Research paper

Plant Regeneration through Somatic Embryogenesis from Young Leaves of *Cinnamomum kanehirae* Hayata

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【Summary】

Embryogenic calli of *Cinnamomum kanehirae* Hayata, are yellowish-white, compact, and granular, which were induced from young leaves, and were maintained on half-strength macro- and micro-elements of MS media containing 1 mg L^{-1} 6-Benzyl aminopurine (BA) plus 0.5 mg L^{-1} 1-naphthalene acetic acid (NAA). Embryogenic calli were cultured at 5℃ for 14 d on woody plant medium (WPM) with no plant growth regulators, and then somatic embryos appeared on the surface of these calli after they were transferred to 25℃ for another 6 wk. Of somatic embryos, 31.4% germinated that were cultured on WPM supplemented with 0.2 mg L^{-1} GA_3 and 150 ml L^{-1} coconut milk in 2 mo and then transferred to medium without plant growth regulators for another 10 mo with 2-mo intervals of subculturing. Regenerating plantlets of 5 cm in height were acclimated and transplanted to a greenhouse with an 83% survival rate. Subsequently, these plantlets were transferred to the Botanic Garden of the National Museum of Natural Science for 3 yr, after which the diameter at breast height was 6 cm, and the height was 4 m.

Key words: somatic embryo, *Cinnamomum kanehirae* Hayata, regeneration, callus.

Chen YC, Chang C. 2009. Plant regeneration through somatic embryogenesis from young leaves of *Cinnamomum kanehirae* Hayata. Taiwan J For Sci 24(2):117-25.

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Received August 2008, Accepted March 2009. 2008年8月送審 2009年3月通過。

研究報告

牛樟幼葉培養體胚發生與植株再生

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摘 要

從牛樟幼葉誘導生成黃白色、硬實粒狀的胚性癒傷組織，穩定地繼代培養於含1 mg L^{-1} BA及0.5 mg L^{-1} NAA之基礎培養基(CI medium)。牛樟胚性癒傷組織移入未添加生長調節劑之WPM培養基並置於5℃環境下培養14天，再移到25℃的環境下培養6週，可於癒傷組織表面觀察到體胚形成。牛樟各種型態之體胚同時移入含0.2 mg L^{-1} GA_3 and及150 ml L^{-1}椰子水的WPM培養基2個月後，再移入不含生長調節劑之WPM培養基10個月，每2個月繼代一次，有31.4%體胚發芽長出胚根及胚芽而再生成小植株，其中僅正常型態體胚能順利發芽，畸型體胚則逐漸褐化。株高5公分的再生株經馴化及移植後具有83% (33/40)的存活比率，再生株於國立自然科學博物館植物園展覽區栽培3年，胸徑達6 cm，株高4 m。

關鍵詞：體胚、牛樟、再生、癒傷組織。

陳盈君、張正。2009。牛樟幼葉培養體胚發生與植株再生。台灣林業科學24(2):117-25。

INTRODUCTION

Cinnamomum kanehirae Hayata was once a dominant broadleaf tree species, endemic at elevations of 200~2000 m in the mountains of Taiwan (Liao 1996). It was an important source of essential oils, terpineol, perfume, medicines, and cabinet wood. However, this species has become very rare and endangered by overcutting (Chang et al. 2002). At present, the conventional methods of propagation by cutting, seeds, and layering are very slow and do not guarantee homogeneity. The small seed yield from wild trees is associated with difficulties of pollination and damage consumption by birds. Although vegetative propagation was established from cuttings, the percentage of root formation was insufficient. The percentages of root formation of cuttings of this species collected from 14-yr-old trees were only 20~26%, even though they were treated with 2000~4000 ppm indolebutyric acid (IBA) (Kao and Huang 1997).

Plant tissue culture represents an ideal and reliable way to proliferate this rare species. In spite of micropropagation of *Cinnamomum* species having previously been published (Govinden-Soulange et al. 2007), information on the tissue culture of *C. kanehirae* is scanty. Chang et al. (2002) demonstrated the embryo culture of this species and revealed that tissue culture plants had better tree forms, with more-erect stems and more fork-stems than rooted cuttings of clones. Tsai (2004) investigated callus induction from spring buds and stem segments on MS basal medium supplemented with 3 mg L^{-1} 6-Benzyl aminopurine (BA) and 4 mg L^{-1} 2,4-D in the dark for 3 wk. The calli were maintained and proliferated but no plantlets regenerated during the culture process.

