatic embryogenesis is the principal developmental pathway in the regeneration of plants from cultured tissues of woody species [1] and provides a high potential for propagation by synthetic seeds. Other applications of somatic embryogenesis include disease and virus elimination, as well as germplasm conservation. Efficient plant regeneration from somatic embryos also provides a foundation for crop improvement, including somatic hybridisation and genetic transformation. For crops such as cocoa, a limiting factor is the lack of a reliable procedure for somatic embryogenesis and plant regeneration from a wide range of genotypes.

In cocoa, the first reports of somatic embryogenesis [2,3] utilised immature zygotic embryo tissues, but these procedures were of little use for clonal propagation. Litz [4] was able to induce embryogenesis from cocoa leaf explants but without plant regeneration. The successful production of somatic embryos from non-sexual tissues and their conversion into plants [5,6] indicated a low efficiency of embryogenesis, which was strongly influenced by genotype [7]. This study investigated somatic embryogenesis from 52 cocoa accessions.

2. Materials and methods

2.1. Plant materials

Plant materials were obtained from bud grafted field-grown clonal plants at Kota Kinabalu, Sabah, and Sg. Wangi Estate, Perak, Malaysia.

2.2. Culture conditions

Unopened flower buds (4–6 mm in length) were surface sterilised by washing with tap water, then immersing in 70% ethanol for 5 min, 10% commercial bleach (5.25% (v/v) sodium hypochlorite) with 0.10% sodium dodecyl sulphate (SDS) for 20 min, and 1% bleach with 0.10% SDS for 20 min. Buds were then rinsed three times for 5 min each rinse in sterile reverse osmosis water. Staminodes were extracted, using scalpels and forceps, following removal of sepals and petals. For callus initiation, staminodes were cultured in 90 mm diameter plastic Petri dishes containing 30 ml of callus initiation (MIM41n) medium. Dishes were sealed with

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Parafilm and incubated for 3–4 weeks in the dark at 25±2 °C. Calli were then subcultured onto 30 ml of differentiation (MEM22a) medium for 6–8 weeks in the dark with transfer to the same medium after 4 weeks. MIM41n medium consisted of Driver and Kuniyuki (DKW) basal salts (Duchefa, Biochemie BV, The Netherlands), 20 g l^{-1} glucose, Murashige and Skoog (MS) [8] vitamins, 2 g l^{-1} 2,4-dichlorophenoacetic acid (2,4-D), 0.1 g l^{-1} N^6-[2-isopentenyl]adenine (2-iP), 500 mg l^{-1} glutamine and 2 g l^{-1} Phytagel (Sigma), pH 5.5. MEM22a medium consisted of DKW salts, MS vitamins, 50 mg l^{-1} glutamine and 2 g l^{-1} Phytagel, pH 5.5. Cotyledonary stage somatic embryos were removed from the calli and maintained on MEM22a medium with subculture every 2–3 weeks. Somatic embryos (>4 mm in length) with clearly defined shoot and root axes were transferred individually to 50 ml of MGM medium for germination. MGM medium consisted of 1/5× DKW salts, 5 g l^{-1} sucrose, 10 g l^{-1} glucose, 100 mg l^{-1} myo-inositol, 2 mg l^{-1} glycine, 2.2 g l^{-1} Phytagel (Sigma), pH 5.8. Media were dispensed onto 250 ml glass jars (Biocraft, Singapore) and covered with Suncaps (Sigma) for ventilation. The cultures were kept in the light (16 h photoperiod, daylight fluorescent tubes, 25 μmol m^{-2} s^{-1}) at 25±2 °C.

2.3. Effect of physiological age and different floral parts on embryogenesis

Three different physiological ages of the flower were used: 1–2-week-old (unopened, about 3–4 mm in length), 2–3-week-old (unopened, about 5–6 mm in length) and 3–4-week-old (opened flowers). Three different floral parts were studied: anthers, staminodes, and petals. The experiment was carried out in triplicate.

