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SHORT COMMUNICATION

Leaf callus induction and suspension culture establishment in lychee (*Litchi chinensis* Sonn.) cv. Huaizhi

Xin-Ye Ma · Gan-Jun Yi · Xue-Lin Huang · Ji-Wu Zeng

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Abstract This study reports a protocol for leaf callus induction and suspension culture establishment in lychee cv. Huaizhi. The results showed that 12-day-old leaf explants cultured under a photoperiod of 16/8 h with their adaxial side touching the medium were the optimum conditions for leaf callus induction. Globular embryos were formed when the induced calli were kept on the callus induction medium without 2,4-D for 24 weeks. Friable calli were induced after 2–3 subcultures at 4 weeks intervals on the Murashige and Skoog medium supplemented with 3 mg/L IAA and 2 mg/L BAP. Suspension culture was established when these friable calli were subcultured six times in liquid callus induction medium.

Keywords Explant orientation · Friable calli · Globular embryos · Leaflet age · Leaflet position · Light regime · Lychee

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X.-Y. Ma · X.-L. Huang (✉)
The Key Laboratory of Gene Engineering of the Ministry of Education, School of Life Sciences, Zhongshan (Sun Yat-sen) University, 510275 Guangzhou, People's Republic of China
e-mail: huangxl@mail.sysu.edu.cn

Present Address:
X.-Y. Ma
The Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences and Peking Union Medical College, 100193 Beijing, China

G.-J. Yi (✉) · J.-W. Zeng
Fruit Research Institute, Guangdong Academy of Agricultural Science, 510640 Guangzhou, People's Republic of China

Abbreviations

BAP	6-Benzylaminopurine
2,4-D	2,4-Dichlorophenoxyacetic acid
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
KT	Kinetin
NAA	α-Naphthalene acetic acid

Introduction

Lychee (*Litchi chinensis* Sonn.) is an important economic fruit crop, which has gained acceptance locally and internationally due to its unique taste and flavor. However, its commercial production has been hindered by several problems including lack of suitable commercial cultivars with high production yields, regular bearing and wide harvesting season (Ghost and Bhavan 2001). The protocol of plant regeneration in vitro may be used to accelerate the release of new cultivars. Lychee plants have been regenerated with slow progress from anthers to immature embryos in early (Amin and Razzaque 1995; Fu and Tang 1983; Kuang et al. 1996; Yu and Chen 1997; Yu et al. 2000; Zhou et al. 1996), partially at least attributed to the strong time restriction of explants collection. The feasibility of leaf culture (Puchooa 2004; Raharjo and Litz 2007) demonstrated recently can be hopeful to facilitate the experiment of this kind. However, there is not yet the detailed report in China where owned abundant germplasm resources of lychee.

It this article, we attempted leaf culture with an elite local lychee cultivar Huaizhi in Guangdong Province to fill the gaps, and concentrated on calli induction and suspension culture establishment. Calli are propitious to proliferation and morphogenesis in vitro for their dedifferentiated and juvenile state, while suspension culture with fast growth,

well dispersity and fine texture have even more uses in biotechnology (Chawla 2004).

Materials and methods

Explants preparation

Seeds of 'Huaizhi' were potted in a mixture of garden soil and peat (2:1, v/v) under greenhouse conditions at 26 ± 2°C and 80% relative humidity. The plants were watered at weekly intervals and fertilized with a solution of half-strength Murashige and Skoog (1962) (MS) inorganic salts monthly. The 2-year-old juvenile plants were used as donors for leaf explants.

The apical leaflets in a branch of compound leaves were selected as starting materials for the leaf explants (Fig. 1). The median part of the leaflet was cut into 1 cm^2 segments, which were then used as explants. Before culture, the leaf explants were first surface-sterilized by washing with running tap water for 30 min, and disinfected for 15–20 min with 1.5% sodium hypochlorite and 3–5 drops of Tween 80. Finally, they were rinsed three times with sterile distilled water.