This study proposes a somatic embryogenesis system by callus induction, subculture, and regeneration. This system is promising for use in investigating embryo induction and development of *C. kanehirae* for propagation.

MATERIALS AND METHODS

Callus induction and maintenance

Seeds of *C. kanehirae* collected from a mother tree in the Botanic Garden (National Museum of Natural Science, Taichung, Taiwan) were packaged in moist sphagnum moss at 5℃ for 3 mo of cold stratification. Seeds which germinated in a mixture of peatmoss, perlite, and vermiculite (1:1:1 v/v/v) and plants with heights of 5~15 cm were used as the explants. The hypocotyls, petioles, and leaf veins were surface-sterilized with 2% NaOCl supplemented by 1 drop of Tween 20 for 15 min and then were rinsed with sterilized water 3 times. These explants were sliced into lengths of 0.5 cm, cut cross-wise to make many wounds, and placed horizontally on the surface of callus-induction (CI) medium. The CI medium contained half-strength salts of MS (Murashige and Skoog 1962) medium with 0.5 mg L^{-1} nicotinic acid, 0.5 mg L^{-1} pyridoxine HCl, 0.1 mg L^{-1} thiamine HCl, 100 mg L^{-1} myo-inositol, 170 mg L^{-1} NaH_2PO_4, 20 g L^{-1} sucrose, 0.5 mg L^{-1} NAA (1-naphthalene acetic acid), and 2.8 g L^{-1} gelrite. All media were adjusted to a pH of 5.2 prior to autoclaving (15 min at 121℃; 1 kg cm^{-2}). BA (0, 2.5, and 5 mg L^{-1}) was added to the CI medium to investigate the effects of callus induction. Cultures were maintained in the dark at 25±1℃ for 2 mo. Subsequently, 0.5 g of calli was subcultured with serial combinations of BA and NAA (0, 1.0, 2.5, and 5.0 mg L^{-1} BA and 0, 0.5, and 1.0 mg L^{-1} NAA), and the cultures were stored in the dark. The increase in fresh weight and morphogenesis of calli were recorded after 1 mo of culturing.

Induction of somatic embryos

The leaf-derived calli cultured on CI basal medium supplemented with 1.0 mg L^{-1} BA were transferred to the same liquid medium with the gelrite removed. Liquid cultures were maintained on a rotary shaker at 125 rpm with a light intensity of 100 lux for a 16-h photoperiod. After 3 wk of culture, fractions sieved through a 40-mesh filter (cell dissociation sieve-tissue grinder kit, Sigma CD-1, St. Louis, MO, USA) were transferred to the same liquid medium for another 3 wk. Masses that could not pass through the 40-mesh were transferred to semisolid woody plant medium (WPM) (Lloyd et al. 1981) and incubated at 5℃ in darkness for 14 d. Then, the cultures were moved to artificial light conditions with a 16-h photoperiod of 2000 lux with a light source of daylight fluorescent tubes (FL-30D/29, 40 W, China Electric, Taipei, Taiwan) at 25±1℃ by subculturing at 2-mo intervals in WPM. The total number of somatic embryos was recorded during the period.

Germination of somatic embryos

In total, 375 somatic embryos in different stages of development were cultured on WPM with or without 0.2 mg L^{-1} GA_3 and 150 ml L^{-1} coconut milk for 2 mo. Those somatic embryos which did not germinate were transferred to new WPM without GA_3 or coconut milk every 2 mo. The definition of germination was the radicle protruding and plumule growth. When small plantlets reached 0.5~1.0 cm in height, they were separated with forceps and transferred to WPM without plant growth regulators. The cultures were exposed to 4000 lux of artificial light with a light/dark cycle of 16/8 h at 25±1℃. The regenerants were cultured on medium until they were moved to a greenhouse.

