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In vitro regeneration of 'Feizixiao' litchi (*Litchi chinensis* Sonn.)

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A simple efficient *in vitro* plant regeneration system was developed by indirect somatic embryogenesis of 'Feizixiao' litchi (*Litchi chinensis* Sonn.). Pollen in the anther of monocytes was used to induce callus. Two auxins (naphthalene acetic acid [NAA] and 2,4-dichloriphenoxyacetic acid [2,4-D]), and two cytokines (kinetin [KT] and 6-benzyladenine [BA]) were tested to explore their influence on callus induction. MS medium supplemented with 2.22 µM BA, 2.69 µM NAA, 13.57 µM 2,4-D, and 0.4 g/L LH (lactalbumin hydrolysate) showed the highest callus induction frequency. The callus obtained from anther was subcultured in MS medium containing 4.52 µM 2,4-D to obtain synchronized friable embryogenic callus. Different developmental stages of SEs were obtained from the callus on MS medium containing 6% (w/v) sucrose and different PGRs (plant growth regulators). On MS medium containing 6% (w/v) sucrose and supplemented with 0.54 µM NAA, 23.23 µM KT, 0.4 g/L LH, 0.56 µM inositol, and 10% (w/v) CW (coconut water), a higher number of SEs (globular, heart, torpedo and cotyledonary embryos) was achieved than on other media. Plantlets were established onto half-strength MS medium containing 1.44 µM GA_3 (gibberellic acid) followed by successful acclimatization in the greenhouse. With flow cytometry and chromosome counting, ploidy analysis of regenerated plants revealed that the regenerated plantlets were all diploid. This study is the first report on somatic embryogenesis of 'Feizixiao litchi', providing an opportunity to improve the cultivar by biotechnology methods.

Key words: litchi (*Litchi chinensis* Sonn.), anther culture, callus, regeneration, somatic embryogenesis.

INTRODUCTION

Litchi (*Litchi chinensis Sonn.*), known as "the queen of fruit" (Menzel and Waite, 2005), is an important fruit tree in the tropical and subtropical regions of the world (Menzel, 1983). Given its long reproductive cycle and highly heterozygous genetic background (Litz, 1988; Raharjo and Litz, 2005), new litchi cultivars are difficult to create via conventional breeding methods. Modern breeding techniques such as gene manipulation have the advantages of high efficiency and directional improvement of specific traits, providing a new way for the improvement of litchi cultivars (Das and Rahman, 2012). The establishment of a regeneration system *in*

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vitro for litchi bioengineering breeding is vital. Many efforts have been made regarding the subject, but few successful results have been published. Fu and Tang (1983) reported that they obtained 24 plantlets via organogenesis from pollen callus. Subsequent reports were made on somatic embryogenesis and plantlet regeneration in litchi via the culture of anthers (Deng, 2005; Xie et al., 2006; Wang et al., 2013), immature embryos (Zhou et al., 1993, 1996; Kuang et al., 1997), or protoplasts (Yu et al., 1996). Das et al. (1999) reported a high proliferation rate from mature seeds using two kinds of methods. Yu et al. (2000) achieved somatic embryogenesis and plantlets from 'Xiafanzhi' litchi protoplasts isolated from embryogenic suspensions. Puchooa (2004) used young unvaccinated 'Tai So' leaves as explants to study various factors on the regeneration of the leaf blade and eventually obtained regenerated plants. By 'Heli' anther culture, Guo et al. (2014) obtained somatic embryos with root and no sprout. However, Huang and You (1990) and Yu (1991) and Guo et al. (2014) reported that the stem sprout buds, failed to produce (Huang and You, 1990; Yu, 1991). All the above results showed that the regeneration of different litchi varieties is inconsistent with their medium. The same culture medium plays different roles on the regeneration of different litchi varieties. Therefore, different genetic backgrounds of litchi varieties have a remarkable effect on *in vitro* regeneration ability. To date, only a few cultivars, such as 'Nuomici' (Kuang et al., 1997), 'Xiafanzhi' (Lai and Sang, 2003) and 'Hushanjiaohe' (Fu and Tang, 1983), have been successfully regenerated *in vitro*. 'Feizixiao' is an early maturity variety with a tender, juicy, sweet aril and high and stable yield. *In vitro* regeneration of 'Feizixiao' is potentially a valuable method for conservation, mass propagation, and genetic transformation of this species. Although 'Feizixiao' micropropagation has been described (Deng, 2005), no report exists regarding its *in vitro* plant regeneration by a somatic embryogenesis system.

This study aimed to develop a simple and efficient protocol for efficient plant regeneration rates via somatic embryogenesis from anther explants of 'Feizixiao', as well as to investigate the effects of plant growth regulators on the processes.

