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ORIGINAL PAPER

Somatic embryogenesis and plant regeneration of litchi (*Litchi chinensis* Sonn.) from leaves of mature phase trees

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Abstract Embryogenic cultures were induced from leaflets from new vegetative flushes of mature 'Brewster' litchi trees on B5 medium containing 400 mg l^{-1} glutamine, 200 mg l^{-1} casein hydrolysate, 30 g l^{-1} sucrose, 4.52 μM 2,4-D, 9.30 μM kinetin and 3 g l^{-1} gellan gum in darkness. Embryogenic cultures consisting of proembryonic cells and masses were maintained either on semi-solid MS medium supplemented with 4.52 μM 2,4-D and 0.91 μM zeatin or as embryogenic suspension cultures in liquid medium of the same composition. Maturation of somatic embryos occurred on semi-solid MS medium with 5–20% (v/v) filter-sterilized coconut water in darkness. Recovery of plants from somatic embryos was improved with 14.4 μM GA_3 on half-strength MS medium with 0.2 g l^{-1} activated charcoal under a 16 h photoperiod provided by cool white fluorescent lights (60–80 μmol s^{-1} m^{-2}). Plants have been successfully acclimatized in the greenhouse.

Keywords Lychee · Somatic embryo · Suspension culture

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Abbreviations

BA	Benzylaminopurine
B5	Gamborg et al. (1968)
CW	Coconut water
2,4-D	2,4-Dichlorophenoxyacetic acid
GA_3	Gibberellic acid
MS	Murashige and Skoog (1962)
PEM	Proembryonal mass

Litchi *Litchi chinensis* Sonn., is an important subtropical fruit tree, with annual fruit production of 1.5–2.0 mt (http://www.lycheesonline.com/In_china_Farmers.cfm). The litchi is in the subfamily Sapindoideae of the Sapindaceae family together with other important tropical and subtropical fruit trees species, namely longan (*Dimocarpus longan* Lour.) and rambuttan (*Nephelium lappaceum* L.) (Subhadrabandhu and Stern 2005). All litchi cultivars are derived from selections made from seedling trees derived from openly pollinated trees. Like most tropical and subtropical fruit trees, the species has a long juvenile period (7–8 years). Shelf life, disease resistance (particularly anthracnose), yield, annual bearing and seedlessness are the primary breeding objectives of litchi (Litz and Raharjo 2005).

The efficient regeneration of elite, i.e., mature-phase, cultivars from cell cultures is an important prerequisite for applying biotechnology procedures to improve clonally propagated perennial trees such as litchi. Previously, embryogenic litchi cultures have been induced from immature zygotic embryos of 'Xiafanzhi' and 'Chen Zi' (also known as 'Brewster') (Yu and Chen 1997; Yu et al. 2000; Zhou et al. 1996); however, regeneration of plants from mature phase trees has not been reported. Induction of embryogenic cultures has been reported from leaves of clones of the related fruit tree species longan *Dimocarpus longan* (Litz 1988; Matsumoto et al. 2004) and from soapnut *Sapindus trifoliatus* L. (Desai et al. 1986). The objective of the present study was to develop an efficient protocol for regenerating litchi from mature phase trees via somatic embryogenesis.

Leaflets from the compound leaves of new vegetative flushes of mature (>100-year-old) trees of litchi cultivars 'Brewster' ('Chen Zi') and 'Mauritius' ('Da Zao') from the tropical and subtropical fruit tree germplasm collection of the Tropical Research and Education Center of the University of Florida, Homestead FL were used as explants. Leaflets were dipped in water containing 2–3 drops of Tween-20, rinsed under running tap water for 1 h, disinfested for 10 min in 10% (v/v) commercial bleach with 5 drops of Tween-20, and rinsed 3× with sterile deionized water.