Transplantation of plantlets

Well-developed in vitro plantlets at 5 cm of height were moved to a greenhouse for acclimation for 7 d, then the roots were carefully

washed. The plantlets were then transferred to a dibble tube containing a peatmoss, perlite, and vermiculite (1:1:1 v/v/v) mixture for 1 wk in a growth chamber. Healthy plantlets were moved into a tunnel, and the percentage survival rate of the regenerants was recorded.

Histological and microscopic studies

Representative samples were removed from cultures and put in a formalin, propionic acid, and 70% ethanol (FPA; 1:1:18) solution for at least 4 h at room temperature. Fixed samples were dehydrated in a serial ethanol solution and then infiltrated with paraffin. Serial tissue sections were obtained using a rotary microtome (Reichert Histostat 820, AO Reichert Scientific, Buffalo, NY, USA) and the sections were placed on slides and then oven-dried at approximately 52℃, arranged on slide racks, and treated with a schedule of de-waxing, staining, and mounting. The prepared slides were observed with a microscope (AXIOSKOP 2, Zeiss, Germany).

RESULTS

Callus induction and subculture

The percentage of calli induced was 100% with all 3 kinds of explants (Table 1). BA (1~5 mg L^{-1}) was effective in inducing calli, but the morphology of the calli depended on the BA concentration and the explants. Petioles and hypotocyls of explants cultured on BA at a lower concentration formed yellowish-white compact calli, while brownish and soft calli formed with 5 mg L^{-1} BA. Leaf veins explants produced yellowish-white, compact and pink, soft calli in all BA-containing media. The same results of callus induction were observed in leaves derived from mature trees (data not shown). Some granules arose from the surface of leaf veins on medium containing 1~2.5 mg L^{-1} BA. Adventitious roots formed in primary cultures, and there was no root formation after 1 or 2 generations of subculture.

Leaf-derived calli were maintained by serial subcultures on half-strength MS medium supplemented with BA, NAA, or the 2 combined. The fresh weights of calli were significantly increased in medium with 1 mg L^{-1} BA and 1 mg L^{-1} NAA during 1 mo of culture (Table 2). However, most embryogenic calli were observed on medium supplemented with 1 mg L^{-1} BA and 0.5 mg L^{-1} NAA. Several kinds of calli, including yellowish-brown soft calli, yellowish-white soft or compact calli, and pink soft calli, were observed. Embryogenic calli were yellowish-white, compact, and granular and were stably maintained

Table 1. Percentage of callus induction based on 3 concentrations of BA combined with 0.5 mg L^{-1} NAA

BA[1] (mg L^{-1})	Percentage with calli (%)[2]			Percentage with roots (%)		
	Petiole	Leaf vein	Hypocotyl	Petiole	Leaf vein	Hypocotyl
1.0	100	100	100	57.1	62.5	36.3
2.5	100	100	100	0	75	9.1
5.0	100	100	100	0	0	9.1

[1] The basal medium contained half-strength Murashige and Skoog (1962) salts, supplemented with (mg L^{-1}): myo-inositol (100), nicotinic acid (0.5), thiamine (0.1), pyridoxine HCl (0.5), glycine (2.0), NaH_2PO_4 (170), sucrose (20,000), gerlite (2800), and NAA (0.5), and the pH was 5.2. All cultures were kept in the dark at 25±1℃.

[2] Means of 7~16 replications taken after 1 mo of culturing.

Table 2. Effects of BA and NAA on leaf-derived callus proliferation and morphogenesis

BA (mg L^{-1})[1)]	NAA (mg L^{-1})	Callus proliferation[2)]	Morphogenesis[3)]
0.0	0.0	2.02 c	YC, BC, R
1.0	0.0	3.96 bc	YC, BC, R
2.5	0.0	2.94 bc	YC, BC
5.0	0.0	3.30 bc	YC, BC
0.0	0.5	5.73 abc	BC, R
1.0	0.5	6.81 abc	YWS, EC, PC, R
2.5	0.5	6.35 abc	YWS, PC
5.0	0.5	7.64 abc	YWS
0.0	1.0	7.59 abc	YWS, R
1.0	1.0	11.69 a	YWS, PC
2.5	1.0	7.61 abc	YWS, R
5.0	1.0	8.64 ab	YWS, R

[1)] The basal medium contained half-strength Murashige and Skoog (1962) salts, supplemented with (mg L^{-1}): myo-inositol (100), nicotinic acid (0.5), thiamine-HCl (0.1), pyridoxine HCl (0.5), glycine (2.0), NaH_2PO_4 (170), sucrose (20,000), and gerlite (2800), the pH was 5.2, and cultures were kept in the dark at 25±1℃.