MATERIALS AND METHODS

Plant materials and callus induction

Branches with floral buds of 10-year-old 'Feizixiao' were collected from the base of Hainan Leihu Fruit Ltd. on March 12, 2011. Immature flowers were washed in running tap water to remove dust. They were then dipped into 70% (w/v) ethanol, and 0.1% (w/v) $HgCl_2$, followed by three rinses in sterile distilled water under aseptic conditions. The anthers were separated from the flowers for callus induction.

The anthers were cultured on MS (Murashige and Skoog, 1962) solid (containing 0.7% (w/v) agar) medium with 0.4 g/L LH and 30 g/L sucrose. The medium was supplemented with different concentrations and combinations of plant growth regulators [PGRs: BA (0, 0.89, 2.22, and 4.44 µM), KT (0, 2.32, 4.65, and 9.29 µM), NAA (0, 1.07, 2.69, and 5.37 µM), 2,4-D (0, 4.52, 9.05, and 13.57 µM)] for callus induction. An orthogonal experimental design was used for these experiments with three repeats for each treatment, seven bottles per repeat, and 9 to 11 anthers per bottle. The callus was record after 8 weeks of culture. The callus obtained from anthers was subcultured in MS medium containing 4.52 µM 2,4-D to obtain synchronized friable embryogenic callus.

Somatic embryogenesis

The 18-day-old friable embryogenic calli (light, yellow, vigorous, fine-grained) were transferred to MS medium supplemented with 6% (w/v) sucrose, 0.4 g/L LH, 0.56 µM inositol, 10% (w/v) coconut water, and a combination of auxin NAA (0–0.54 µM) and cytokines KT (13.94, 23.23, and 32.53 µM), ZT (13.68, 22.81, and 31.93 µM), and TDZ (13.62, 22.71, and 31.79 µM). A completely randomized design with 10 replications (0.1 g of fresh callus per replicate) for each treatment was used for these experiments. Different developmental stages of SEs (>0.3 cm) were recorded after 7 weeks of culture.

Plant regeneration

Different developed stages of SEs (>0.8 cm) were transferred to different MS basal media (MS, 1/2 MS) supplemented with various concentrations of GA_3 (0, 1.44, 2.89 and 5.77 µM) and 3% (w/v) sucrose for regeneration. A completely randomized design with 15 replications (4 to 6 explants per replicate) was used for these experiments. Regeneration was recorded after 9 weeks of culture. These explants were incubated under 16 h (lightness)/8 h (darkness) photoperiod (provided by cool-white fluorescent lamps at a photon flux of 27 µmol/m^2s^1) at 26±2°C.

All media were adjusted to pH 5.8 prior to autoclaving at 121°C for 20 min. All explants were incubated in the dark, unless stated otherwise.

Flow cytometry and chromosome counting

The ploidy level of regenerated plantlets was analyzed using flow cytometry. Root tips were chopped and measured using a Cell Lab Quanta SC (Beckman Coulter, CA, USA). Nuclei obtained from the root tip of air layering seedlings were used as a diploid control. Histograms of DNA content were evaluated using Cell Lab Quanta SC MPL Analysis software (Beckman Coulter, CA, USA).

Chromosome counts were carried out on root tip cells of the plantlet pretreated for 3 h with a saturated solution of 1,4-dichlorobenzene. Roots were fixed in 3:1 ethanol-glacial acetic acid, hydrolyzed in 1 M hydrochloric acid, and rinsed three times with distilled water. Each rinsed root tip was placed on a glass slide. The root cap was removed, and the remaining root was stained with a carbol fuchsin solution. Each root tip was moderately squashed as the cover slip was applied to help spread the chromosomes. Micrographs were taken with an FSX-100 microscope camera system (Olympus, Tokyo, Japan).

Specimen preparation for light microscopy

According to the methods described by He (1989) and Lee and Mu (1966), sections of friable embryogenic callus, white opaque SEs, non-friable embryogenic callus, different abnormal SEs, and vitrified somatic embryos were fixed in FAA for 20 h. The specimens were then rinsed three times in 70% (w/v) ethanol. For staining, the

