Research paper

Somatic Embryogenesis and Plant Regeneration from Immature Embryo Cultures of *Cinnamomum kanehirae*

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【Summary】

Cinnamomum kanehirae fruits at various stages of maturity were collected from Wulai (Taipei), Guanwu Forest Recreation area (Hsinchu), and Dasyueshan (Taichung) for an analysis of somatic embryogenesis. Only seeds that developed to the white cotyledonary stage which occupied < 75% in the seed volume could induce embryogenesis. Embryos could germinate, and somatic embryogenesis was induced from immature embryos cultured on WPM medium supplemented with activated charcoal (AC). When cultured on medium with a PGR supplement, embryos began somatic embryo differentiation directly or formed a somatic embryo from an intervening callus. Nodular calli had a better somatic embryo regeneration capacity then friable calli. The best medium for somatic embryogenesis was WM medium containing 0~1 mg/L naphthaleneacetic acid (NAA), 0~0.5 mg/L kinetin, and 1 g/L AC. The optimum medium for multiplication of white-cotyledonary stage somatic embryos was WM medium supplemented with 1 g/L AC, 3 mg/L NAA, and 0.5~1 mg/L kinetin. After 40 d of culture, somatic embryos had increased to 2.3~2.5 times in fresh weight. When cultured on WPM basal medium supplemented with 26.4 mg/L abscisic acid (ABA) and 1.5 g/L AC, respective rates of germination and normal plantlet development for white cotyledonary-stage somatic embryos were 58.9 and 50%. The survival rate of embryogenic plantlets was > 95% after being transferred to a greenhouse. After 6 mo, the plantlets with an average height of 17 cm were transplanted to Yuchi (Nantou) and Yuanshan (Yilan). The average height was 133±31.5 cm 1 yr after transplanting to Yuchi, and was 212±42.9 cm 17 mo after transplanting to Yuanshan.

Key words: *Cinnamomum kanehirae*, immature embryo culture, plant regeneration, somatic embryogenesis.

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研究報告

牛樟未成熟胚培養之體胚發生與植株再生

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摘 要

本研究蒐集台北烏來(Wuli)、新竹觀霧森林遊樂區(GuanWu Forest Recreation area)與台中大雪山(Dasyueshan)地區之不同成熟度的牛樟種子進行胚培養。結果只有在胚發育成白色且小於75%種子體積的未成熟胚可誘導體胚再生。將此未成熟胚培養於WPM添加活性炭培養基，胚可發芽或同時誘導體胚再生(somatic embryogenesis)。培養於添加植物生長調節之培養基可直接分化體胚，或先產生癒傷組織再由癒傷組織再生體胚。癒傷組織之體胚再生率以顆粒狀癒傷組織(nodular calli)較鬆軟癒傷組織(friable calli)高，最佳培養基為WM添加0~1 mg/L NAA, 0~0.5 mg/L kinetin, and 1 g/L AC。子葉期體胚可增殖於WM添加1 g/L AC, 3 mg/L NAA, 0.5 mg/L kinetin培養基，40天可增加2.3~2.5倍。將子葉期體胚培養於WPM添加1.5 g/L AC, 26.4 mg/L ABA有58.9%可發芽，50%可發育成健全小苗。小苗移入溫室培養成活率95%以上，將6個月17 cm左右溫室小苗各60株，栽植於南投魚池(Yuchi, Nantou)12個月，平均苗木高為133±31.5 cm，於宜蘭員山(Yuanshan, Yilan)栽植17個月平均苗高為212±42.9 cm。

關鍵詞：牛樟、未熟胚培養、植株再生、體胚發生。

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INTRODUCTION

Cinnamomum kanehirae Hayata, of the Lauraceae family, is endemic to Taiwan (Liao 1996). Although it was once distributed at elevations of 200~2000 m throughout the island, *C. kanehirae* is currently only found in some scattered remote areas at middle and high elevations due to overcutting of natural forests. *Cinnamomum kanehirae* has great potential in the exclusive furniture market and as carving material (Kao and Huang 1993). Its timber, containing high terpineol, is highly resistant to rot and insect damage, and has a fine sense texture as a well as beautiful cross grain. Moreover, as the only natural host of *Antrodia cinnamomea*, a valuable medicinal fungus (Chang and Chou 1995), the illegal cutting of *C. kanehirae* frequently occurs. As a result, seedling demand for plantations is increasing.

Due to the influence of maturation, the root formation percentage of *C. kanehirae* cuttings is usually very low. The rooting rate of cuttings collected from mother trees in natural forests was < 5% (Wei 1974), while cuttings collected from 14-yr-old ortets was only 20~26% (Huang and Kao 1997). *Cinnamomum kanehirae* faces a high risk of endangerment in the wild due to multiple reasons, such as limitation of pollination between isolated individuals, small seed yields (a high fruit drop rate but few mature fruits), damage from fruit consumption by animals, seed dormancy, and poor natural regeneration (Huang et al. 1996). To conserve and reproduce elite clones of this species, micropropagation was reported to be a successful technique (Chang et al. 2002, 2010).

