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Micromorphology of Somatic Embryo Development of *Castanopsis cuspidata* (Thunb) var. *sieboldii* (Makino) Nakai

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Abstract

In vitro somatic embryogenesis of *Castanopsis cuspidata* var. *sieboldii* was achieved from immature embryo-derived callus and the morphological course of events during embryo development was evaluated by scanning electron microscopy technique (SEM). Embryogenic callus could be initiated from immature embryos and optimum induction of callus was observed on Murashige and Skoog (MS) medium supplemented with 10 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 1 μM benzylaminopurine (BAP). Somatic embryo development was occurred on half-strength basal MS medium supplemented with 2 μM BAP, 0.25 μM naphthaleneacetic acid (NAA). Somatic embryo maturation was accomplished using 1 μM zeatin, 0.25 μM indolebutyric acid (IBA). SEM observations indicated development of an extracellular material at an early stage of differentiation and a course of differentiation leading to development of somatic embryos.

Key words: *Castanopsis sieboldii*, Extra-cellular material, Somatic embryogenesis, Scanning electron microscopy.

Abbreviations

BAP, Benzylaminopurine; 2,4-D, 2,4-Dichlorophenoxyacetic acid; IBA, Indolebutyric acid; MS, Murashige and Skoog; NAA, Naphthaleneacetic acid; SEM, Scanning electron microscopy.

Introduction

Castanopsis cuspidata (Thunb) var. *sieboldii* (Makino) Nakai is an evergreen tree species grown in evergreen broad-leaved forests distributed from the middle of Honshu (Kanto) to Taiwan (Ohwi, 1975; Numata, 1990) Evergreen broad-leaved forests are important forest community peculiar to the warm-temperate and subtropical zone and are dominated by evergreen oaks of the genera *Quercus, Castanopsis* and *Lithocarpus* (Kira, 1991).

For effective utilization of forest resources, there is a demand to produce plus trees for charcoal and good quality timber. Trees with good growth rates, adaptability, stem form, wood quality and resistance to diseases are considered as plus trees. There are considerable obstacles to conventional breeding of this tree species, such as long reproductive cycles, intervals of seed years, problems with seed storage for extended periods and difficulties with vegetative propagation.

For this reason, *in vitro* propagation is one of the essential tools to develop for multiplication of trees with desirable characters. To our knowledge, there are no reports on *in vitro* propagation of *C. sieboldii*. A reliable plant regeneration system through somatic embryogenesis has recognized to be useful for micropropagation, artificial seeds and recombinant gene technology. Knowledge on micromorphological changes of callus is essential to understand organization of cells in callus at different developmental stages and to confirm differentiation pattern either somatic embryogenesis or organogenesis. SEM observation of the development of extracellular material on callus surface is also extremely useful to distinguish embryogenic callus, because extracellular material that appears at the early stages of development was reported to be important factor to regulate plant morphogenesis

(Samaj *et al.*, 1999).

Therefore, the present study was carried out to investigate suitable culture conditions for *in vitro* propagation of *C. sieboldii* through immature embryo culture and to evaluate the differentiation pathway by observing morphological course of events using scanning electron microscopy.

Materials and Methods

Plant material

Immature acorns of *C. sieboldii* were collected from open pollinated trees from different locations of northern forests in the Okinawa Island. Acorns were harvested at 2 weekly intervals during August, September 2000, 2001 and 2002 and were about 10 mm in width and 15 mm in length. Following their removal of the trees, fruits were bulked and cupule was removed before surface sterilization. Acorns were sterilized by washing with tap water with few drops of benzalkonium chloride for 10 minutes, followed by stirring in 70% ethanol for 5 minutes and in a solution of 3% free chlorine (commercial bleach) with two to three drops of Tween 20 (polyoxyethylenesorbitan monolaurate) for 15 minutes. Following surface sterilization, acorns were dissected; zygotic embryos were removed and cultured on callus initiation medium.