Culture medium and conditions

Except for various growth regulators, the solid basal media (SBM) contained MS basal medium, 500 mg/L lactoalbumin hydrolysate (LH), 200 mg/L activated charcoal (AC), 30 g/L sucrose and 7 g/L agar. The components of liquid medium (LM) were the same as relevant solid medium without agar and AC. All media were adjusted to pH 5.8 prior to autoclaving at 121°C for 15 min. Unless stated otherwise, all explants were incubated with adaxial side touching the medium under a 16 h (light)/8 h (dark) photoperiod (provided by cool-white fluorescent lamps at a photon flux of 27 μmol m^{-2} s^{-1}) at 26 ± 2°C.

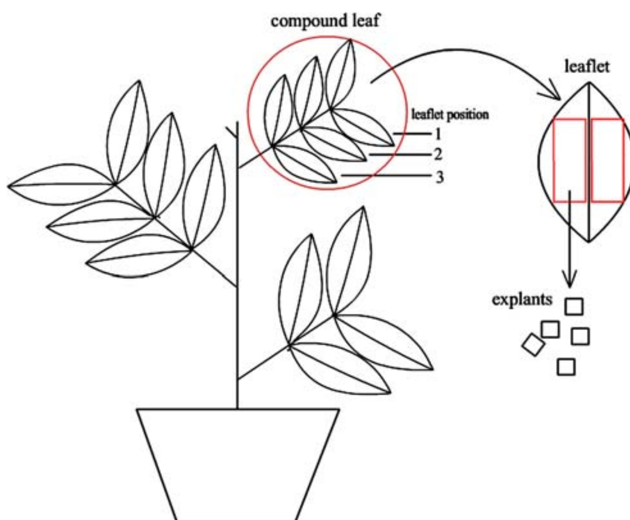

Fig. 1 Schematic diagram of explant preparation

Factors affecting callus induction

The callus induction medium (CIM) was prepared according to Fu and Tang (1983) which contained similar components of SBM, 2 mg/L 2,4-D, 0.5 mg/L NAA, 2 mg/L KT and 200 mg/L AC. To optimize the variables for callus induction, the following sets of experiments were conducted: (1) Effect of age on callus induction: explants were taken from 8-, 12- and 16-day-old leaflets at the same apical leaflet position in the compound leaves. The time when young leaflet was just fully unfolded was taken as day 0. (2) Effect of leaflet position: three kinds of explants were prepared from leaflets at different position in a compound leaf, namely, (1) apical, (2) median and (3) basal position (Fig. 1). Leaflets age was limited to the optimal one, following the results obtained in experiment (1). (3) Effect of explant orientation: two contrary plating orientations, adaxial side of explants either touching the medium or vice versa, were handled parallel. (4) Effect of light regime: explants were cultured individually at a continuous dark condition or a light condition with photoperiod of 16/8 h.

Callus yield (w/w) was measured as the final fresh weight of cultures relative to the initial explants weight. The calli obtained under the optimal experimental conditions were subsequently used for embryo induction on CIM without 2,4-D and suspension culture in liquid CIM.

Suspension culture establishment

Friable calli was induced when leaf calli were transferred to MS medium supplemented with 3 mg/L IAA and 2 mg/L BAP (Puchooa 2004); and further subcultured until calli reached the stage fit for suspension culture, i.e. rapid growth and good dispersibility.

For suspension culture establishment, two gram of friable calli was selected and transferred to a 100 mL Erlenmeyer flask containing 30 mL LM (CIM devoid of agar and AC). The suspension cultures were then placed on a gyratory shaker at 120 revolutions per minute under dark at 26 ± 2°C. Subculture was carried out weekly by adding 20 mL fresh LM medium to 10 mL old suspension cultures containing 3 mL PCV (packed cell volume) calli. At the end of the second subculture, the suspension was filtered through a pre-sterilized, stainless steel sieve of 900 μm. After another four rounds of subcultures, the cytological states of cultures such as shape, size and division frequency, etc. were observed and recorded under an inverted microscope to evaluate the status of suspension culture.