Although embryogenesis may successfully rescue immature embryos (Sharma et al. 1996), somatic embryogenesis procedures in woody plants are recalcitrant and a difficult task (Cangahuala-Inocente et al. 2007). Among over 2000 species recorded in the Lauraceae, the somatic embryogenesis ability of only a few members of this family was studied, including *Sassafras randaiense* (Hay.) Rehd. (Chen and Wang 1985), *Persea americana* Mill. (Wijaksono and Litz 1999, Witjaksono and Litz 2002, Sánchez-Romero et al. 2005), *Laurus nobilis* L. (Canhoto et al. 1999), *Ocotea odorifera* (Vell.) Rohwer (Catarina et al. 2001), *O. catharinensis* Mez. (Viana et al. 2004, Catarina et al. 2005), *C. pauciflorum* Nees (Kong et al. 2009), *C. camphora* (L.) Presl. (Cheng and Ma 1990, Du and Bao 2005, Shi et al. 2010), and *C. kanehirae* (Chen and Chang 2009, Chang et al. 2010). Chen and Chang (2009) investigated *C. kanehira*e somatic embryo regeneration from induced calli of young leaves of germinated seeds, while we reported the preliminary results of somatic embryogenesis and shoot multiplication from immature embryos and young leaves of 10-yr-old seedlings (Chang et al. 2010).

To improve the application of *C. kanehira*e plantlets produced by somatic embryogenesis, this research focused on the effects of seed maturity on embryo germination and somatic embryo induction, as well as establishing a somatic embryo multiplication and plant regeneration system. Fruits at various degrees of maturity collected from Taipei, Hsinchu, and Taichung were used in this study. By using immature embryos for culture, *C. kanehira*e fruits can be harvested earlier before the fruits drop or are eaten. This propagation tool offers an opportunity to develop clonal forestry of high-value rare old trees after progeny and somatic embryo clone tests, and also facilitates investigation of the physiology of embryo development and gene transfer applications.

MATERIALS AND METHODS

Plant materials

Green immature and blackish-purple mature fruits were collected from Wulai (Taipei) in July 2010, and Wulai, Guanwu (Hsinchu), and Dasyueshan (Taichung) in June~November 2012. After recording the size and embryo development status, the fruits were used for embryo culture.

Embryo culture

Pericarps of mature fruits were removed in advance. Both mature and immature fruits were cleaned and sterilized with 70% (v/v) ethanol for 2 min, followed by 3% (v/v) sodium hypochlorite in an ultrasonic bath for 20 min. Under laminar flow and after being rinsed with sterile distilled water 4 times, seed coats were carefully removed from sterilized fruits using a scalpel and tweezers. Embryos with 2 white cotyledons were then excised and placed on WPM (Lloyd and McCown 1981) basal medium supplemented with 30 g/L sucrose and 1.5 g/L activated charcoal (AC) (called WA medium). Embryo germination and morphogenesis during 2 mo of culture were recorded.

Fruits from Wulai were also used for a study investigating effects of the medium and its components on callus induction and somatic embryogenesis. Immature embryos were cultured on WPM, B_5 (Gamborg et al. 1968), or 1/2 MS (half-strength concentrations of the major salts of MS medium, Murashige and Skoog 1962) containing 30 g/L sucrose, 150 ml/L coconut milk (CM), 0.5 g/L glutamine, and a set of combinations of naphthaleneacetic acid (NAA) with benzyladenine

(BA) or kinetin. Three replications of 10 embryos per treatment were evaluated. Callus induction and somatic embryogenesis during 2 mo of culture were recorded.

Somatic embryogenesis and multiplication

One gram (fresh weight) of embryo-derived calli or cotyledonary somatic embryos was cultured on WPM basal medium supplemented with 30 g/L sucrose, 150 ml/L CM, 0.5 g/L glutamine (called WM medium) with 0 or 1.5 g/L AC and a set of combinations of NAA and kinetin (0, 1, 3, and 4 mg/L NAA and 0 and 0.5 mg/L kinetin) in Petri dishes. Each treatment was replicated 3 times. Fresh weight and numbers of cotyledonary somatic embryos derived in 40 d of culture were recorded.

Somatic embryo germination and plantlet regeneration and transplantation

Somatic embryos with cotyledons were cultured on WPM basal medium supplemented with 26.4 mg/L abscisic acid (ABA) or 1.5 g/L AC. Three replications of 30 somatic embryos per culture dish/treatment were evaluated. The number of embryos germinated (defined as the production of 2 or more true leaves and the development of roots over 0.3 cm) in 40 d of culture, and the number of plants developed (defined as a normal plantlet with root, stem, and leaves developed) in 50 d of culture were recorded.

Germinated somatic embryos were then cultured in culture tubes. When having reached 3~4 cm in height, and after being washed with water to remove any agar, small plantlets were transferred to plug trays filled with sterilized no. 2 perlite and vermiculite (1:1 v/v). To maintain moisture, plug trays were placed on a plastic basket with some water retained in the bottom and covered with a plastic cloth for 1 wk in a growth chamber. Plug trays were moved to a greenhouse with a fog irrigation system (15 s of fog/10 min) for another 2~3 wk, and then transferred to black plastic pots filled with vermiculite and peat (1:1 v/v) supplemented with 3.33 g/L slow-release solid fertilizer (N:P:K = 14:12:14, Chisso-Asahi Fertilizer, Tokyo, Japan) under 20% shade. After being transplanted for 6 mo in 2013, 60 plantlets (17±3.1 cm in height) were transferred from the greenhouse to Yuanshan (Yilan) and Yuchi (Nantou). The growth and height of plantlets were recorded after 17 mo in Yilan and 12 mo in Nantou.

Medium preparation, culture conditions, and data analysis

All media were adjusted to pH 5.7±0.05 using 1 N KOH or HCl. Agar (Difco agar) at 7.5 g/L was added to the media before autoclaving for 15 min at 121℃ (1.05 kg cm^{-2}). Explants were cultivated in 12-cm flat-bottom culture tubes (1 explant/tube) containing 12 ml of medium for the embryo culture and plantlet growth study, while otherswere cultivated in 25 ml of medium in 9-cm Petri dishes for the somatic embryogenesis, multiplication, and germination study. Culture tubes and Petri dishes were maintained in a growth chamber at 25±2℃ and a 16-h photoperiod provided by fluorescent light (45 μE $m^{-2}s^{-1}$).