2.4. Effects of genotype on callus type and embryogenesis

Anther and staminode tissues from the 52 clones evaluated (Table 1) were assessed for the percentage of explants-producing callus and their morphology (compact white/friable yellow) on MIM41n medium after 4 weeks, and the percentage of calli-producing somatic embryos on MEM22a medium after 6 weeks. At least 100 explants were cultured per clone.

Table 1
Effect of floral parts and age on embryogenesis

Physiological age	Anther	Staminode	Petal
1 2 Weeks old	0.2	1.6	0
2 3 Weeks old	3.3	6.2	0
3 4 Weeks old (open)	0	0	0

The flowers were taken from clone KKM19. The figures represent the percentage of explants-producing embryos and are the mean of three different experiments.

2.5. Effects of different basal media on embryogenesis

Embryogenically competent calli of clones AMAZ12 and CAB64 were transferred to MEM22a medium, and to MS, Woody Plant Medium (WPM) [9], and B5-based media [10], the latter three media having the same concentration of sucrose and vitamins as MEM22a medium. Each evaluation consisted of 25 calli with three replicates.

2.6. Effects of phytohormones on somatic embryogenesis

Staminode calli of clones AMAZ12 and CAB64 were transferred after 3–4 weeks culture on MIM41n medium to MEM22a medium (control) and to MS medium with 2 mg l^{-1} gibberellic acid (GA_3), 0.01–0.1 mg l^{-1} 2,4-D, and 0.01–0.2 mg l^{-1} thidiazuron (TDZ) for induction of embryogenesis.

2.7. Cytological analyses of regenerated plants

Excised root tips (2 cm length) from three regenerants of clones AMAZ12, AMAZ15/15 and from five seed-grown (control) plants were examined to determine their somatic chromosome complement. Cytological analyses were carried out as described by Andras et al. [11]. Chromosome preparations were photographed using a Nikon Microphot SA microscope.

2.8. Statistical analysis

Means and standard errors (S.E.M.) were used throughout and statistical significance among values were assessed using ANOVA [12] incorporating the post hoc Tukey–Honestly significantly difference (Tukey-HSD) test using a commercial statistical software package (Minitab®). A probability of <0.05 was considered significant.

3. Results

3.1. Effect of physiological age of flowers and different floral parts

Among the different physiological ages of the flowers used, unopened, 2–3-week-old flower buds yielded the highest percentage of explant-producing embryos (Table 1). Staminodes were the best explants for embryogenesis compared with anthers and petals.

3.2. Effect of genotype on callus type and embryogenesis

The percentage of calli-producing embryos ranged from 0.5 to 5.8 (Table 2). The only clones studied that produced somatic embryos were EET75 (0.5%), EET397

Table 2
Embryogenic potential different cocoa clones

No.	Clone	Explant callusing (%)	Compact white callus (%)	Yellow friable callus (%)	Explant-producing embryos (%)
1	EET75	91.2	100	0	0.5
2	ICS39	87.7	100	0	0
3	EET94	98.9	100	0	0
4	CC18	84.8	96.4	3.6	0
5	ICS40	60.0	99.4	0.6	0
6	CC39	80.1	66.1	33.9	0
7	GS39	77.6	100	0	0
8	EET397	69.2	87.3	16.7	5.8
9	ICS89	69.4	80.2	19.8	0
10	NA33	XL?	96.2	3.8	0
11	UF10	40.4	77.7	22.3	0
I?	R23	18.9	90.1	9.9	0
13	KKM19	90.0	62.3	37.7	0
I4	LAF17	60.0	100	0	0
15	R113	32.7	93.2	6.8	0
16	SIC32	70.8	76.8	23.2	0
17	R44	78.3	84.9	15.1	0
18	ICS88	hQO	93.4	6.6	0
19	UIT2	h7.8	100	0	0
20	KKM4	91.7	92.6	7.4	0
21	KKM7	97.3	100	0	0
22	KKM15	81.7	92.6	7 4	0
23	CC10	80.0	74.3	25 7	0
24	GS36	74.3	85.1	24.9	0
25	GS29	78.6	100.0	0	0
26	MHP?	89.0	80.0	20.0	0
27	CC80	51.0	46.4	53.6	0
28	SIAL93	78.0	64.6	35.4	0
29	UF168	16.0	100.0	0	0
30	IMC67	74.3	34.8	65.2	0
31	PBC170	85.7	58.8	41.2	0
32	PBC123	95.7	65.9	34.1	0
33	PBC113	43.6	100.0	0	0
34	PBC137	88.7	25.0	75.0	0
35	PBC159	91.0	99.5	0.5	1.0
36	PBC179	92.3	68.5	21.5	0
37	EET65	87.7	90.9	9.1	0.5
38	PBC162	82.7	72.3	27.7	0
39	PBC130	89.7	85.3	14.7	0
40	PBC140	86.0	32.1	67.9	0
41	PBC208	89.7	31.3	68.7	1.4
42	PBC128	84.6	18.8	81.2	0
43	DRI	78.7	17.5	82.5	0
44	ICS48	95.0	87.1	12.9	0
45	ICS100	91.0	98.8	1.2	0
46	EET308	-35.7	96.2	3.8	0
47	IMC14	90.3	4.9	95.1	0
48	AMAZ15/15	47.9	63.0	37.0	4.7
49	PA107	85.3	50.7	49.3	0
50	BE10	69.5	44.6	55.4	0
51	CAB64	83.4	70.2	29.8	4.8
52	AMAZ12	97.1	83.5	16.5	2.6