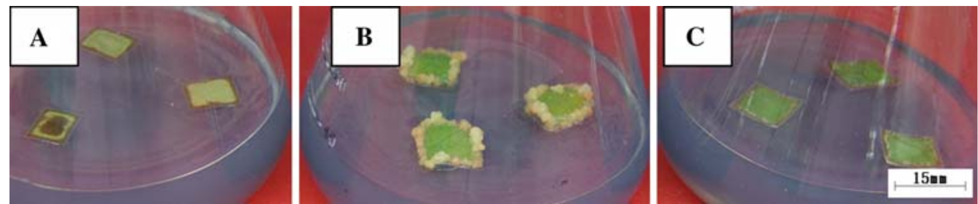

Fig. 2 Influence of leaflet age on callus induction of leaflet from 2-year-old lychee (cv. Huaizhi) juvenile plants. **a–c** Explants at 8 (**a**), 12 (**b**) and 16 (**c**) days

Table 1 Effect of leaflet age, leaflets position, explants orientation and light regime on callus induction of leaf from lychee (cv. Huaizhi) juvenile plants

Factors	Callus yield[a] (g/g explants)
Leaflet age (day)	
8	2.33 ± 0.58 c
12	14.67 ± 2.08 a
16	5.67 ± 1.53 b
Leaflet position	
Apical position	14.67 ± 2.08 a
Median position	13.33 ± 1.53 a
Basal position	11.33 ± 2.52 a
Explant orientation	
Adaxial side up	4.33 ± 0.58 b
Adaxial side down	16.67 ± 2.08 a
Light regime	
Light with photoperiod	16.67 ± 2.08 a
Constant dark	7.33 ± 1.53 b

[a] The values were represented as mean ± SE; with each factor as unit, means within this column followed by the same letter were not significantly different at $P = 0.05$ (Duncan's multiple range test)

Histological investigation

The cultured samples were fixed in FAA (90% of 70% ethanol, 5% of 40% formaldehyde, 5% glacial acetic acid), dehydrated through an ethanol-xylol series before they were embedded in paraffin wax. The samples were sectioned at 7–8 µm, stained in safranin-fast green and examined under an inverted microscope. As for suspension cultures, drops of samples were transferred directly to a slide and observed under an inverted microscope.

Data analysis

All the treatments were carried out in 3 replicates with 30 explants each. Data were subjected to analysis of variance and significance comparison among means by Duncan's multiple range test at $P = 0.05$.

Results and discussions

Callus induction from leaflet

Influence of leaflet age on callus induction

After culturing for 4 weeks, yellow or light yellow calli (Fig. 2) were only observed from the 12th day old explants. The yield obtained was also significantly higher than the younger or older ones (Table 1). This observation was supported by several studies which showed that young leaves of a certain age were the only ideal explants (Das and Rout 2002; Gu and Zhang 2005) because such leaves may contain the largest proportion of competent cells whose fate could be easily altered under in vitro conditions (Perez-Tomero et al. 2000). Therefore, the results indicated that the 12th day was the optimal age for maximum callus induction.

Influence of leaflet position on callus induction

No significant differences in callus yield were observed in explants taken from different leaflet positions (Table 1). Moreover, the appearances of the calli were similar (Fig. 3). This result showed that leaflet position was not a determining factor on callus induction.

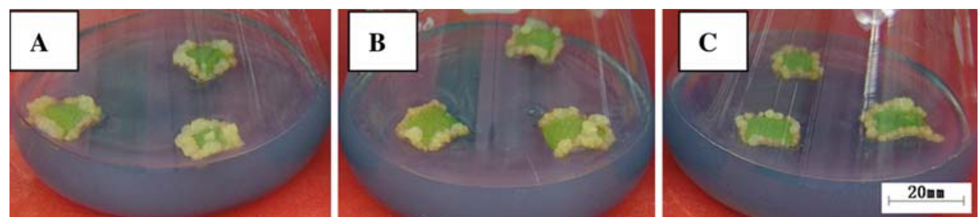

Fig. 3 Influence of leaflet position on callus induction of leaflet from 2-year-old lychee (cv. Huaizhi) juvenile plants. **a–c** The explants in three flasks are prepared separately from the apical (**a**), median (**b**) and basal (**c**) leaflet position