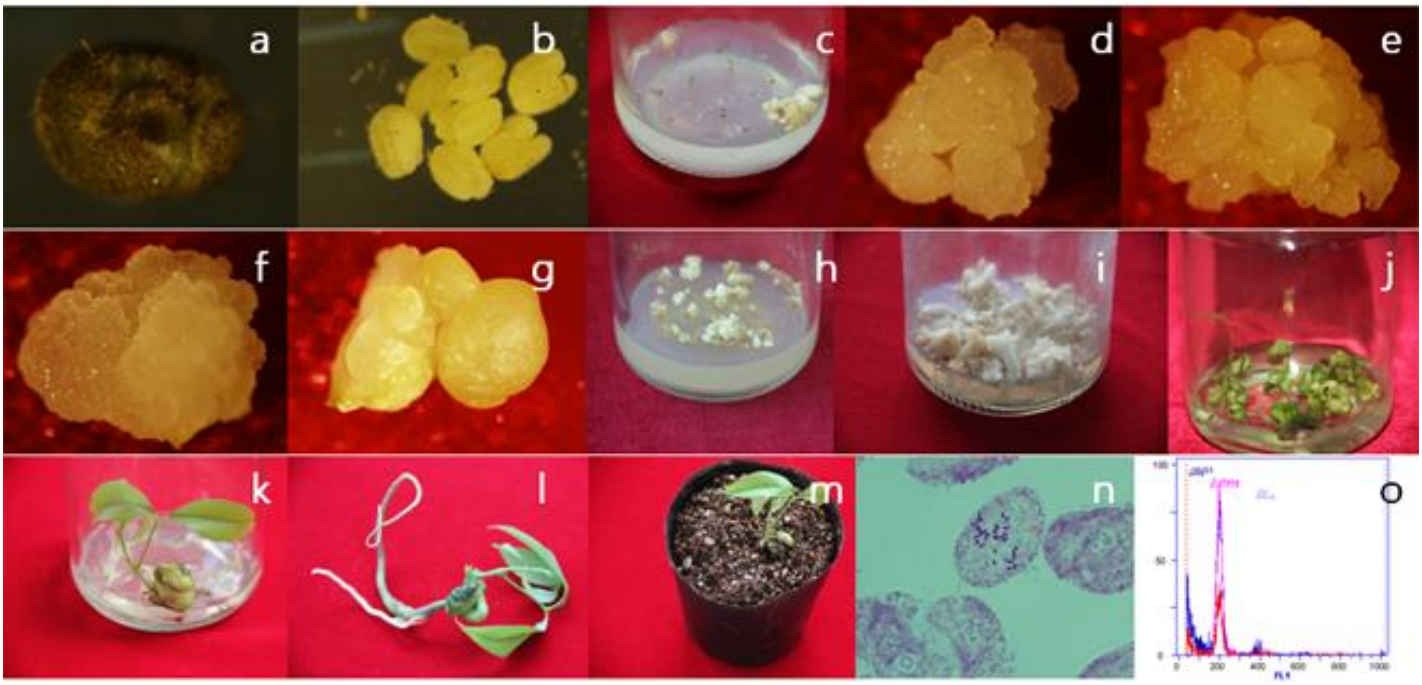

Figure 1. *In vitro* plant regeneration by indirect somatic embryogenesis from anthers of 'Feizixiao': (a) and (b) Immature flower buds and yellow anther (×5.6). (c) Different friable embryogenic callus developed. (d-g) Microscopic photograph of developmental stages of friable embryogenic callus (×2.5). (h and i) Induction of white somatic embryos on MS medium supplemented with 0.54 µM NAA, 23.23 µM KT, 4 g/L LH, 0.56 µM inositol, and 10% (w/v) CW. (j-l) Germination into plantlets in MS medium supplemented with 1.44 µM GA_3. m Acclimatized litchi plantlets in 1:1:1 (v/v/v) vermiculite: sand: coconut chaff mixture. n and o Determination of ploidy level of regenerated plants by flow cytometry and chromosome counting, displaying 2n = 2x = 30.

specimens were stained in Ehrilich's dyeing liquid. The specimens were dehydrated in a range of different concentrations of dimethylbenzene and embedded in different concentrations of paraffin. The specimen block was roughly trimmed to exclude excess plastic matrix and expose the surface of the specimen. The roughly trimmed block was mounted into a Reichert Ultracut microtome, and 5 to 6 µM sections were cut using glass knives.

Statistical analysis

Data were statistically analyzed using ANOVA and are presented as the mean ± standard error of all independent experiments. Treatment means were separated using Duncan's multiple range test at the 5% probability level and analyzed using DPS (version 2.0, Tang, 2010).

RESULTS AND DISCUSSION

Induction of embryogenic callus

Embryogenic calli were achieved from anther explants on MS medium, 3% (w/v) sucrose, and various PGRs in combination. One callus formed at the yellowish anthers after approximately 3 weeks of culture, other calli developed in all treatments, except the control, after 6 weeks (Figures 1a to c). The first callus was more light yellow in color, heavily watery, and non-granular (Figure 1d). The other was non-friable callus. Other calli emerged after 6 weeks. These calli were light yellow, compact, friable, tiny and/or nodular, and pre-embryogenic (Figures 1e to g). Amin and Razzaque (1995), Yu and Chen (1997), Puchooa (2004), Rahario and Litz (2007), Ma et al. (2009), Xu and Lai (2013), and Guo et al. (2014) also reported a similar type of callus development in other litchi cultivars. Similar phenomena were also found in longan (Lai et al., 1997), 'Premier' honey peach (Lai et al., 2008), *Heavea* (Tan et al., 2009), and *Pinus koraiensis* (Wang et al., 2015).