Induction medium was B5 (Gamborg et al. 1968) major salts, MS (Murashige and Skoog 1962) minor salts and vitamins, 100 mg l^{-1} myo-insitol, 400 mg l^{-1} glutamine, 200 mg l^{-1} casein hydrolysate and 30 g l^{-1} sucrose, and supplemented with 4.52 mM 2,4-dichlorophenoxyacetic acid (2,4-D) and a cytokinin, 8.88 μM benzylaminopurine (BA) or 9.30 μM kinetin or 9.12 μM zeatin. Medium was solidified with 3 g l^{-1} Gel-Gro™ gellan gum. The cytokinins were filter-sterilized and added to autoclaved plant growth medium. The sterile media were dispensed in 50 ml aliquots into 100 × 20 mm sterile plastic Petri dishes. Twenty leaflet explants were inoculated abaxial side down on induction medium in each Petri dish, which were then sealed with Parafilm®. The cultures were incubated in darkness at 26 ± 1°C. During the first 2 weeks after explanting, the tissue was monitored daily for contamination, and clean explants were transferred onto fresh induction medium. Explants were subcultured onto fresh induction medium after 8 weeks. Each auxin: cytokinin combination consisted of 9 or 10 dishes. The responses of explanted leaflets to inductive conditions were measured after 8–10 weeks. Data were expressed as the percentage response ± standard error. The square roots of percentage data were arcsine transformed, and were analyzed by ANOVA using SAS 9.1 (SAS Institute, Cary NC USA). The significance of cultivar and medium effects and their interaction was determined.

Embryogenic cultures which consisted of proembryonal cells and masses (PEMs), were maintained on semi solid medium consisting of half strength MS major salts, MS minor salts and vitamins, 100 mg l^{-1} myo-insitol, 30 g l^{-1} sucrose, 4.52 μM 2,4-D, 0.91 μM zeatin and 4 g l^{-1} Agargel (Sigma). There were six inocula in each 100 × 20 mm Petri dish, each of which consisted of ca. 80–100 mg of PEMs (15 mm diam). There were 9–10 Petri dishes for each treatment, and the experiment was repeated 2×. The Petri dishes were sealed with Parafilm and were incubated in darkness at 26 ± 1°C. PEMs were subcultured onto fresh medium of the same formulation at 4-week intervals.

To initiate embryogenic suspension cultures, 400 mg of 14-day-old 'Brewster' PEMs was inoculated into 40 ml liquid maintenance medium in each 125 ml Erlenmeyer flask. There were 6 replicated flasks. The flasks were sealed with aluminum foil and Parafilm. Suspension cultures were maintained on a rotary shaker at 125 rpm under ambient conditions in the laboratory at 26 ± 1°C, and were subcultured at 2-week intervals. At each subculture, suspensions were passed through sterile, nylon filtration fabric (1.6 mm opening), and an inoculum consisting of ca. 400 mg of the larger fraction size (>1.6 mm) was added to each 125 ml Erlenmeyer flask containing 40 ml fresh liquid maintenance medium. Growth parameters of suspension cultures were determined from settled cell volumes (SCV) and fresh weight of PEMs during a 6-week period. Data were analyzed by ANOVA and regression equations for SCV and fresh weight were determined.

Somatic embryo development was initiated from embryogenic cultures from semi solid and liquid maintenance medium. Suspension cultures were passed through sterile, nylon filtration fabric 2 weeks after subculture, and the small fraction was used. PEMs were also harvested from embryogenic cultures 4 weeks after subculture on semi-solid maintenance medium. PEMs were plated on semi-solid MS medium containing 45 mg l^{-1} sucrose, 0, 5, 10, 15 and 20% (v/v) filter-sterilized coconut water (CW) and 3 g l^{-1} gellan gum dispensed in 100 × 20 mm

Petri dishes. There were 11–13 replicates for each treatment and the control, and the experiment was repeated 2×. Data were analyzed for significance by the chi-square test using SAS 9.1. CW was obtained from freshly harvested immature coconuts and was filter-sterilized before adding to autoclaved medium. Approximately 200 mg PEMs was plated in each Petri dish, and spread evenly on the surface of the medium. The cultures were incubated in darkness at 26 ± 1°C, and somatic embryo maturation was recorded after 8–10 weeks.

In vitro conditions for recovery of plants were determined by transfer of mature, white-opaque somatic embryos ≥10 mm diameter to plant recovery medium. Recovery medium consisted of semi-solid MS medium with 30 mg l^{-1} sucrose, with or without 14.4 µM GA_3, and solidified with 3 g l^{-1} gellan gum. Twelve somatic embryos were transferred into each Petri dish (100 × 20 mm) containing 50 ml of plant recovery medium. There were 12–13 Petri dishes for teach treatment and the control, and the experiment was repeated 2×. Following shoot and root emergence, plantlets were transferred individually into sterile glass containers (90 × 55 mm) containing 60 ml semi solid MS basal medium with 30 g l^{-1} sucrose, 100 mg l^{-1} activated charcoal and 4 g l^{-1} Agargel. Growing conditions were 26 ± 1°C with a 16-h photoperiod (60–80 µmol s^{-1} m^{-2}) provided by cool white fluorescent lights. After 6–10 weeks, plantlets with 3–5 leaves and well-developed root systems were transplanted into pots containing Pro-mix BX and Perlite (1:1) and were acclimatized under intermittent mist for 2 weeks in a greenhouse under ambient temperature.