Media composition

Basal medium consisted of Murashige and Skoog (MS) mineral salts, vitamins and amino acids (Murashige and Skoog, 1962) supplemented with 3% sucrose and 0.9% agar. The zygotic embryos were initially cultured on callus initiation medium consisted of basal medium supplemented with various concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D; 0, 0.1, 1, 10, 30 μM) and benzylaminopurine (BAP; 0, 0.1, 1, 10 μM). The pH was adjusted to 5.8-6.0 with 0.1N NaOH.

Embryogenic calli induced were subcultured onto medium containing half-strength MS mineral salts, full strength vitamins, 100 μM Fe-Na-EDTA, 3 μM glutamine, 3% sucrose and 0.9% agar, and supplemented with combinations of BAP (2, 4, 8 μM) and naphthaleneacetic acid (NAA; 0.25 μM). Calli with developing somatic embryos were transferred to medium consisting of half-strength MS mineral salts, full strength vitamins, 100 μM Fe-Na-EDTA, 3 μM glutamine, 3% sucrose and 0.9% agar, and supplemented with 1 μM zeatin in combination with 0.25 μM indolebutyric acid (IBA). Shoots germinated from somatic embryos were transferred to medium that consisted of half-strength MS mineral salts, full strength vitamins supplemented with 150 μM Fe-Na-EDTA, 1 μM zeatin 3% sucrose and 0.9% agar.

Callus initiation experiments were conducted with 24 replicates per treatment and repeated 3 times in each year and experiments conducted for 3 years. Experiments on somatic embryogenesis were conducted with 20 replicates per treatment. ANOVA analysis was performed using SAS program (SAS Institute, Cary, NC, USA) using pooled data of each year. LSD (P=0.001) was used for multiple comparisons.

Culture conditions

Petri dishes of callus initiation cultures were incubated in the dark at 25℃. After transfer to the embryo development medium, petri dishes were placed under 16 hrs photoperiod of 6000-lux fluorescent light at 25℃. Shoots were transferred to culture tubes and maintained under16 hrs photoperiod of 6000-lux fluorescent light at 25℃.

Scanning electron microscopy

For scanning electron microscopy (SEM), callus pieces at various developmental stages were processed according to the procedure described by Bobak *et al.*, (1999). Samples were fixed in 2%(v/v) glutaraldehyde in 0.1 M potassium phosphate buffer for 3 hrs at room temperature and the calli were then rinsed with the same buffer and post-fixed with 1%(w/v) osmium tetroxide in the same buffer for 1 hr at 4℃. Following fixing, the samples were dehydrated in ascending concentrations of ethanol (viz., 30%, 50%, and 70%) for 10 min at 4℃ in each concentration and finally in 100% ethanol at room temperature for 10 min. The samples were stored in 100% acetone at room temperature overnight. Dehydrated callus samples were critical point dried in a critical point drier JCPD-3 (JEOL, Japan) using CO_2 as the transition fluid (Anderson, 1951). The samples were mounted on brass stubs with double adhesive carbon tape and gold coated in a Fine Coat-Ion Sputter JFC 1100 (JEOL, Japan) for 3 min. Micromorphology of the samples was observed under high vacuum conditions at 10-12 kV using a JSM-5600 LV scanning electron microscope (JEOL, Japan) equipped with a back-scattered electron detector.

Results and Discussion

Various 2,4-D concentrations (0.1, 1, 10 μM) in combination with various BAP concentrations (0.1, 1, 10 μM) were capable of promoting proliferation of calli, but the percentage of embryos that produced compact calli and size of the callus varied

Table 1 Percentage of embryos producing compact calli in different 2,4-D and BAP concentrations after 3 weeks of culture

BAP (μM)	2,4-D (μM)			
	0	0.1	1	10
0	0	20.8 ± 0.19	8.3 ± 0.14	16.7 ± 0.19
0.1	4.2 ± 0.07	29.2 ± 0.19	45.8 ± 0.26	81.9 ± 0.14
1	4.2 ± 0.07	54.2 ± 0.07	65.2 ± 0.14	91.7 ± 0.14
10	0	29.2 ± 0.26	41.7 ± 0.07	76.7 ± 0.10

Mean ± standard deviation of three repeated experiments with 24 replicates per treatment

among the different combinations of growth regulators (**Table 1**). Callus proliferation in a wide range of growth regulator concentrations may be due to the actively growing nature of the explant at the time of sampling.