Different combinations of PGRs were used to test their effect on the induction of embryogenic callus. The experiment revealed significant differences in the number of callus produced among treatments, indicating that the combination and concentration of PGRs exerted a significant effect on callus induction (Table 1). LSD test revealed that a significantly higher number of callus was obtained when anthers were cultured in medium L9 (2.22 µM BA, 2.69 µM NAA, and 13.57 µM 2,4-D), followed by medium L8 (0.89 µM BA, 9.29 µM KT, 2.69 µM NAA, and 4.52 µM 2,4-D) and medium L3 (4.65 µM KT, 2.69 µM NAA, and 9.05 µM 2,4-D). No significant difference was observed among L4, L12, L14, and L15, which produced fewer FECs compared with the other media. Medium L1, which contained no added PGRs, produced no callus. Interestingly, medium L12, in which callus induction was low, produced callus similar to

Table 1. Effect of growth regulators combination on callus induction of litchi of 'Feizixiao'.

Medium	Growth regulators (μM)				Frequency of callus (%)	Types of callus
	BA	KT	NAA	2,4-D		
L1	0	0	0	0	0	Black, die
L2	0	2.32	1.07	4.52	79.52±0.16^{abcd}	CII b
L3	0	4.65	2.69	9.05	82.31±0.17^{abc}	CII b
L4	0	9.29	5.37	13.57	60.47±0.13^{de}	CII a
L5	0.89	0	1.07	9.05	77.75±0.18^{abcd}	CII b
L6	0.89	2.32	0	13.57	63.26±0.20^{abcd}	CII b
L7	0.89	4.65	5.37	0	66.49±0.20^{cde}	CII a
L8	0.89	9.29	2.69	4.52	87.07±0.13^{abc}	CII b
L9	2.22	0	2.69	13.57	93.06±0.12^{a}	CIII
L10	2.22	2.32	5.37	9.05	70.27±0.09^{cde}	CII b
L11	2.22	4.65	0	4.52	80.13±0.18^{abcd}	CII b
L12	2.22	9.29	1.07	0	44.28±0.09^{e}	CII a
L13	4.44	0	5.37	4.52	77.46±0.12^{ab}	CIII
L14	4.44	2.32	2.69	0	59.28±0.13^{de}	CII a
L15	4.44	4.65	1.07	13.57	56.83±0.15^{e}	CII a
L 16	4.44	9.29	0	9.05	79.82±0.16^{abcd}	CII ^{b}
K1	1556.19	1737.98	1562.62	1190.48		
K2	2062.14	1906.43	1808.81	2269.40		
K3	2014.29	2000.48	2252.14	2171.19		
K4	1913.93	1901.67	1922.98	1915.48		
R	18.07	9.38	24.63	38.53		

Results are mean ± SD derived from 7 bottles / Repeated, 9 to 11 anthers / bottle. Means followed by the same letter do not differ significantly (p=0.05) as indicated by one-way ANOVA followed by Duncan's comparison test. CII a: Light yellow, compact, loose, coarser particles, many pre-embryos; CII b: Light yellow, compact, loose, Tiny and coarse particles, a little pre-embryo; CIII: Light yellow, hard, more tiny particles (Lai et al., 1997). K1, K2, K3 represents the sum of the indicators for each level of each factor repeated seven times, K1 represents the value sum of "1" level corresponding to the test index. BA: K1= (L1+L2+L3+L4)*7; KT: K1= (L1+L5+L11+L16)*7; R represents Range. Range = the maximum value of average yield- the minimum value of the average yield. BA: R= (K2-K1)/ (7*4); KT: R= (K3-K1)/ (7*4).

those in medium L9. These observations suggested that certain auxin-cytokinin ratios in medium may be necessary for callus induction from anther culture of litchi. In this study, R value revealed that 2,4-D was most effective in FEC induction, followed by NAA, BA, and KT. The effect of 2,4-D on FEC induction was also observed in other litchi species (Fu and Tang, 1983; Zhou et al., 1993). Yu and Chen (1997) reported that induction of embryogenic callus from immature embryos after NAA, instead of 2,4-D, was a failure. Su et al. (2004) showed that the callus in medium without 2,4-D did not survive the following experiment. Many species confirmed that FEC induction is influenced by 2,4-D, such as in pine (Yu et al., 2011), in longan (Lai et al., 1997), and *Hevea brasiliensis Mull. Arg* (Huang et al., 2014).

In the same medium, continuous EC subculture will lead to embryonic loss, thus, the addition of KT and $AgNO_3$ in the medium synchronized embryogenesis (Lai et al., 1997). The regulated EC differentiated into somatic embryogenesis after 18 days of culture. In the present study, three different combinations of PGRs were used in SE formation.