[2)] Approximately 0.5 g of callus proliferation was measured as the final fresh weight divided by the initial fresh weight. Means of 4 replicates with the same letters do not significantly differ at $p < 0.05$.

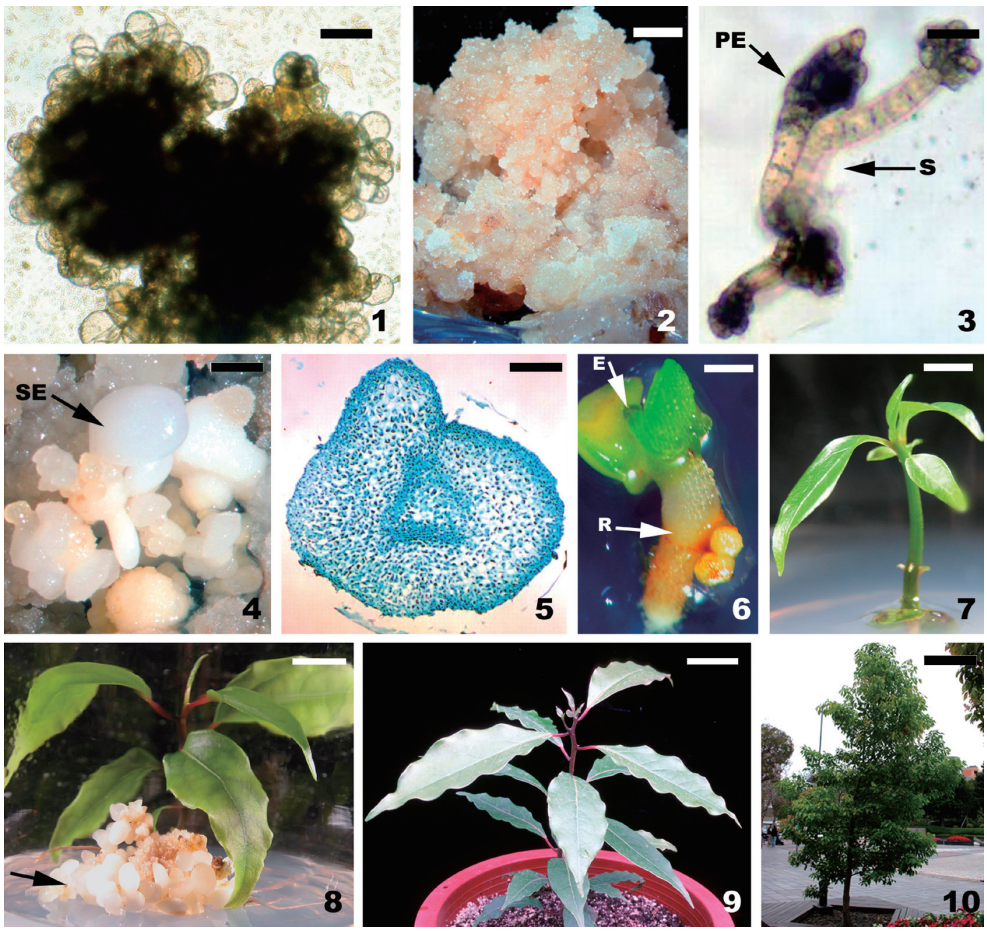
[3)] YC, soft yellowish calli; BC, soft brown calli; R, root; YWS, soft yellowish and white calli; EC, embryogenic calli, yellow-white, compact, and granular calli; PC, soft pink calli.

on medium containing 1 mg L^{-1} BA plus 0.5 mg L^{-1} NAA.