At high concentrations of 2,4-D (30 μM), browning of the explants resulted in poor development of callus. The highest callus proliferation was observed in medium containing 10 μM 2,4-D with 0.1 or 1 μM BAP (significantly different from all treatments at $p<0.05$). The BAP concentration was not as critical as the 2,4-D concentration, since media containing 0.1, 1 or 10 μM BAP in combination with 10 μM 2,4-D all resulted in high callus develop-ment (not significantly different at $p<0.01$). Therefore, considering the above two factors, medium with 10 μM 2,4-D and 1 μM BAP was selected as the optimum medium for callus induction, because the percentage of embryos producing compact calli and the sizes of the calli were largest in that medium.

The immature embryos (about 1-2 mm in width and 4-5 mm in length) of *C. sieboldii* obtained from actively growing acorns developed into yellowish compact embryogenic callus 2-3 weeks after cul turing on MS medium containing 0.1, 1, 10 μM 2,4-D in combination with 0.1, 1, 10 μM BAP concentrations (**Fig. 1A**). On the other hand, white and friable calli were initiated from embryos obtained from fully-grown acorns (2-3 mm in width and 5-6 mm in length). These calli initiated after about 5 weeks from the seed coat of the embryo and could not be maintained by subculturing (**Fig. 1B**). Therefore, the stage of the embryo is a critical factor for embryogenic callus initiation. The immature embryos of actively growing acorns are capable of pro ducing embryogenic callus. In *C. sieboldii* flowering occurs in March or April and the acorns take about 18 months to mature. The acorns remain very small until August, grow into full size during August to September, and mature in October. Therefore the acorns are at the actively growing stage during the sampling time. Das *et al.,* (1997) also reported that the development of embryogenic callus of *Dalbergia sissoo* (Indian rose wood) was dependent on the age of the zygotic embryo explant.

The two types of callus showed distinct characteristic of cellular arrangements when observed under SEM. Yellowish compact calli consisted of small globular closely associated cells, while the white friable calli consisted of elongated and highly disassociated cells (**Fig. 2A, B**). Complimentary to these results, similar micromorphological observations of cellular arrangements of compact and friable calli of sugarcane were observed by Rodriguez *et al.,* (1995).

Embryogenic yellowish, compact calli were subcultured onto fresh media in order to stimulate embryo development. Mineral salt concentration was reduced to a half and, the BAP concentration was increased, while 2,4-D was replaced with NAA in order to evaluate the effect of growth regulators on differentiation. Rapid callus proliferation with nodular somatic embryo development resulted on medium with a low BAP concentration (2 μM) while at the highest BAP concentration (8 μM), callus developed abnormal leafy structures (**Table 2**). On the medium with 2 μM BAP and 0.25 μM NAA, about a four-fold increase in callus size was observed and most of the calli developed somatic embryos after 5weeks (**Fig. 1C**). The increased cytokinin concentration may have resulted in differentiation of embryos. Complementary to these results, Vieitez (1995) observed that both the high salt media and high growth regulator levels resulted in compact-calli and abnormal shoot development, while a low concentration of a cytokinin (2.2 μM) with an auxin at lower concentration than the cytokinin (0.05-0.25 μM) resulted in proliferation of embryogenic callus in chestnut.

Development of somatic embryos was evaluated by observing calli under SEM. About one week after subculture of calli, presence of a prominent extra-cellular layer was observed (**Fig. 3A**). An extra-cellular layer on the callus surface was also seen in Coffee (Sondahl *et al.,* 1979), African rice