The pH of all media was adjusted to 5.7. Media were sterilized by autoclaving at 1.1 kg cm^2 at 121°C for 20 min.

Responding leaflet explants of 'Brewster' and 'Mauritius' were initially swollen and brown, and a translucent, friable callus developed after three to 4 weeks (Fig. 1a). For both cultivars, the optimum initial response occurred with 2,4-D and kinetin (Table 1), and the effect of medium was highly significant (Pr > *F* < 0.0001). Induction of embryogenic cultures only occurred from 'Brewster' leaflets (Table 1 and Fig. 1b). The optimum induction medium also contained 2,4-D and kinetin, and the cultivar effect was highly significant (Pr > *F* <

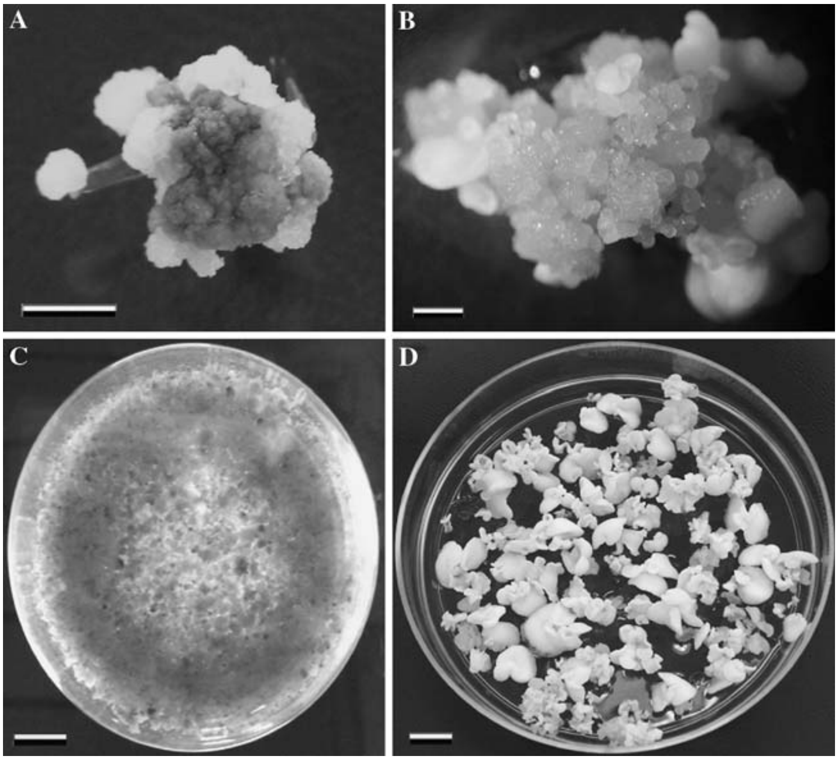

Fig. 1 Somatic embryogenesis of 'Brewster' litchi from leaflet explants. (**A**) Initial explant response on induction medium showing swelling and callus formation 4 weeks after culturing, bar = 5 mm; (**B**) Embryogenic culture 8 weeks after explanting, bar = 2 mm; (**C**) Embryogenic suspension culture (2-wk-old) in 125 ml Erlenmeyer flask, bar = 1 cm; (**D**) Somatic embryos 8 weeks after plating on maturation medium, bar = 1 cm

Table 1 Effect of BA, kinetin and zeatin with 2,4-D on induction of embryogenic cultures from leaflet explants of litchi 'Brewster' and 'Mauritius' after 8–10 weeks[1]

Cytokinin	'Brewster'		'Mauritius'	
	% responsive (± std error)	% embryogenic (± std error)	% responsive (± std error)	% embryogenic (± std error)
BA	8.10 ± 2.67	2.67 ± 1.69	10.59 ± 2.29	0
kinetin	16.53 ± 1.48	11.71 ± 1.51	19.08 ± 2.28	0
zeatin	10.76 ± 2.20	4.73 ± 1.84	3.46 ± 1.52	0