Somatic embryo formation

Somatic embryogenesis was achieved from EC on MS medium, 6% (w/v) sucrose, and various PGRs alone or in combination. The response of SEs to three different media was extremely variable, especially at five weeks after culture initiation (Figures 1 and i). Simon et al. (2007) also reported a similar type of embryo development in 'Brewster'. Duncan's multiple range test indicated that the medium supplemented with KT and NAA produced a significantly higher number of SEs compared with the other two media; the average SE induction numbers were 489, 344 and 101 FW/g in medium supplemented with KT and NAA, ZT and NAA, and TDZ and NAA, respectively. In this study, SE shapes varied after 7 weeks in treatments (Table 2). The embryo development stages observed were double cotyledon, no cotyledon embryo, single cotyledon, multi-cotyledon embryo, flake embryo,

Table 2. Effect of growth regulator combination on somatic embryogenesis of 'Feizixiao'.

Medium	Number	Growth regulators (μM)				Frequency of embryo formation (%)			
		NAA	KT	ZT	TDZ	Cotyledonary	Double-stages	Different stages of embryo	Means
T1	1	0	23.23	0	0	12.1±1.67^{ab}	1.9±2.50^{abc}	86±2.60^{efg}	314
T2	2	0.54	13.94	0	0	12.7±1.92^{ab}	2.5±2.48^{abc}	84.8±1.73^{g}	118
T3	3	0.54	23.23	0	0	13.3±2.50^{a}	2±2.18^{abc}	84.7±3.81^{g}	489
T4	4	0.54	32.53	0	0	11.2±1.38^{bc}	3.5±3.02^{a}	85.3±3.75^{f}	143
T5	5	0	0	22.81	0	11.1±0.79^{bc}	1.1±1.78^{c}	87.8±1.94^{def}	181
T6	6	0.54	0	13.68	0	11.9±1.15^{abc}	0.9±0.74^{bc}	87.2±1.30^{efg}	109
T7	7	0.54	0	22.81	0	12.8±1.81^{ab}	2±0.99^{ab}	85.2±2.06^{g}	344
T8	8	0.54	0	31.93	0	10.5±1.73^{cd}	1.5±1.61^{abc}	88±2.63^{de}	133
T9	9	0	0	0	22.71	9.2±1.10^{de}	0±0.00^{d}	90.8±1.10^{bc}	87
T10	10	0.54	0	0	13.62	8.7±1.48^{ef}	0±0.00^{d}	91.3±1.48^{bc}	23
T11	11	0.54	0	0	22.71	6.9±1.16^{g}	0±0.00^{d}	93.1±1.16^{a}	101
T12	12	0.54	0	0	31.79	7.7±1.36^{fg}	0±0.00^{d}	92.3±1.36^{ab}	117

Results are mean ± SD derived from 10 bottles (each 0.1 g FEC). Means followed by the same letter do not differ significantly (p=0.05) as indicated by one-way ANOVA followed by Duncan's comparison test. Different stages of embryo: globular embryos, heart-shaped stage, torpedo-shaped embryos and cotyledonary embryos.