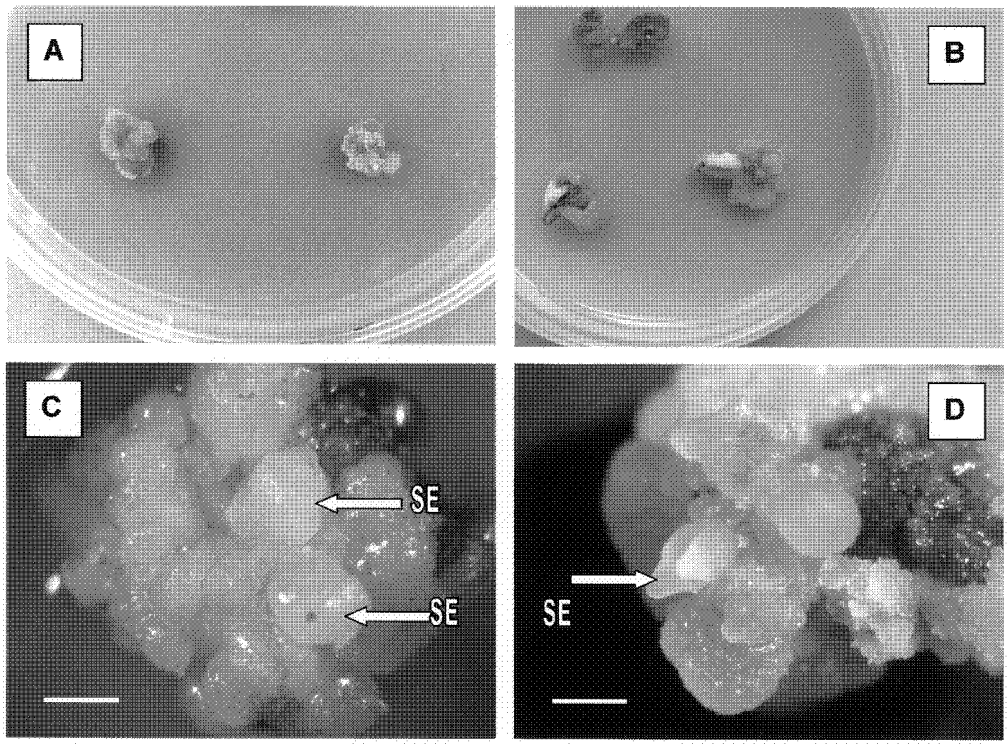

Fig. 1 Somatic embryogenesis in *Castanopsis sieboldii.* (A) Embryogenic callus on medium containing 10 μm 2,4-D and 1 μm BAP. (B) Development of friable callus from zygotic embryos. (C) Somatic embryo (SE) development on medium containing 2 μM BAP and 0.25 μM NAA (bar: 1 mm). (D) Somatic embryo maturation (SE) on the medium with 0.25 μM IBA and 1 μM zeatin (bar: 1 mm).

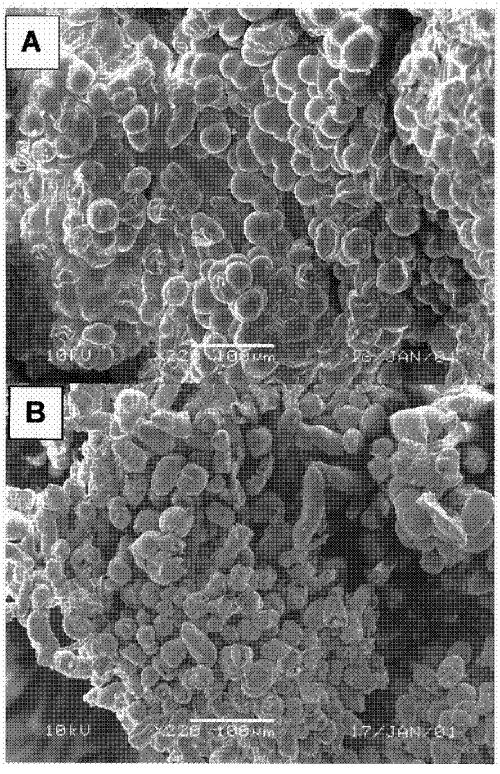

Fig. 2 Distinct characteristic features of callus. (A) Closely packed round shaped cells of embryogenic callus. (B) Loosely packed elongated cells of friable callus.