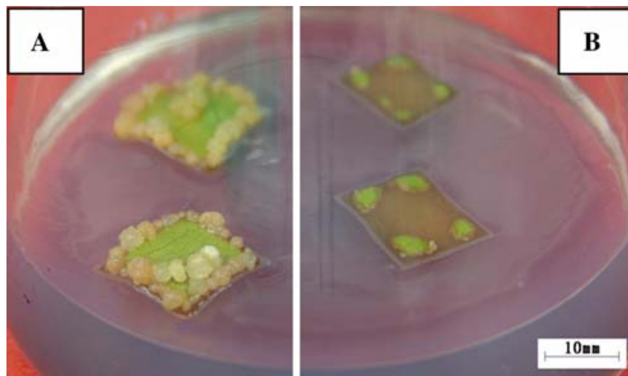

Fig. 4 Influence of explants orientation on callus induction of leaflet from 2-year-old lychee (cv. Huaizhi) juvenile plants. **a**–**b** The explants cultured with the adaxial surface touching the medium (**a**) or the other way round (**b**)

Influence of explant orientation on callus induction

The explants with adaxial side facing down produced yellow and light yellow calli (Fig. 4), and calli yield was significantly higher than that with adaxial side facing up (Table 1). In contrast, Raharjo and Litz (2007) showed that callus induction from the leaf explants of lychee cv. Brewster were more successful with adaxial side up. Although no comparative experiments were carried under their described culture conditions, this disparate observation was most probably due to genotype differences (Chen and Dribnenki 2002; Zhang et al. 1998).

Influence of light regime on callus induction

Explants under light regime with photoperiod of 16/8 h produced more calli than that under constant dark (Table 1), and the color of calli was brighter in color compared to those cultured under the latter condition (Fig. 5). However, better induction under dark conditions than light was observed in lychee cv. Brewster (Raharjo and Litz 2007)—plausibly due to genotypic differences as mentioned above.

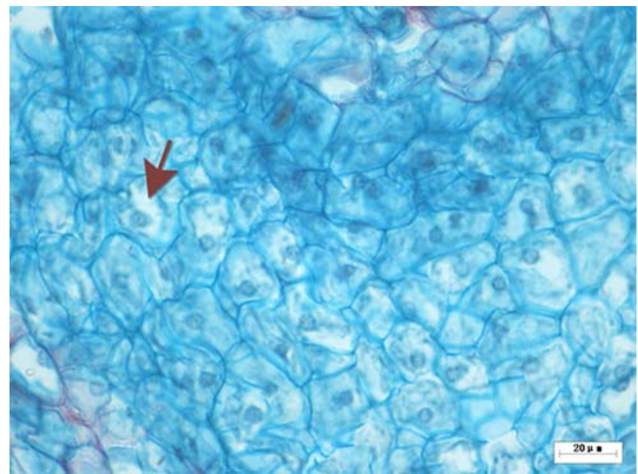

Fig. 6 Histological observation on calli from leaf explants under optimal culture conditions, *arrow* shows the cell with embryogenic characteristic—large nucleus and active cell division (×600, *bar* = 20 μm)

Globular embryo induction from calli

Histological observation showed that the leaf calli comprised large amount of cells with embryogenic characteristics, such as large nuclei and active cell division (Fig. 6). After 24 weeks of cultivation on CIM without 2,4-D, the calli formed globular-like structures which was determined to be globular embryos by histological examination (Fig. 7).

Suspension culture of the friable calli

After the leaf calli were transferred and subcultured 2–3 times on the MS medium with IAA and BAP, friable calli were obtained (Fig. 8a). When these friable calli were subcultured 6 times, the suspension cultures (Fig. 8b) comprised mostly uniform cell masses of small size and with active division (Fig. 8c). It was apparent that the suspension culture from leaf calli was established successfully here.

In conclusion, this study has implicated that genotypic difference is a potential factor for leaf culture in lychee.