Each cocoa clone was screened once due to the seasonal availability of flowers ($n > 100$). Percentage of explants callusing was recorded following 4 weeks culture of staminode explants on MIM41n medium.

(5.8%). PBC159 (1.0%), EET65 (0.5%), PBC208 (1.4%), AMAZ15/15 (4.7%), CAB64 (4.8%), and AMAZ12 (2.6%). Two types of callus were generally observed: compact white and yellow friable (Fig. 1A and B).

3.3. *Effect of basal salt composition on embryogenesis*

Results were variable between clones. AMAZ12 gave the highest percentage of calli-producing embryos on

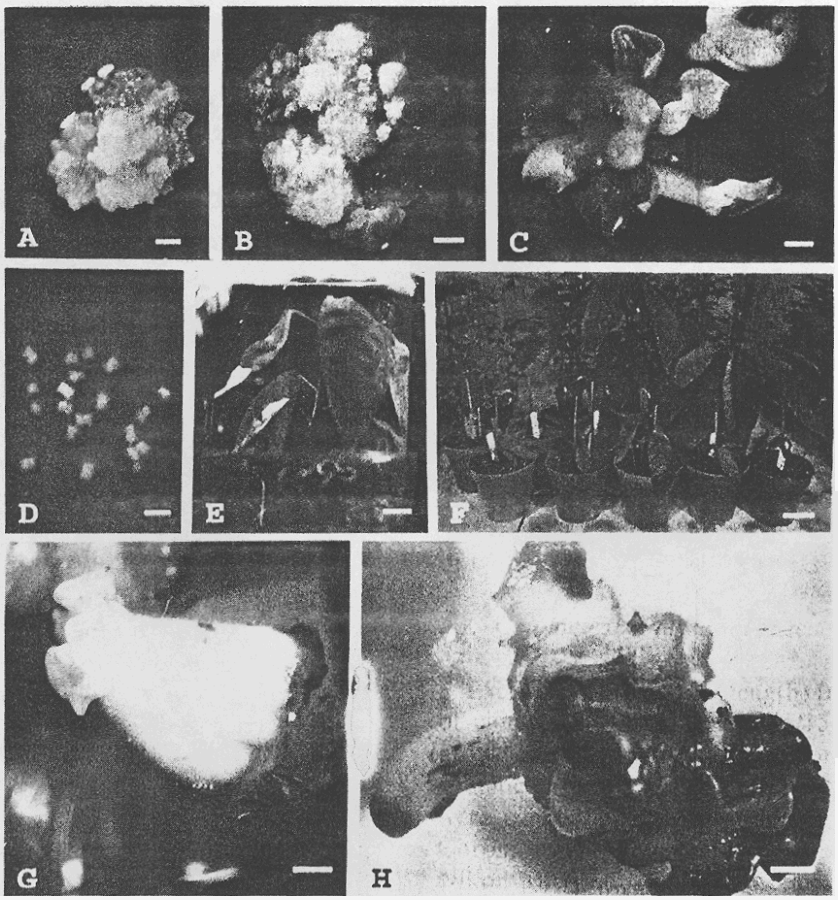

Fig. 1. Somatic embryogenesis of cocoa from staminode tissues: (A) approximately 3–4-week-old yellow friable callus from clone AMAZ12 cultured on MIM41n medium (bar = 1.2 mm), (B) approximately 3–4-week-old compact white callus from clone AMAZ12 cultured on MIM41n medium (bar = 1.2 mm), (C) approximately 3–4-week-old somatic embryos differentiated from staminode callus of clone AMAZI? on MEM22a medium (bar = 2.0 mm), (D) cytological analysis of a root tip cell of a regenerated plantlet of clone AMAZ15/15 ($2n = 2x = 20$) (bar = 10 μm), (E) germination of a somatic embryo (approximately 8–12-week-old) of clone SIAL93 on MGM medium (bar = 11 mm), (F) acclimatised plants from four different clones (AMAZ12, AMAZ15/15, CAB64 and SIAL93) (bar = 5.5 cm), (G) fusion of hypocotyls on a somatic embryo (approximately 6–8-week-old) of clone AMAZ12 on MEM22a medium (bar = 1.5 mm), and (H) multiple cotyledons of a somatic embryo (approximately 6–8-week-old) of clone CAB64 on MEM22a medium (bar = 1.5 mm).

DKW basal medium, whereas for clone CAB64 this was on MS-based medium (Table 3).

3.4. Effect of 2,4-D and TDZ on the induction of somatic embryogenesis

The tire of 0.01 mg l^{-1} 2,4-D + 0.01 mg l^{-1} TDZ in differentiation medium was found to give the highest .percentage of calli-producing embryos for both clones studied (Table 4). This was approximately a sevenfold increase over the control for clone CAB64 and a sixfold increase over the control for AMAZ12.