Data were analyzed with general linear models (GLMs) of SAS software (SAS Institute 1995).

RESULTS AND DISCUSSION

***C. kanehirae* fruit and embryo development**

Due to the high fruit drop rate, only very few purple to blackish-purple mature *C. kanehirae* fruits with fully developed embryos were available. We observed 2 *C. kanehirae* individuals in June~November 2012 in Wu-

lai. In total, 148 and 131 fruits of these 2 trees were recorded (Table 1). Fruits began to drop in May. Fruit setting rates of the 2 trees recorded later were 47.3 and 38.17% on 1 June, 4.73 and 2.29% on 6 July, and only 2.03 and 0.76% on 31 July. By the end of August, no fruit remained on the trees. The appearances of the dropped fruits were usually normal. However, inside the seeds, the embryos might be at different developmental stages, such as an empty seed with undeveloped embryos (Fig. 1a) and embryo hypoplasia (Fig. 1b). Fruits with normally developed embryos also dropped for unknown reasons (Fig. 1c, d).

Not many *C. kanehirae* fruits were collected at the 3 sites in 2012, due to their unreachable height and high drop rate. At Dasyueshan, 71 green fruits were collected from 3 trees in August. At Wulai, 20 green fruits were collected early each month in June~August. However, no fruits were available after mid-August since almost all of the

Table 1. Observation of 2 *Cinnamomum kanehirae* tree fruit numbers in the Wulai area

Tree	Total fruits	Total fruits (%)			
	1-May	1-June	6-July	31-July	30-Aug.
Tree 1	148	70 (47.30)	7 (4.73)	3 (2.03)	0 (0)
Tree 2	131	52 (38.17)	3 (2.29)	1 (0.76)	0 (0)

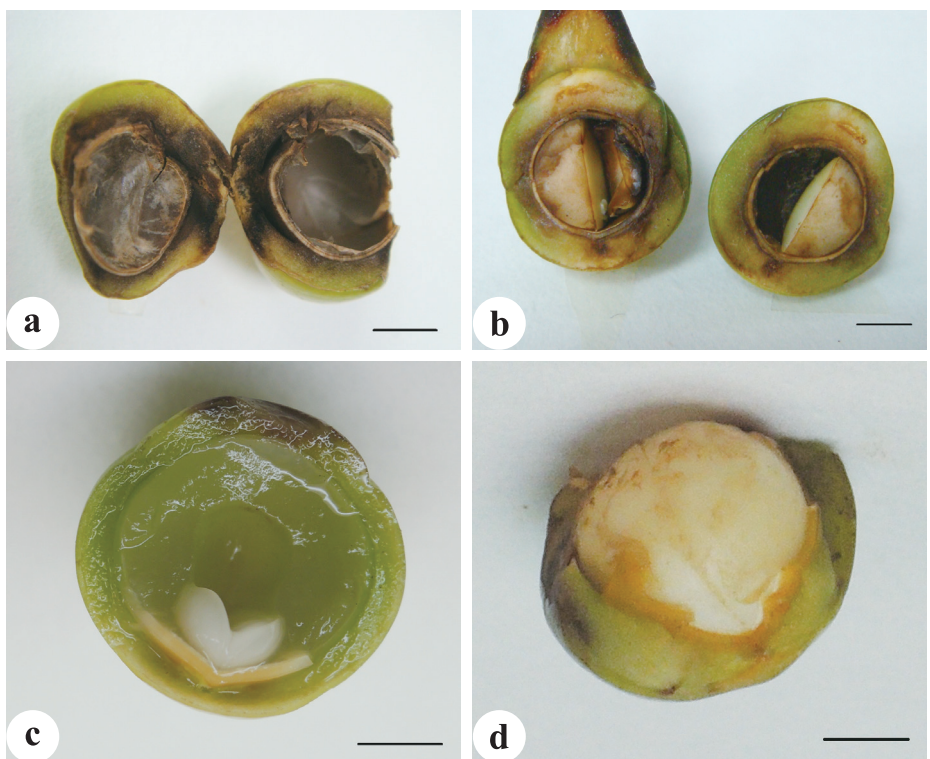

Fig. 1. Embryo development of *Cinnamomum kanehirae* seeds. a, Empty seed; b, embryo hypoplasia; c and d, normal embryo developed: the embryo occupied < 50% (c) and ~100% (d) of the volume inside the seed. (bar = 0.2 cm).

fruits had dropped. At Guanwu, 780 green fallen fruits were picked up off the forest floor after typhoon Saila passed in early August. Later, 249, 162, and 26 green, greenish-brown, and black-purple dropped fruits were respectively collected in September, October, and November.

Fruits were divided into 3 groups based on their diameter, i.e., < 0.8 cm (small), 0.8~1.3 cm (middle), and > 1.3 cm (large) (Fig. 2a). All fruits were larger than 0.8 cm, except 1.3% of fruits collected from Guanwu in August. Fruits at Wulai were the largest among all sites, followed by those at Dasyueshan. The phenology of fruit development was varied at different sites. Over 90% fruits were > 1.3 cm starting in July at Wulai, while 80.3% were > 1.3 cm at Dasyueshan in August. Fruits at Guanwu developed relatively more slowly. About 67% of fruits were 0.8~1.3 cm in September and October, and all had developed into large ones by November.