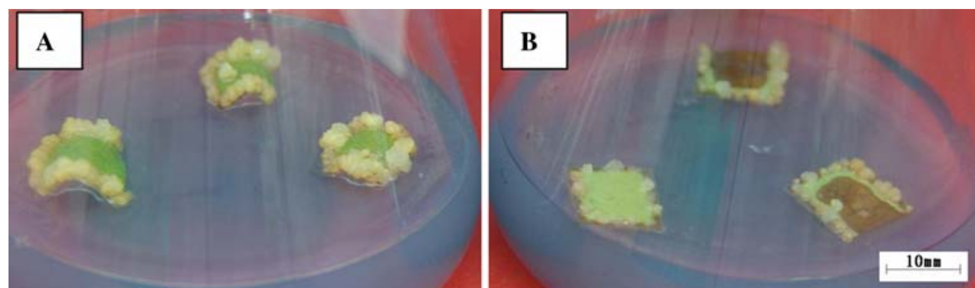

Fig. 5 Influence of light regime on callus induction of leaflet from 2-year-old lychee (cv. Huaizhi) juvenile plants. **a**–**b** The cultures were kept respectively under light conditions with a 16/8 photoperiod (**a**) and constant dark condition (**b**)

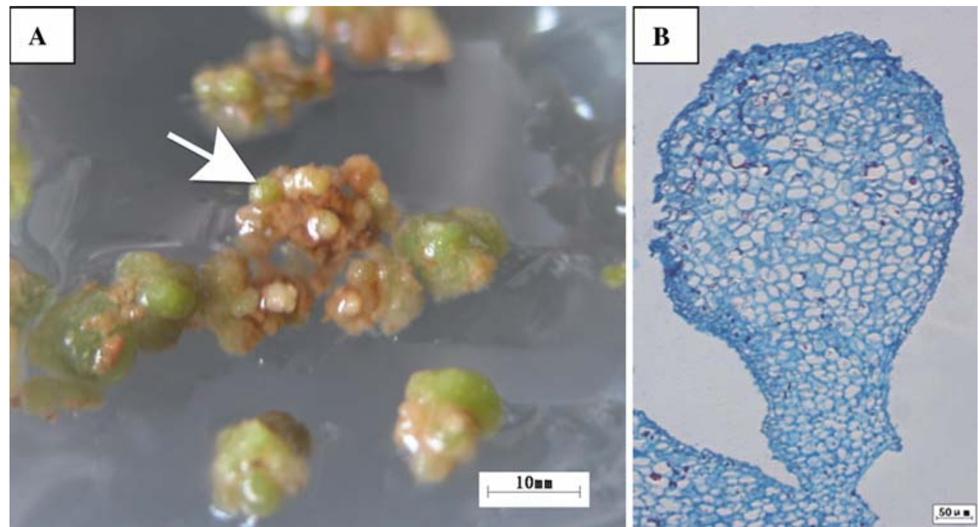

Fig. 7 Globular embryos formed from calli of lychee (cv. Huaizhi) leaves. **a** The globular-like structure, **b** histological observation on globular-like structure—globular somatic embryo (×100, *bar* = 50 μm)

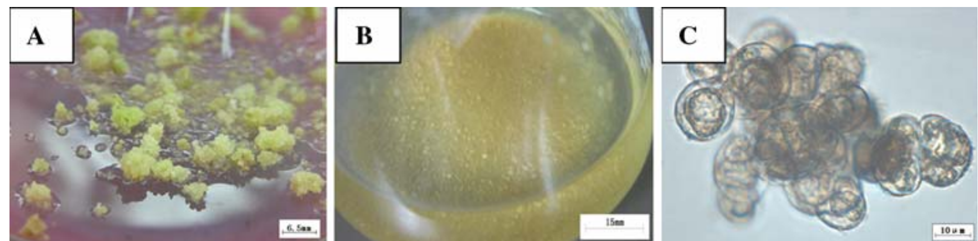

Fig. 8 Suspension culture established from leaf calli of lychee (cv. Huaizhi). **a** Friable calli, **b** the suspension culture initiated from friable calli, **c** observation on cell masses in suspension cultures (×600, *bar* = 10 μm)

Nevertheless, the procedure described here has provided a preliminary step towards regenerating 'Huaizhi'. Further research is needed to determine the optimal conditions for globular embryos initiation and plantlet recovery.

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