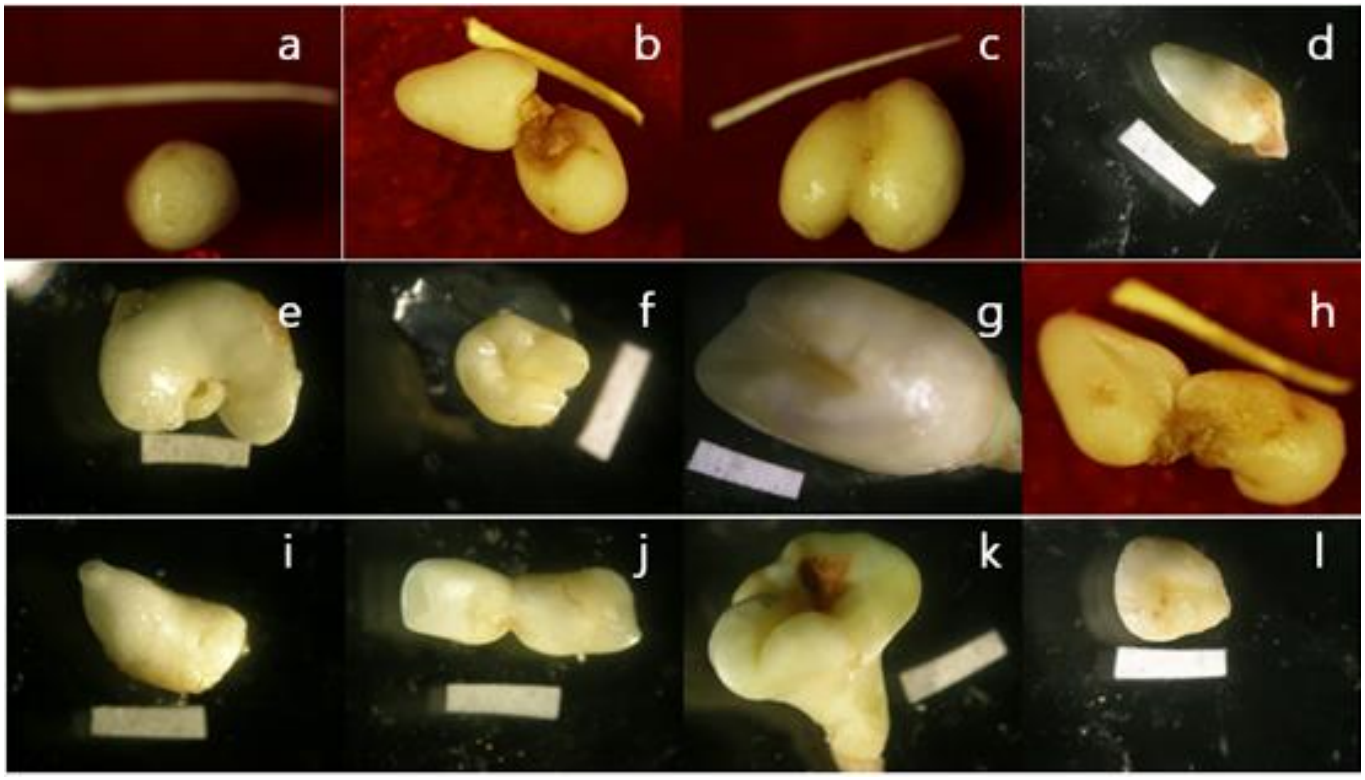

Figure 2. Microscopic photograph of developmental stage of somatic embryos. (a-d) Globular, heart, torpedo, and cotyledonary embryos. (a, d) (×2.5) and (b, c) (×2). Bar, 6 mm. No cotyledon, single cotyledon, multi-cotyledon, flake, goblet, conjoined, trumpet and mass embryo (×2.5). Bar, 3 mm.

conjoined embryo, goblet embryo, trumpet embryo, and mass embryo (Figure 2). Similar patterns of embryo development stages were reported for other plant species (Kuang et al., 1996; Tan et al., 2011).

These experiments resulted in significant differences in the number of SEs produced among treatments,

Table 3. Effect of combination of different basic medium with different regulator on regeneration.

Medium	Number	Basal medium	Growth regulators (μM)	Number of culture	Number of root	Number of regeneration	Frequency of root (%)	Frequency of regeneration (%)
		MS	GA3					
R1	1	1	0	88	12	1	13.78±9.58^{b}	1.11±4.30^{b}
R2	2	1	1.44	91	9	2	10±15.17^{b}	2.22±5.86^{ab}
R3	3	1	2.89	71	14	5	19.67±22.87^{b}	6.67±12.34^{a}
R4	4	1	5.77	76	8	0	10.44±14.74^{b}	0±0^{b}
R5	5	1/2	0	71	16	0	23.67±25.15^{b}	0±0^{b}
R6	6	1/2	1.44	73	50	1	68.33±10.97^{a}	1.33±5.16^{b}
R7	7	1/2	2.89	81	12	1	15.33±22.46^{b}	1.33±5.16^{b}
R8	8	1/2	5.77	85	20	0	22.89±21.41^{b}	0±0^{b}

Results are mean ± SD derived from 15 bottles / Repeated, 4 to 6 anthers / bottle. Means followed by the same letter do not differ significantly (p=0.05) as indicated by one-way ANOVA followed by Duncan's comparison test.

indicating that the combination and concentration of PGRs had a significant effect on SE yield (Table 2). For double cotyledonary embryos, Duncan's multiple range test revealed a significantly higher number of SEs when FECs were cultured in medium T3 (0.54 μM NAA and 23.23 μM KT), followed by medium T7 (0.54 μM NAA and 22.81 μM ZT) and medium T2 (0.54 μM NAA and 13.94 μM KT). No significant differences were observed among T8, T9, T10, T11 and T12 media which produced fewer SEs compared with the other media. Interestingly T1 and T4, with only cytokinin (KT or ZT) and no auxin (NAA) added, produced smaller and fewer SEs but medium T9 displayed contrasting results. These observations suggested that certain auxin-cytokinin ratios in medium may be necessary for SE formation.

Plant regeneration

Successful plant regeneration on the plant induction medium was achieved from post-somatic embryogenetic developmental stages: Globular, heart, torpedo, cotyledonary, and plumule embryos. Most double cotyledonary and conjoined mature embryos germinated to produce complete plantlets after 4 weeks in culture. Most abnormal embryos failed to germinate on the entire medium.