(Brisibe *et al.,* 1992), *Pinus nigra* (Jasik *et al.,* 1995), Indica rice (Basu *et al.,* 1997) and in *Drosera rotundifolia* (Bobak *et al.,* 1999) which appears almost simultaneously with early symptoms of differentiation. They have identified this layer as a polysaccharide layer. This layer probably supports movement of nutrients through the callus surface, compatible with its wet appearance (Maeda *et al.,* 1988). The extracellular material that appears at the early stages of development seems to be important for plant regeneration from calli in culture (Bobak *et al.,* 1999).

Symptoms of differentiation were observed about 2 weeks after subculture of callus. Division of the callus mass into small clusters with compact cell arrangement was observed at this stage (**Fig. 3B**). Reduction of the extra-cellular layer into a thin layer gave a wrinkled appearance to the callus surface (**Fig. 3C**). Rupture of the extra-cellular layer and multiplication of surface cells were evident when calli were observed 3 weeks (**Fig. 3D, E**). Brisibe *et al.* (1992) reported that in callus of African rice, enlargement and multiplication of the surface cells resulted in the rupture at various sectors of the continuous polysaccharide layer, leading to formation of a fibrillar network, which connected the surface cells of the callus. After 4

Table 2 Percentage of calli producing somatic embryos in different BAP and NAA concentrations after 4 weeks of culture

Growth regulators (μM)		Percentage of calli forming globular somatic embryos (%)	Number of embryos per callus
BAP	NAA		
2	0.25	81.67 ± 0.08	8.08 ± 2.0
4	0.25	8.33 ± 0.06	1.75 ± 0.96
8	0.25	0 (leafy structures)	0 (leafy structures)

Mean ± standard deviation of three repeated experiments with 20 replicates per treatment