3.5. Abnormality of somatic embryos

Although most of the embryos obtained were normal. i.e. with an embryo axis (hypocotyls) and two cotyledons (Fig. 1C), some of the embryos were abnormal. with fusion of hypocotyls (Fig. 1G) or multiple cotyledons (Fig. 1H). These embryos do not germinate normally. but are capable of producing secondary embryos on subsequent cultures on MEM(22a) medium.

Table 3
Effect of basal medium on the induction of somatic embryogenesis

Basal salt in medium	Percent calli-producing one or more embryos (mean ± S.E.M.)	
	AMAZI?	CAB64
MS	0	11.0 ± 3.0
DKW (MEM22a)	28.0 ± 10.2*	0
WPM	13.0 ± 2.4	5.0 ± 2.0
B5	0	0

Results are based on replicates of 25 calli per basal medium. Each treatment was repeated at least three times. Data were collected following 6 weeks culture on each treatment.

* $P < 0.05$ compared with other treatment mean value.

Table 4
Effect of 2,4-D and TDZ on the induction of somatic embryogenesis from cocoa genotypes CAB64 and AMAZ12

Treatment	Percent calli-producing somatic embryos (mean ± S.E.M.)	
	CAB64	AMAZ12
MS basal medium (control)	8.8 ± 3.9	7.0 ± 7.0
MS basal medium + 2 mg l^{-1} GA_3 + 0.01 mg l^{-1} 2,4-D + 0.01 mg l^{-1} TDZ	62.0 ± 14.0*	41.0 ± 15.2
MS basal medium + 2 mg l^{-1} GA_3 + 0.10 mg l^{-1} 2,4-D + 0.01 mg l^{-1} TDZ	0	4.0 ± 4.0
MS basal medium + 2 mg l^{-1} GA_3 + 0.01 mg l^{-1} 2,4-D + 0.10 mg l^{-1} TDZ	36.0 ± 23.4	14.0 ± 8.3
MS basal medium + 2 mg l^{-1} GA_3 + 0.10 mg l^{-1} 2,4-D + 0.10 mg l^{-1} TDZ	0	0
MS basal medium + 2 mg l^{-1} GA_3 + 0.01 mg l^{-1} 2,4-D + 0.20 mg l^{-1} TDZ	16.0 ± 4.0	8.0 ± 8.0

Results were based on 25 calli per medium per clans: each treatment was repealed at least three times. Data were collected following 6 weeks culture for each treatment.
* $P < 0.05$ compared with control mean value.