Induction and germination of somatic embryos

Cell aggregates (Fig. 1) were sieved out on a 40-mesh filter, cultured on WPM semi-solid medium, transferred to a refrigerator at 5℃ for 2 wk, and then cultured at 25℃ under 2000 lux of artificial light for another 6 wk. The cell aggregates were then subcultured on new semi-solid WPM for 8 wk, and embryogenic calli formed (Fig. 2). Pre-embryos with suspensors which a group of cell developed from the fertilized ovum (Fig. 3) were derived from them and white globular somatic embryos appeared after 6 wk of culturing (Fig. 4). Histological and morphological observations indicated that the developing somatic embryos exhibited no detectable vascular connections with the mother explant and contained a vascular system (Fig. 5), which is one of the most important characteristics of somatic embryo development.

Somatic embryos began to germinate earlier on WPM compared with those cultured on the WPM supplemented with 0.2 mg L^{-1} GA_3 and 150 ml L^{-1} coconut milk, and slowly turned brown. The germination percentage was very low during the first 2 mo. However, these somatic embryos germinated stage by stage after they were transferred to WPM without plant growth regulators. The percentage of germination increased to 11.7% (16/137) and 31.4% (43/137) at 6 and 12 mo, respectively. Compared to 2 mo of GA_3 treatment, there was no obvious difference in the somatic embryo germination rate in the first period, but it continually rose for another 10 mo on WPM with subculturing at 2-mo



Figs. 1~10. Somatic embryogenesis and plant regeneration of *Cinnamomum kanehirae*.
1. Embryogenic cell aggregates with compact cytoplasm (bar = 52 µm).
2. Embryogenic calli were yellowish-white, compact, and granular and were stably maintained on medium containing 1 mg L^{-1} BA and 0.5 mg L^{-1} NAA (bar = 1.3 mm).
3. Pre-embryo (PE) with suspensor (S) observed within the embryogenic calli (bar = 30 µm).
4. White globular somatic embryos (SE→) which appeared on the surface of calli cultured on WPM at 5℃ for 14 d and then transferred to 25℃ for 6 wk (bar = 0.49 mm).
5. Histological section showing the vascular system of a somatic embryo (bar = 100 µm).
6. Somatic embryo with 2 cotyledons which have turned green, an elongated epicotyl (E), and a radical root (R) (bar = 0.3 mm).
7. True leaves expanded and a whole plantlet was established (bar = 0.8 cm).
8. Plantlet derived from a somatic embryo grew well, and some secondary somatic embryos (→) had spontaneously developed on the surface of the calli which formed on the base of the plantlet (bar = 12 mm).
9. Plantlet transferred to a greenhouse and was 20.4 cm height after 5 mo of culturing (bar = 4.9 cm).
10. A regenerant 3 m in height and 6 cm in DBH after of 3 yr in the Botanic Garden (bar = 9 cm).

Table 3. Percentage of somatic embryos germinating on the 2 different media

Medium	Percent (%) somatic embryos germinating[3)]		
	1 mo	6 mo	12 mo
WPM[1)]	2 (5/238)[a]	10.9 (26/238)[a]	18.5 (44/238)[a]
WPMCG1[2)]	0 (0/137)[a]	11.7 (16/137)[a]	31.4 (43/137)[b]

[1)] Different stages of somatic embryos were cultured on WPM.

[2)] WPM basal media supplemented with 0.2 mg L^{-1} GA_3 and 150 ml L^{-1} coconut milk. Somatic embryos were cultured on GA_3-supplemented medium for 2 mo and then transferred to WPM basal medium.

[3)] The formula is presented below, and the data were compared with the standard number (1.96). The amount > 1.96 shows a significant difference between the treatments.