Usually, *C. kanehirae* seeds become mature in October or November. During embryo development, the size of cotyledons can easily be identified and can be an indicator of the seed development stage. All fruits at Wulai were already of a middle size in June; however, no white cotyledons (WE) developed until July (60%). In August, all fruits at Wulai had WE. At Guanwu, the proportion of fallen fruits with developed WE reached 96.4% in August, dropped to 25.5~45.1% in September and October, but increased to 57.7% in November. The falling of fruits in August was very likely caused by a typhoon, and the strong winds may have blown both sound and unsound fruits down. In September and October, fruits fell mainly due to abnormal development (empty seeds with undeveloped embryos or embryo hypoplasia). In November, as both abnormally developed and mature fruits might fall naturally, the proportion of WE increased.

According to the percent of cotyledon volume within a seed, embryo development was divided into 4 stages: 1. mature embryos (fruit completely mature, seed filled up with

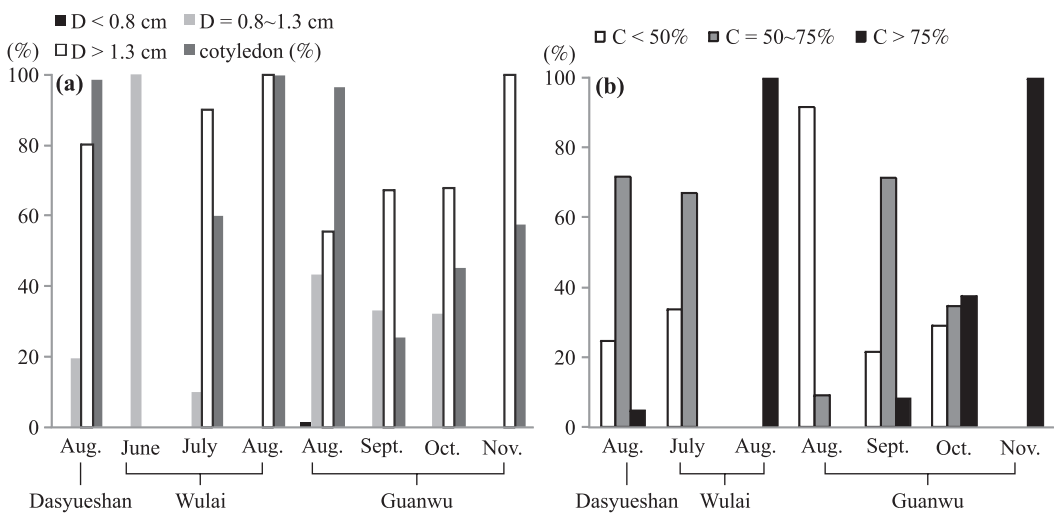

Fig. 2. Percentage of fruit size and cotyledon formation of *Cinnamomum kanehire* in different months. (a) Fruit size and percentage of cotyledon formation. (D, fruit diameter) (b) Percent of cotyledon volume (C) within a seed. Fruits were collected from Dasyueshan, Wulai, and Guanwu.

the cotyledon, the endosperm having disappeared, and the fruit coat purple to purplish-black); 2. immature embryos A (EA) (cotyledon occupying > 75% of the seed volume, and the endosperm occupying the rest, Fig. 1d); 3. immature embryo B (EB) (cotyledon occupying 50~75% of the seed volume); and 4. immature embryo C (EC) (cotyledon occupying < 50% of the seed volume, Fig. 1c). Fruits were green at all 3 immature embryo stages.

When comparing embryo developmental stages at different places (Fig. 2b), percentages of EA, EB, and EC fruits at Dasyueshan in August were 4.3, 71.4, and 24.3%, respectively, while all fruits at Wulai were at the EA stage. Embryo development was the slowest at Guanwu. In August, 8.6 and 91.4% of fruits were at the EB and EC stages. No fruit at the EA stage was observed until Sepetmber, while all fruits had developed to the EA stage in November.

From fruit development observations at the 3 different areas, the development of WE could not be determined by fruit sizes. However, the development of embryos could be estimated by season with a little variations yearly according to our field observations at Wulai for 10 yr. Cotyledons began to develop approximately in late June and had completed development by early August. Based on our survey of 2012, in July at Wulai and in August in central mountain areas, fruits with WE were favorable material for embryo germination and somatic embryogenesis.

Embryo observations and culture of fruits at various developmental stages

In total, 402 embryos dissected from sterilized fruits collected from 3 areas were cultured on WA medium. Among all collected fruits, there were only 5 mature seeds (Table 2). Over 96.7% of mature embryos and immature ones at the EA stage germinated within 1 mo. There were 3 responses of cultivated immature embryos at the EB or EC stages: somatic embryogenesis, embryo germination (Fig. 3a), and embryo germination plus somatic embryogenesis which occurred on the cotyledon (Fig. 3b) or root (Fig. 3c). Results of immature embryo germination of fruits from Wulai and Dasyueshan were similar, as around 1/4 of immature embryos at the EB stage geminated, and no germination of those occurred at the EC stage. For fruits from Guanwu, germination rates were 84.6 and 54.5% for immature embryos at the EB or EC stages, respectively. The somatic embryogenesis ability was better for embryos at the earlier developmental stage with no

Table 2. Effects of different developmental stages of *Cinnamomum kanehirae* embryos on their germination and somatic embryogenesis ability as cultured in WPM plus 30 g/L sucrose and 1.5 g/L activated charcoal medium for 2 mo. Seeds were collected from Dasyueshan (D), Wulai (W) and Guanwu (G)