3.6. Cytological analyses of regenerated plants

The somatic chromosonie nuniber of regenerated plants of clones AMAZ12 and AMAZ15 is 20 (Fig. ID), similar to the chromosonie numbers obtained from seed-derived plants.

3.7. Development and growth of somatic embryos-derived plants

Some somatic embryos produced roots and developed into normal shoots (Fig. 1E) on MGM medium. Expanded leaves were observed after 2 months in culture. The growth of somatic embryo-derived plants was similar to seed-derived plants. exhibiting an orthotropic growth habit. Once the plants developed a healthy root and shoot system (approx. 2 months after culture on MGM medium). they were transferred to soil and acclimatised to ex vitro conditions (Fig. IF).

4. Discussion

In our studies, 2–3-week-old flowers gave the best frequency of embryogenesis with staminode explants. We also observed that cocoa flower explants collected at the beginning of the rainy season, especially after prolonged dry period, tend to have a higher efficiency of embryogenesis. The general health of the mother plant and season of the year also affected embryogenesis frequencies in cocoa.

Calli were mainly of two types: compact white or yellow friable. though other types may occasionally be seen. Chatelet et al. [13] reported four different types of calli induced from immature seed explants on medium with various concentrations of 2.4-D and BAP: fluffy, white callus, hyperhydric translucent callus. mucous-like callus. and brown soft callus. The control of callus types by different hormones was not studied in their experiment. However. we observed the influence of genotype on callus type. Some clones like AMAZ12 and SIAL93 produce a mixture of yellow friable and compact white calli, whereas clones ICS39 and GS29 produced solely compact white calli. Genotypic effect on the quality of callus produced was also observed in sorghum [14]. Eight clones were capable of undergoing embryogenesis using the same media. The frequency of somatic embryo production is not high: all clones were below 10%. The ability to form somatic embryos is believed to be under genetic control, and individual genotypes within a species can differ in their ability to undergo somatic embryogenesis [15].

In our study with different carbon source. the percentage of callus-producing embryos in clones AMAZ15/15 and AMAZ12 were highest in development media with maltose (data not shown). Elhag et al. [16] reported that cocoa embryogenesis from callus was sensitive to carbon source. They found that embryogenesis increased in all the three clones studied when sucrose was replaced by glucose or fructose. When sucrose is replaced by other carbon sources, embryogenesis is enhanced in other plants also [17,18]. Maltose may be broken down more slowly than sucrose and consequently provides a readily metabolisable carbon source over a longer period of culture. Maltose probably also affects the process of enibryogenesis physiologically, rather than as an energy supply. since its effeot can occur at low concentrations [19].

MS-based media have been typically used for cocoa somatic embryogenesis [5,13,20]. DKW [21] minerals were initially developed for in vitro propagation of walnut, and were found to be the best medium for somatic embryogenesis for many species [22]. DKW medium provided a significantly higher concentration of calcium, sulphur, and magnesium compared with MS medium. These elements are probably essential for cocoa somatic embryogenesis and differentiation. In walnut, embryo induction was best on DKW medium for most species, except for *Juglans nigra*, which preferred WPM medium [23].

Abnormalities of somatic embryos were frequently observed. Alemano et al. [24] divided embryos intothree categories. Category l consisted of normal embryos that exhibited clear bipolarity. well-defined shoot and root apices, and cotyledons. Category 2 was embryos that were fused at the cotyledons or hypocotyls. The third category was embryos that had a protoderm but did not

show bipolar orpanisation. Normally these abnormal embryos (categories 2 and 3) do not germinate into plantlets. The frequency of germination of somatic embryos and conversion into plants is still low. Further research is needed to improve this area.

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