Source	DF	Mean	F	Pr>*F*
[1] Dependent variable = % response				
Cultivar	1	0.00462159	0.31	0.5816
Medium	2	0.22876671	15.22	<0.0001
Cultivar*medium	2	0.09460699	6.30	0.0036
Dependent variable = % embryogenic response				
Cultivar	1	0.55986662	61.64	<0.0001
Medium	2	0.08813151	9.70	0.0003
Cultivar*medium	2	0.08482394	9.34	0.0003

0.0001). The effects of medium and the cultivar:medium interaction on induction of embryogenic cultures were also significant (Pr < *F* 0.0003). Embryogenic 'Brewster' cultures consisted of PEMs and globular and early heart stage somatic embryos on semi solid maintenance medium containing 2,4-D and zeatin. In contrast, later stages of development, i.e., late heart and torpedo stage somatic embryos, occurred on induction media containing kinetin and BA. Embryogenic 'Mauritius' cultures failed to be induced (Table 1), and a rapidly growing non-morphogenic callus was initiated.

Embryogenic 'Brewster' suspension cultures in liquid induction medium containing 2,4-D and zeatin consisted of small cell aggregates, PEMs and globular somatic embryos (Fig. 1c). The proliferation of 'Brewster' PEMs under maintenance conditions was by repetitive somatic embryogenesis, with development of somatic embryos arrested at the globular stage. 'Brewster' embryogenic suspension cultures demonstrated logarithmic growth as measured by increased settled cell volume (SCV) and increased fresh weight during a 3-week period following culture establishment. A lag phase characterized growth of suspension cultures after week three (Fig. 2). There was no significant change in SCV and fresh weight between weeks four and six. Under

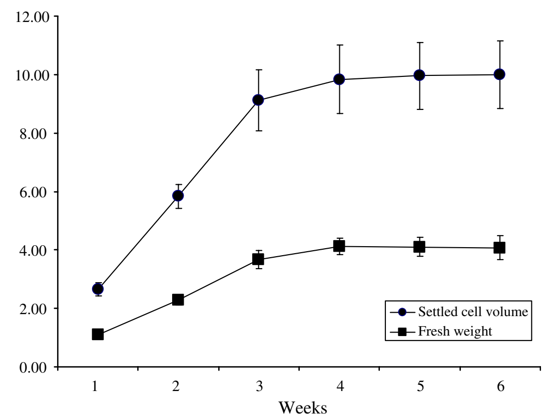

Fig. 2 Growth (fresh weight and SCV) of embryogenic 'Brewster' litchi suspension culture in 40 ml liquid medium in 125 ml Erlenmeyer flasks (mean ± standard error). Y-axis represents fresh weight and settled cell volume (SCV). ($y = a + b/x^{0.5}$; $r^2 = 0.6059279453$ for SCV; $y = a + b/x^{0.5}$; $r^2 = 0.7252149293$ for fresh weight)

optimal growth conditions and with 2-week subculture intervals, 'Brewster' SCV increased approximately 5-fold during each 4-week subculture interval.

Two weeks after plating litchi PEMs on somatic embryo maturation medium, globular, heart, torpedo and white-opaque cotyledonary stage somatic embryos developed (Fig. 1d). Somatic embryos achieved their maximum size, approx. 1.0 cm diameter, 6–8 weeks following subculture on maturation medium. The total number of somatic embryos, the number of large white-opaque somatic embryos >1.0 cm diameter and the number of <0.5 cm diameter somatic embryos produced in each Petri dish were significantly affected by the presence and concentration of CW in the maturation medium (Table 2) ($Pr > X^2 < 0.0001$). The largest number of somatic embryos, irrespective of their quality, occurred on medium with 5 and 10% (v/v) CW; whereas, 10 and 20% (v/v) CW was optimum for the development of large 1.0 cm diam opaque-white somatic embryos.

Mature white-opaque somatic embryos (approx. 1.0 cm diameter) became green on plant recovery medium following their transfer to light conditions under a 16-h photoperiod. Emergence of the radicle followed by growth of the tap root was succeeded by shoot elongation 2–6 weeks after transfer to light conditions (Fig. 3a). The frequency of occurrence of somatic embryos with at least one shoot and one root was higher on medium with 14.4 μM GA_3 (46.7 ± 5.0) than on medium without GA_3 (9.2 ± 3.5). Apparently normal plantlets with 2–3 compound leaves were obtained 6 weeks after root emergence (Fig. 3b). Survival of plants after transfer to the greenhouse was approximately 38% (Fig. 3c).