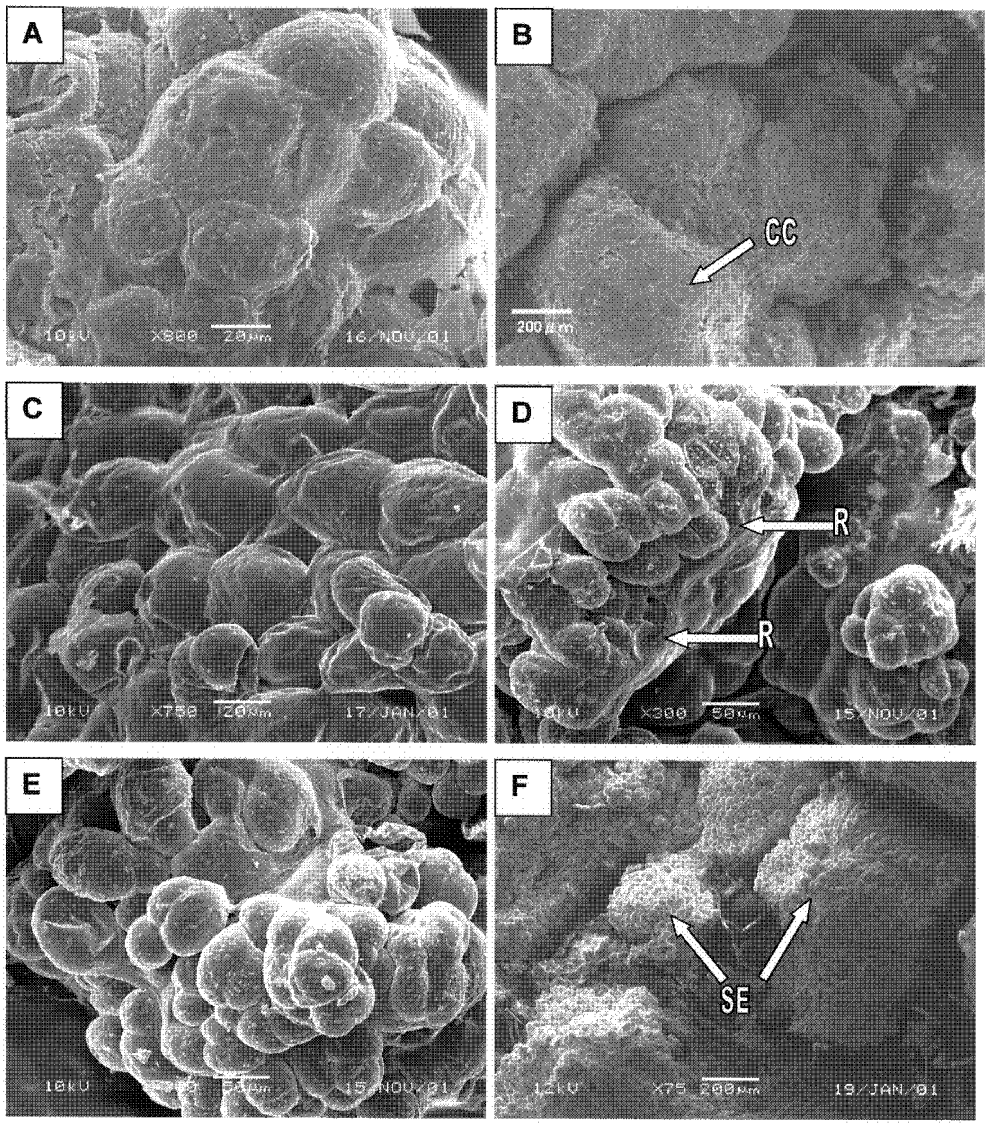

Fig. 3 Scanning electron microscopy of callus differentiation of *Castanopsis sieboldii*. (A) Development of an extracellular layer. (B) Differentiation of callus mass into compact clusters (CC). (C) Reduction of extracellular material giving a wrinkled appearance to the surface layer. (D) Rupture (R) of the extracellular material. (E) multiplication of surface cells. (F) Callus at the initial stage of somatic embryo (SE) development.

weeks calli developed many globular nodules on the surface, suggesting somatic embryo development (**Fig. 3F**).

Since zeatin was reported to be suitable for shoot initiation (Luis *et al.*, 1999; Wakita *et al.*, 1996), nodular calli were subcultured on to an embryo maturation medium with a combination of zeatin and IBA. Maturation of somatic embryos was observed about 3 weeks after embryogenic callus was transferred to the medium containing 1 μM

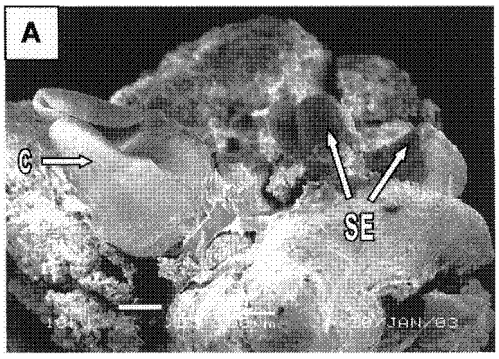

Fig. 4 *In vitro* plantlet development of *Castanopsis sieboldii*. SEM observation of somatic embryo maturation on the medium with 1 μM zeatin and 0.25 μM IBA (Cotyledon-C, Somatic Embryos -SE). (B) Shoot (S) and root (R) growth on the medium with 150 μM Fe-Na-EDTA and 1 μM Zeatin (bar: 1 cm).

zeatin and 0.25 μM IBA (**Fig. 1D**). An increase in the size of the callus was not observed on this medium. This may be due to reduction of endogenous auxin activity, due to the removal of auxin in the medium.

From SEM observations, embryo maturation was evident on callus surface about 2 weeks after transferring callus on to the maturation medium (**Fig. 4**). The embryo maturation appeared to be not well synchronized, as different developmental stages of somatic embryos were observed at the same time.

Some of the mature embryos germinated to produce shoots in the same medium but the number of shoots produced is low. About 12% of the callus cultured on somatic embryo development medium produced shoots. Therefore further investigations are necessary for synchronize germination of somatic embryos for clonal propagation.

Conclusion

In this study, we showed that embryogenic callus initiates from immature embryos obtained from acorns at the onset of actively growing stage. According to the micro-morphological course of events, regeneration occurred through embryogenesis under the present culture conditions. Further improvement in to media composition is required to synchronize the shoot development from somatic embryos.

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