$$|p_1 - p_2| \cdot \sqrt{p_1 q_1 \cdot n_1^{-1} + p_2 q_2 \cdot n_2^{-1}}^{-1} \leq 1.96$$

intervals, and the total germination number of somatic embryos with GA_3 process was greater than those cultured on WPM without GA_3 treatment (Table 3). There were some abnormal somatic embryos, such as multi-cotyledons or single cotyledons, and they slowly turned brown and did not germinate. Normal somatic embryos had a round shape, were white and had a solid structure; they germinated with 2 green cotyledons and a radicle and then the leaves expanded (Figs. 6, 7). Plantlets derived from somatic embryos grew well on the new WPM, and some secondary somatic embryos spontaneously developed on the surface of calli formed on the base of the plantlets (Fig. 8). These secondary somatic embryos could also develop into complete plantlets after they were transferred to WPM. Five-centimeter plantlets were acclimated in the growth chamber, and after being transferred to the tunnel, whole plants were established (Fig. 9). The survival rate was 83% (33/40), and the tallest plant was 4 m in height and 6 cm of diameter at breast height (DBH) after 3 yr in the field (Fig. 10).

DISCUSSION

In a review of studies of trees of the Lauraceae, primary explants for somatic embryogenesis focused on zygotic embryos of *Sassafras ramdaoemse* (Hay.) Rehd (Chen and Wang 1985); *C. camphora* (L.) Presl (Cheng and Ma 1990, Du and Bao 2005); *Persea americana* (Mooney and van 1987, Pliego-Alfrao and Murashige 1988, Wijaksono and Litz 1999a, b); *Laurus nobilis* L. (Canhoto et al. 1999); and *Ocotea odorifera* Me (Catarina et al. 2001) and somatic embryos of *O. catharinensis* Mez. (Catarina et al. 2003). In this work, the indirect somatic embryogenesis of *C. kanehirae* through the calli derived from young leaf explants might be a good source of micropropagation for this species.

Germination of somatic embryos of the Lauraceae is difficult, and percentages are very low, at approximately 0% for *L. nobilis* (Canhoto et al. 1999), 0~5% for *P. americana* (Wijaksono and Litz 1999b), and very low for *C. camphora* (Cheng and Ma 1990) and *P. americana* (Mooney and van 1987). Recently, somatic embryogenesis and plant regeneration from protoplast of *Cinnamomum* were reported, and the efficiency could reach 17.5%, which is quite high compared to other plants. In this work, somatic embryos treated with GA_3 for 2 mo germinated after they were moved to WPM with no plant growth regulators. After 12 mo of culturing, the highest percentage was 31.4% and was

significantly higher than untreated ones. The results were also superior to other previous studies, but the germination of somatic embryos was not synchronized. Although the germination of somatic embryos was not regular, we obtained radical root seedlings with a high transplantation rate (83%) in the greenhouse, and the growth capability was strong.

Secondary somatic embryos of the Lauraceae were differentiated on the following media: MS basal medium, MS plus 1.0 mg L^{-1} BA and 0.1 mg L^{-1} NAA, MS containing many combinations of GA_3 and BA (or kinetin), and medium plus 2,4-D. The formation of secondary somatic embryos can proceed by proliferation of calli in former somatic embryos, and directly on the cotyledon surface of previously formed somatic embryos (Canhoto et al. 1999). Somatic embryos of avocado that developed directly from planted zygotic embryos grew larger, and formed a few secondary somatic embryos from the base of each one (Witjaksono and Litz 1999a). In the experiments performed herein, secondary somatic embryos developed from calli that had formed on the base of somatic embryo-derived plantlets. These secondary embryos were spontaneously produced on the WPM basal medium with no plant growth regulators, and could regenerate into new plantlets. Secondary embryogenesis represents an excellent system and has the potential for large-scale propagation, especially on medium free of plant growth regulators.

In conclusion, embryogenic calli derived from young leaves of *C. kanehirae* were induced on BA-supplemented medium. These calli were cultured on plant growth regulator-free WPM and then stored at 5℃ for 14 d. Somatic embryos formed on the surface of the calli and could then be regenerated into healthy plantlets on the same medium. Cold treatment and GA_3 supplementation had positive effects. Secondary somatic embryos formed into calli from the base of the regenerants. A somatic embryogenesis system for *C. kanehirae* was successfully established to produce this rare species in an efficient and economic manner.

ACKNOWLEDGEMENTS

The authors would like to thank the Council of Agriculture, Taipei, Taiwan, for financially supporting this research under contract no. 94 AS-5.1.4-FD-Z2. Mr. Chen Jien-Nan is appreciated for his assistance with the histological work.

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