Embryos	Percent of cotyledon volume within a seed (%)	No. of total cultured embryos			Germination (%)			Embryogenesis (%)			Germination and embryogenesis (%)		
		D	W	G	D	W	G	D	W	G	D	W	G
Mature embryos	100%	0	0	5	-	-	100	-	-	0	-	-	0
Immature embryos (EA)	> 75	3	20	30	100	100	96.7	0	0	0	0	0	0
Immature embryos (EB)	50~75	50	8	65	22.0	25.0	84.6	26.0	37.5	46.2	12.0	12.5	38.5
Immature embryos (EC)	< 50	17	4	200	0	0	54.5	58.9	50.0	57.5	0	0	12.5

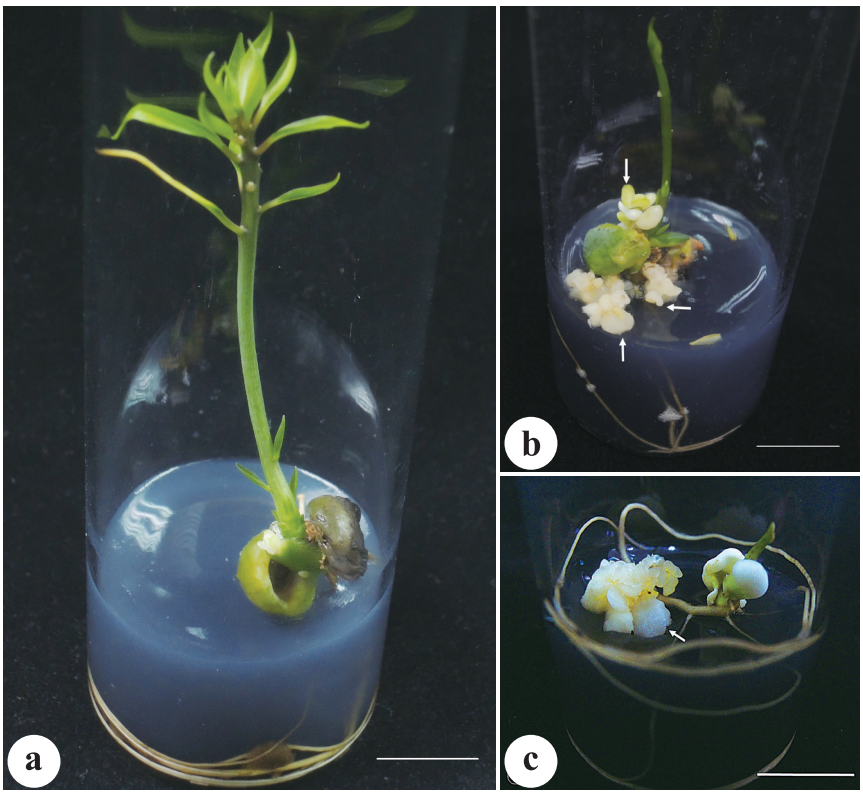

Fig. 3. Embryo culture on WPM + 30 g/L sucrose + 1.5 g/L activated charcoal. a, Embryo germination; b and c, embryo germination and somatic embryogenesis on the cotyledon (b) or on the root (c). (bar = 1 cm).

significant difference among fruits from the different areas. The embryogenesis frequency was 26~46.2% for immature embryos at the EB stage, and 50~58.9% for those at the EC stage. Explants from fruits of either the EB or EC stage at Guanwu had higher rates of germination plus somatic embryogenesis than those from the other areas.

In our previous studies (Chang et al. 2002, 2010), we stated that the cotyledon volume within a seed was closely related to the ability of a seed to germinate. In this study, we also found that only those seeds with a cotyledon occupying > 75% in volume (i.e., at the EA stage) could completely germinate. Inducing germination through culture from embryos at an early developmental stage (EC) was not feasible, although seeds from Guanwu at the EC stage had 54.5% germination in this study. Various factors, such as heredity, provenance, and climate, might affect the components of the embryo or endosperm and in turn have contributed to differentiation at Guanwu.

Effects of basal culture medium and plant growth regulators (PGRs) on embryo culture

Immature embryos dissected from fruits collected at Wulai in 2010 and 2012 were cultured on 11 different media for 2 mo. Both the medium composition and embryo developmental stage influenced embryo morphogenesis (Table 3). Nearly mature fruits

Table 3. Effect of basal medium plus 30 g/L sucrose, 150 ml/L coconut milk, 0.5 g/L glutamine, and plant growth regulators on callus induction and somatic embryogenesis from immature embryo culture of *Cinnamomum kanehirae*. Seeds were collected from Wulai

Basal medium	Plant growth regulators (mg/L)			Percentage of callus induction (%)				Percentage of somatic embryogenesis (%)[1)]			
				2012	2012	2012	2010	2012	2012	2012	2010
	NAA	Kinetin	BA	EA[2)]	EB[2)]	EC[2)]	EB[2)]	EA[2)]	EB[2)]	EC[2)]	EB[2)]
WPM	0	0		0	0.0	0.0	0.0	0	41.7	41.7	33.3
WPM	3	0.5		0	75.0	83.3	75.0	0	0.0	0.0	8.3
WPM	3	1.0		0	58.3	83.3	50.0	0	16.7	8.3	16.7
WPM	3		0.5	0	66.7	66.7	66.7	0	8.3	8.3	8.3
WPM	3		1.0	0	41.7	33.3	50.0	0	0.0	8.3	0.0
WPM	1	0.5		0	41.7	41.7	41.7	0	25.0	25.0	16.7
WPM	1		0.5	0	41.7	50.0	41.7	0	0.0	8.3	8.3
1/2 MS	3	0.5		0	75.0	83.3	83.3	0	8.3	8.3	8.3
1/2 MS	3	1.0		0	75.0	75.0	83.3	0	16.7	16.7	8.3
B_5	3	0.5		0	50.0	41.7	-	0	0.0	0.0	-
B_5	3		0.5	0	50.0	33.3	-	0	0.0	0.0	-

[1)] Means of 12 replications taken after 2 mo of culturing.