Half-strength solid MS medium produced a higher frequency of rooting (23.67%) but a lower frequency of germinated plantlets, whereas MS medium produced a lower frequency of rooting (13.78%) and well-germinated plantlets (1.11%) (Table 3). The frequency of rooting in half-strength solid MS medium was higher than that in full-strength solid MS medium in *Pyrus communis L.* (Predieri et al., 1999). Different salt concentrations would change the osmotic pressure, thereby affecting nutrient absorption and release substances into the medium. Perhaps the decrease in the concentration of nitrogen in half-strength solid MS medium stimulates rooting.

Meanwhile 2.89 μM GA_3 produced higher frequency of rooting (68.33%) but lower germination (1.33%) in half-strength solid MS medium, whereas full-strength solid MS medium produced lower frequency of rooting (19.67%) and well germinated plantlets (6.67%). The beneficial effect of gibberellins on somatic embryo germination has been reported in other plant species. For example, in *Clematis*, enhanced frequency of shoot development and greater internode elongation resulted from the influence of GA_3 (Wang et al., 2014). Compared with other studies on shoot bud differentiation such as *Quercus* (Vengadesan and Pijut, 2009) and *Ammopiltanthus mongolicus* (Yang et al., 2014), in this paper, the tap root of litchi produced black spots (1.5 cm), which ultimately led to plant death. Thus, GA_3 promoted rooting, inhibited root growth, and suppressed lateral root growth.

Successful germination and plant regeneration via SEs have been reported with combinations of KT and NAA in ginger and buffel grass (Lincy et al., 2009; Carloni et al., 2014). After germination, GA_3 is required for plant development in buffel grass. Well-developed plantlets were separated (Figure 1l) and then transferred to plastic pots containing a 1:1:1 (v/v/v) vermiculite: sand: coconut chaff mixture. The plantlets were successfully acclimatized in a greenhouse (Figure 1m) with a 100% survival rate.

The present work is the first report on plant regeneration via indirect somatic embryogenesis from anther explants of 'Feizixiao' litchi. Promising plant regeneration from SEs via indirect somatic embryogenesis was remarkably influenced by PGRs. The system will be helpful for conservation, mass clone propagation, production of bioactive compounds, and genetic transformation studies.

Ploidy level of regenerated plant

All the plantlets were diploid. Ploidy levels of regenerated plants were determined by flow cytometry, and chromosome numbers were determined by chromosome counting (Figures 1n and o). 'Feizixiao' litchi has a