Although somatic embryogenesis of litchi has previously been reported from zygotic embryos (Yu and Chen 1997, 1998; Yu et al. 2000), this is the first report of de novo regeneration of litchi from an elite cultivar. Induction conditions for embryogenic litchi cultures from leaves of mature phase trees are similar to those described in other studies involving Sapindaceae species, including longan (Litz 1988; Litz et al. 2005) and soapnut (Desai et al. 1986). Although this study only involved two cultivars, 'Brewster and 'Mauritius', the induction response was strongly cultivar-dependent. Embryogenic longan cultures have been induced from several cultivars, including 'Kohala', ''Number 11', 'Wong Special', etc., and have not demonstrated a similar cultivar-dependent response (Litz 1988; Litz and Raharjo 2005),

Table 2 Effect of CW on recovery of opaque-white somatic embryos (SE)[1]

CW conc (%)	Number of SE	Abnormal SE > 0.5 cm	>0.5 cm SE	<0.5 SE	Total
0	Observed	18	57	916	991
	Expected	43.423	131.91	815.67	
5	Observed	50	123	2409	2582
	Expected	113.14	343.68	2125.2	
10	Observed	150	481	2156	2787
	Expected	122.12	370.97	2293.9	
15	Observed	130	377	1633	2140
	Expected	93.77	284.85	1761.4	
20	Observed	129	411	1846	2386
	Expected	104.55	317.59	1963.9	
Total		477	1449	8960	10886

[1] Statistic	DF	Value	Probability
Chi-square	8	425.3297	<0.0001
Likelihood ratio chi-square	8	485.6708	<0.0001
Mantel-Haenzel chi-square	1	263.6970	<0.0001
Phi coefficient		0.1977	
Contingency coefficient		0.1939	
Cramer's V		0.1398	

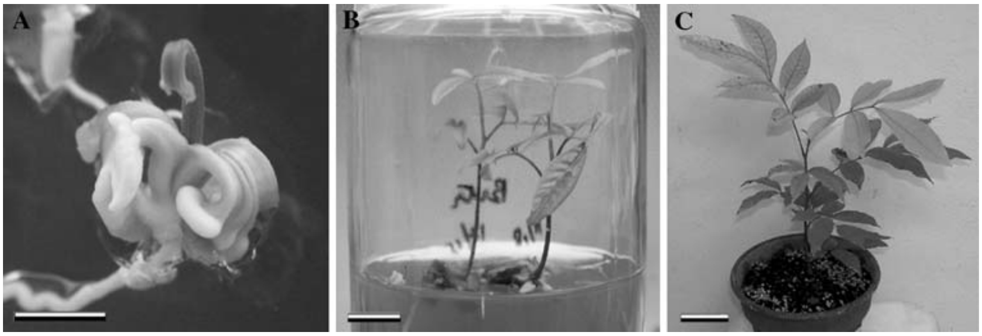

Fig. 3 Plant recovery from somatic embryos of 'Brewster' litchi. (**A**) Germinating somatic embryo on medium with GA_3 bar = 1 cm; (**B**) Plantlets 6 weeks after germination, bar = 2 cm; (**C**) Recovered plant (4 months after transfer ex vitro, bar = 5 cm)

although longan is closely related to litchi. Unlike the litchi (Ding et al. 2000, 2001), longan has not undergone intensive selection during domestication, and is considered to be more heterogenous.

Yu and Chen (1997), working with embryogenic cultures derived from zygotic embryos of litchi, reported that embryogenic cultures maintained on semi-solid medium consisted of a mixture of PEMs and maturing somatic embryos. Silver thiosulfate was used to inhibit somatic embryo maturation and obtain friable cultures, and was also incorporated into liquid maintenance medium (Yu and Chen 1997). No such medium modification was necessary in the current study in order to maintain embryogenic cultures either on semi solid or in liquid medium. In contrast, induction and maintenance medium supplemented with 2,4-D and zeatin suppressed somatic embryo development. CW has been critical for the efficient recovery of good quality large, mature opaque-white somatic embryos of large seeded tropical fruit species (DeWald et al. 1989; Lai et al. 2000; Litz 1988; Witjaksono and Litz 2002; Yu et al. 2000).

Somatic embryogenesis of litchi from leaves of mature trees is critical for using genetic transformation and in vitro mutagenesis to improve existing cultivars. Litz et al. (2005) demonstrated that embryogenic litchi cultures derived from zygotic embryos could be genetically transformed. Therefore, this study is critical for future efforts to transform certain litchi cultivars with genes that mediate certain horticultural traits.

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