[2)] Cotyledon volume with a seed was EA: > 75%, EB: 50~75%, EC: < 50%.

(i.e., at the EA stage) had higher germination rates when cultured on PGR-free WA medium (100%, Table 2) than on WM medium (> 60%, data not shown). No morphogenesis could be induced with PGR supplementation, and explants later turned brown. Embryos at the EB and EC stages began expansion after being cultured for 1~2 mo, followed by callus formation, somatic embryo differentiation, or turning brown and dying. Except on PGR-free media, callus induction rates were 33.3~83.3%, with the highest on WPM and 1/2 MS supplemented with 3 mg/L NAA and 0.5~1 mg/L kinetin at the EC stage.

Most somatic embryos were induced after embryo explant browning. There were 2 types of response: somatic embryogenesis from calli which was initiated from cotyledons (Fig. 4a) and somatic embryogenesis directly from the cotyledon (Fig. 4b). Results at the EB and EC stages were similar. The highest frequency of somatic embryo regeneration (33.3~41.7%) was obtained with WPM medium containing no hormones, followed by WPM media with 1 mg/L NAA and 0.5 mg/L kinetin.

WPM and 1/2 MS were more suitable basal media than B_5 for callus induction and somatic embryogenesis from immature embryo culture. In other words, low-salt medium is suggested for *C. kanehirae* embryo culture. Chen and Chang (2009) also reported that embryogenic calli of *C. kanehirae* were induced from young leaves on 1/2 MS media containing 1 mg/L BA and 0.5 mg/L NAA.

Secondary somatic embryogenesis and multiplication of calli

There are 2 types of callus induced from *C. kanehirae* immature embryos: friable calli (grayish, soft, and watery, Fig. 4c) and nodular calli (whitish-yellow, Fig. 4d). Results of somatic embryos arising from the 2 types of callus on various media are presented in Table

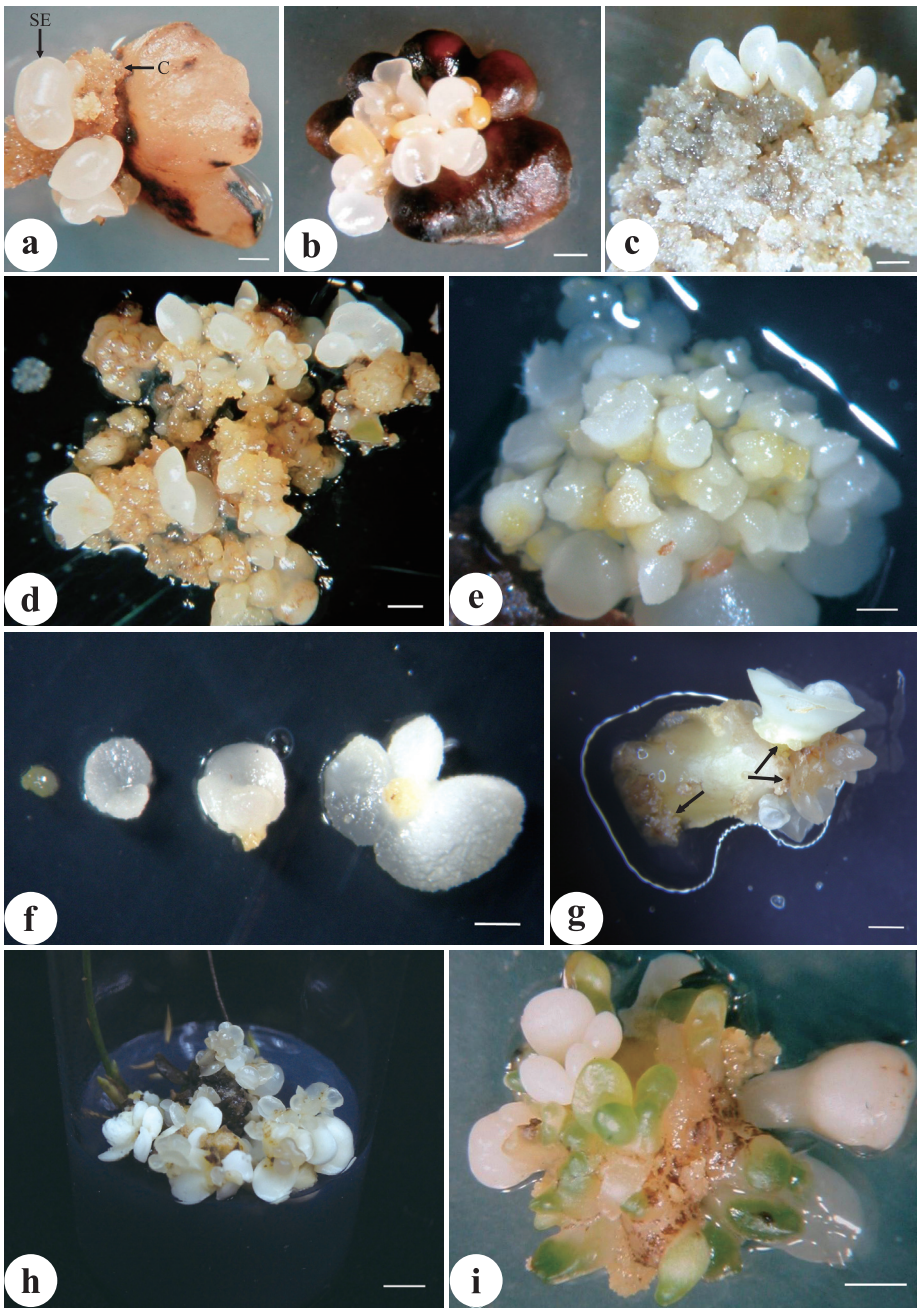

Fig. 4. Different types of somatic embryogenesis of *Cinnamomum kanehirae*. a, Somatic embryogenesis (SE) from a cotyledon callus (C); b, somatic embryogenesis directly from a cotyledon; c, somatic embryogenesis from a friable callus; d, somatic embryogenesis from a nodular callus; e, numerous single somatic embryos from a nodular callus; f, different stages of somatic embryogenesis; g, secondary somatic embryos (arrow) formed on primary somatic embryos; h, transparent and white cotyledonary-stage somatic embryos; i, somatic embyos which turned green before germination. Bar = 0.2 cm.