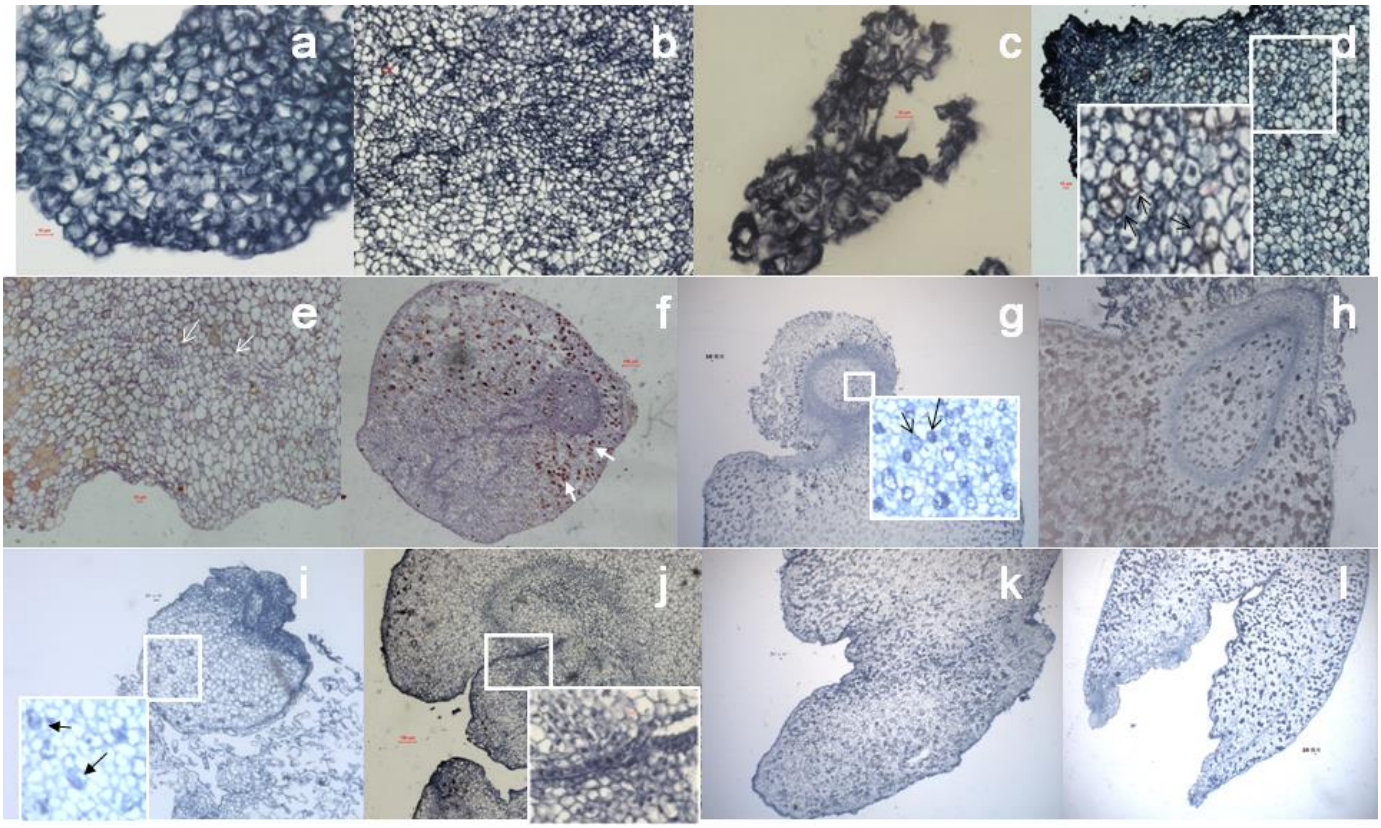

Figure 3. Histological observation of different calli and developmental stage of somatic embryos. (a) The NFEC cytoplasm was sparse or almost absent, and the cell nucleus was relatively small. (b) Cell walls are relatively thick and deeply dyed. (c) FECs that split vigorously contained more embryonic cells, which had small, high nuclear/cytoplasm ratios. (d-j) One embryonic cell divided several time to form multicellular proembryo. The development of the multicellular proembryo was followed by proembryo formation of globular embryo, heart embryo, torpedo embryo, and cotyledon embryo. g Vitrified somatic embryo, arrow indicates tannins. k and l Different developmetal stages of malformation embryo.

chromosome numbers of 2n = 2x = 30. The same chromosome numbers were confirmed in the diploid plants.

Somatic embryogenesis

Histological observations via microscopy showed that different FECs exhibited various morphologies. In general, these FEC cell walls were thick and deeply dyed (Figure 3a). The FECs that were split vigorously contained more embryonic cells, which were small with high nuclear/cytoplasm ratios (Figure 3b). The NFEC cytoplasm was sparse and even disappeared, whereas the cell nucleus was relatively smaller (Figure 3c) compared with the control.

One embryonic cell divided several times to forming a multicellular proembryo. The development of multicellular proembryos was followed by proembryo formation of globular embryo, heart embryo, torpedo embryo, and cotyledon embryo (Figures 3d to j). The somatic embryogenesis of several species was similar to that of litchi; these species included *Eucalyptus globules* and *E. saligna* × *E. maidenii* (Corredoira et al., 2015), *Hevea brasiliensis* (Wang, 2004), and *Liriodendron* hybrids (Chen et al., 2012). Embryo development was similar to zygote embryo development. In this paper, the litchi embryonic cells initially appeared in the interior and then continue to develop somatic embryos (Figure 3d). Therefore, somatic embryogenesis was of similar origin and occurred for single cells. A similar method was also observed with other woody species, such as 'Honghezi' longan (Chen, 2001). However, this phenomenon was different from that in other species, such as *Quercus variabilis* (Zhang et al., 2007), oil palm (Kanchanapoom and Domyoas, 1999), and *Quercus robur* (Corredoira et al., 2006). Zeng et al. (2002) reported that embryogenic cell 'Yuanhong' litchi is of a different origin. Lai and Sang (2003) reported that litchi in low 2,4-D is of a different origin.

Normal somatic embryos are ivory, whereas treated somatic embryos are vitrified. Histological observations showed that the early normal somatic embryonic cells were of uniform size, compact, possessed thick and cell

walls, and deep dyes; starch grains were evenly dispersed within the cell (Figures 3 and i). Moreover, the cambium was obvious. Starch grains accumulated near the earth in mature white cotyledon embryo cells, and bud primordial were obvious (Figure 3j). Vitrified somatic embryos exhibited different cell stages, and the cells were loosely arranged with thin cell walls. A small number of cell walls underwent autophagy and contained numerous brown tannins (Figure 3f).

Different developmental stages, either bud primordium or asymmetric leaf primordium, were lacking in deformed embryos. Moreover, abnormalities in the procambium prevented further development (Figures 3k and l).

Conflict of Interests

The authors hereby declare that no conflict of interest exists among them.

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