4. Nodular calli had a better somatic embryo regeneration capacity, especially on WM medium containing 0~1 mg/L NAA and 0~0.5 mg/L kinetin than friable calli. In contrast, a high concentration of NAA promoted the development of somatic embryos from friable calli, such as medium with 4 mg/L NAA and 0.5 mg/L kinetin. After 40 d of culture, over 20 cotyledonary somatic embryos had formed from 1-g nodular calli on average, which were 10.3 embryos more than from friable calli. If nodular calli continued to develop on WM medium containing 1 mg/L NAA, 0.5 mg/L kinetin, and 1.5 g/L AC for 60 d, more than 50 somatic embryos might form (Fig. 4e).

Generally, development of somatic embryos appeared to progress through globular-, heart-, and torpedo-shaped to the cotyledon stage, followed by embryo maturation and the germination process. All developmental stages were observed during somatic embryogenesis of *C. kanehirae*, and embryos at different stages might simultaneously be observed on the same medium (Fig. 4f).

As to proliferation, secondary embryos formed from the meristem of primary embryos or cotyledons derived from somatic embryos at the cotyledonary stage (Table 4). New secondary somatic embryos were whitish and transparent and later became milky-white in color (Fig. 4g, h). The fresh weight was measured to assess the somatic embryogenesis rate, since embryos simultaneously existed at multiple stages. Most secondary embryos were observed on WM medium supplemented with 1 g/L AC, 3 mg/L NAA, and 0.5~1 mg/L kinetin. After 40 d of culture, somatic embryos had increased from 1 g to 2.3~2.5 g. Although somatic embryos cultured on medium with 4 mg/L NAA weighed more, part of the weight was from white calli which had no differentiation ability.

Beneficial effects of AC on somatic embryogenesis were reported (Perera et al.

Table 4. Effects of different combinations of plant growth regulators with/without activated charcoal (AC) on the increase in fresh weight (FW) and numbers of cotyledonary somatic embryos derived from different calli and the CSE multiplication of *Cinnamomum kanehirae*

Plant growth regulators (mg/L)		AC (g/L)	Callus growth and CSE induction				Multiplication of CSE	
			Friable calli		Nodular calli			
NAA	kinetin		FW (g)[1)]	No. of CSEs[2)]	FW (g)	No. of CSEs	FW (g)	Callusing[4)]
0	0	0	0.9d[3)]	0e	1.1e	7.7c	1.3d	-
3	0.5	0	3.0a	0.7de	3.4a	6.3c	2.1c	++
4	0.5	0	3.5a	2.3d	3.3a	5.7c	2.7b	+++
0	0	1.5	0.9d	1.7d	1.6d	> 20a	2.0c	-
1	0.5	1.5	1.8c	6.7b	1.9c	> 20a	1.9c	-
3	0.5	1.5	1.7c	5.3b	1.9c	15.3b	2.3bc	-
3	1	1.5	2.0bc	4.0bc	2.0c	17b	2.5bc	-
4	0.5	1.5	2.3b	9.7a	2.5b	6.3c	3.0a	++
4	1	1.5	2.6b	4.3bc	2.5b	8.3c	3.1a	+++

[1)] FW, Fresh weight measured after 40 d of culture; initial FW = 1 g.

[2)] CSEs, somatic embryos with a cotyledon.

[3)] Means in a column followed by the same letter do not significantly differ ($p > 0.05$) according to Duncan's test.

[4)] -, +, ++, +++, nil, small, medium, and large calli, respectively.

2007). Similar results were obtained in the present study. AC improved the somatic embryogenesis frequency from immature embryo culture and the production of secondary somatic embryogenesis of *C. kanehirae*. However, Shi et al. (2009) reported that AC, although improving the vigor of somatic embryo growth, had no significant effect on somatic embryo formation in *C. camphora*.

Somatic embryo germination and plantlet transplantation

Results of the germination test of embryos with white cotyledons cultured on WA medium are shown in Table 5. After about 10 d of culture of somatic embryos, white cotyledons had turned green (Fig. 4i) before germination (Fig. 5a). Not all germinating somatic embryos had converted into plantlets with both functioning roots and shoots. Some turned brown and died, while some repeatedly produced calli. The best-suited medium for somatic embryo germination and plantlet growth was WPM containing AC and ABA, on which respective rates of germination and normal plantlet development were 58.9 and 50%. On WPM medium containing only AC or ABA, germination rates were 36.7~37.8%, and normal plantlet development rates were 23.3~25.6%.

Table 5. Effects of activated charcoal (AC) and abscisic acid (ABA) on germination and normal plant development of *Cinnamomum kanehirae* somatic embryos cultured on WPM medium for 40 d (germination) and 50 d (plant development)

AC (g/L)	ABA (mg/L)	Germination[1)] (%)	Normal plantlets (%)
1.5	0	37.8b[2)]	25.6b
0	26.4	36.7b	23.3b
1.5	26.4	58.9a	50.0a

[1)] Germination was defined as at least 1 pair of leaves sprouting out and root elongation of more than 0.3 cm.

[2)] Means in a column followed by the same letter do not significantly differ ($p > 0.05$) according to Duncan's test.

Somatic embryogenesis has been induced successfully; in only a very few species of the family Lauraceae. Except in *C. pauciflorum* (with an 82% germination rate and a 63% normal plantlet development; Kong et al. 2009), all germination rates in other species were < 50%, such as 0% in *Laurus nobilis* (Canhoto et al. 1999), 0~5% in avocado (*Persea americana*) (Wijaksono and Litz 1999), and 31.4% in *C. kanehirae* (Chen and Chang 2009). Germination of *C. camphora* was problematic (Cheng and Ma 1990), and the germination rate was only 17.5% (Du and Bao 2005). Shi et al. (2010) reported that 44.2~50.4% of somatic embryos were able to germinate with normal shoots but a poor root system.

The presence of 0.5 mg/L ABA could improve somatic embryo germination of *C. camphora* (Shi et al. 2010). When supplementing GA (gibberellin) on the medium, the germination frequency of somatic embryos from *C. kanehirae* young leaves improved from 18.5 to 31.4% (Chen and Chang 2009). We also found that supplementing AC and ABA on the medium could increase somatic embryo germination and plantlet development.

After being transferred to WA medium, germinated embryogenic plantlets grew well. Some secondary somatic embryos may have developed on the base of the plantlet stem (Fig. 5b). Since there were no detectable vascular connections between developing somatic embryos and mother explants, plantlet growth and transplantion were not affected. This charateristic of somatic embryo devel-

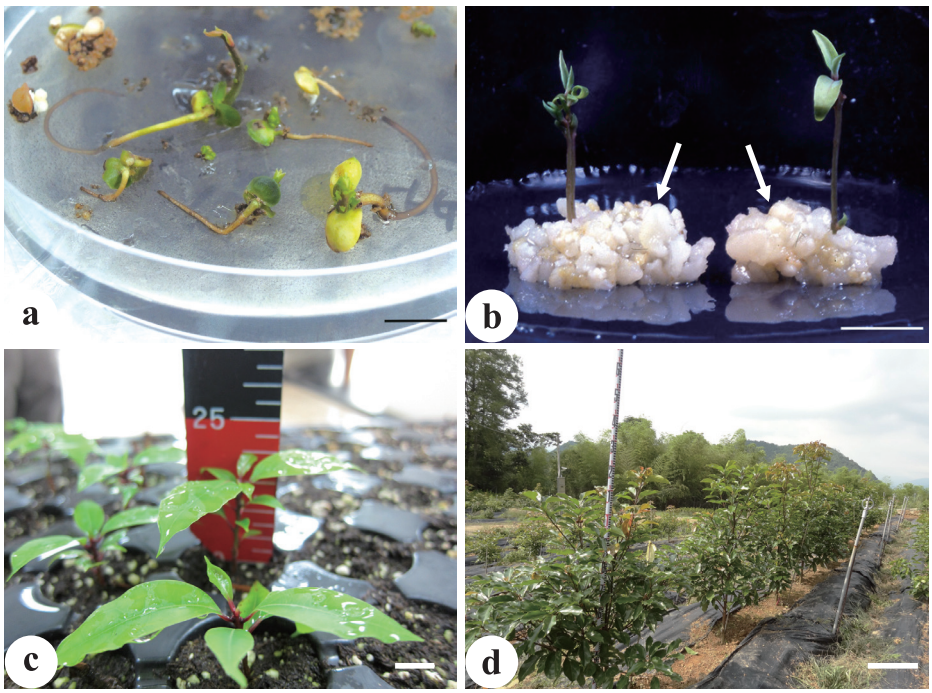

Fig. 5. Plant regeneration from somatic embryos of *Cinnamomum kanehirae*. a, Plantlets from single embryos with ture leaves and well-developed roots; b, some secondary somatic embryos (arrows) developed on the base of the stem; c, regenerated plantlets transplanted into pots grew normally in the greenhouse; d, 1-yr-old plantlets grew uniformly at Yuchi Township, Nantou County. Scales in a~c = 1 cm, d = 20 cm.

opment was also observed in *C. kanehirae* young leaves (Chen and Chang 2009). After being transferred from a culture tube to a growth chamber and then to a greenhouse for acclimation (Fig. 5c), the survival rate of small plantlets was > 95% when finally moved to shaded rooms for 1 mo. After 6 mo, plantlets with an average height of 17±3.1 cm were transplanted to Yuchi and Yuanshan. The average height was 133±31.5 cm after transplanting to Yuchi for 1 yr (Fig. 5d), and was 212±42.9 cm after transplanting to Yuanshan for 17 mo. Plantlets grew vigorously.

CONCLUSIONS

The study has established a somatic embryo regeneration system for *C. kanehira*e by immature embryo culture. Since fruit size was not related to embryo development, it could not be an indicator of embryo development. Only seeds with a white cotyledon occupying < 75% in volume could induce somatic embryogenesis. There are 2 types of somatic embryogenesis: direct and indirect. In direct somatic embryogenesis, the somatic embryo occurs directly from initial explants. In indirect somatic embryogenesis, the embryo is formed from the production of either an intervening callus, cotyledon or radical of a germinated embryogenic plantlet. Secondary somatic embryogenesis might develope on the base of the plantlet stem. When supplementing AC and ABA on the medium, somatic embryos at the white cotyledonary stage turned green, germinated, and developed into

plantlets which then grew fast and vigorously after being transplanted from a greenhouse to the experimental site.

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