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Comparative Anatomy of Embryogenic and Non-Embryogenic Calli from *Pimpinella brachycarpa*

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Anatomical differences between embryogenic and non-embryogenic calli of *Pimpinella brachycarpa* were investigated by light microscopy and electron microscopy. Initial callus tissue emerged from explants after 14 d of culturing. The embryogenic calli (EC) were firm, rather opaque, and light yellow in color. The cells usually formed small, compact clusters. Non-embryogenic calli (NEC), however, were friable, semitransparent, and yellow or gray. These formed relatively larger and loosely held clusters. Scanning electron microscopy showed that EC were composed of individual compact and spherical cells that were rather regular in size and approximately 20 μm long. All were tightly held together and appeared to organize globular embryos. In contrast, the NEC comprised elongated and loosely held cells that were approximately 50 μm long. Tubular and u-shaped NEC cells protruded irregularly, and were of varying heights along the cell aggregates. Transmission electron microscopy of the EC revealed typical eukaryotic cytoplasmic components, including nuclei, mitochondria, and vacuoles in the cytoplasm enclosed by an electron-transparent cell wall. Based on the numerous ribosomes within the cytoplasm, these cells appeared to be well-organized and metabolically active. The NEC cells were much larger and more highly vacuolated than those of the EC. In ultrathin sections, the former seemed to be almost devoid of other cellular contents except for plastids and nuclei. Furthermore, EC and NEC showed different regeneration capacities in their somatic embryo formation. Most EC produced hyperhydric somatic embryos, followed by normal somatic embryos; whereas only a few shooted or rooted somatic embryos arose from the NEC.

Keywords: callus, differentiation, electron microscopy, embryo, regeneration

Somatic embryogenesis plays an important role in clonal propagation. When integrated with conventional breeding programs and molecular and cellular biological techniques, this method provides a valuable tool for accelerating the pace of genetic improvements in commercial crop species (Stasolla and Yeung, 2003). The application of somatic embryogenesis to micropropagation requires an abundance of embryogenic calli (EC) (Moon et al., 2006). Mass production of EC from plant explants has always been difficult because the EC grow slowly while the faster-developing non-embryogenic calli (NEC) dominate the cultures. Because both types often appear in the same tissue (Kaur et al., 1992), a new protocol is needed that selectively promotes EC proliferation. In several *Gramineae* species analyzed by Nabors et al. (1983), EC present nodular features and a smooth surface while the NEC are rough, friable, and translucent. Similar characteristics have been observed in the EC and NEC from *Daucus carota* (Jimenez and Bangerth, 2001). In other *in vitro* culturing system, such as for *Oryza sativa* (Oinam and Kothari, 1995), *Pisum sativum* (Stirn and Jacobsen, 1987), and *Arabidopsis thaliana* (Ikeda-Iwai et al., 2002), coloration has been used as a criterion for selecting EC. Likewise, embryogenic and non-embryogenic tissues from *Coffea arabica* calli are discernible based on their color, with the former being brown and hard while the latter are pale and friable. This correlation between callus appearance and embryogenic competence also has been confirmed by histological studies (Quiroz-Figueroa et al., 2002).

The productivity of somatic embryos depends on the quality of EC cultures (Ibaraki et al., 1998). Their embryogenic potential must be sustained throughout the maintenance phase to ensure stable production. Although this potential depends upon genotype, it can also change with culture period, and is affected by medium composition and environmental conditions (Lee et al., 2006).

Through microscopy, one can easily identify proembryogenic masses, which, in several plant systems, share similar structural features. For example, they may consist of small and highly cytoplasmic cells that often have an accumulation of starch within the plastids (Yeung, 1995). In contrast, non-embryogenic cells are large and vacuolated. The rate of culture growth may be used as an index for evaluating embryogenic potential (Ibaraki and Kurata, 2001). Differences in growth characteristics between embryogenic and non-embryogenic tissues have been reported in the suspension cultures of maize (Stirn et al., 1994), carrot (Ibaraki and Kurata, 1997), and sweetpotato (Zhang et al., 1996).

Various types of callus tissue can be obtained from the *in vitro* culturing of explants. Profumo et al. (1986) have described three types of calli, as isolated by specific propagation methods on solid or liquid media. These include 1) friable EC that can produce numerous embryoids, 2) forerunner calli that differ from the friable type in their color and consistency, and 3) NEC that grow actively but are unable to develop embryoids, even after more than a year of sub-culturing. Interestingly, this differentiation is based on performance that originally occurred on media supplemented with the same nutritional and hormonal substances. Currently, two types of calli are used in classifications of EC and NEC. Differences in their growth characteristics have been reported in suspension cultures of *Zea mays* (Stirn et al.,

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1994) and *D. carota* (Smith and Street, 1974).

Pimpinella brachycarpa (Kom.) Nakai (known as "Chamnamul" in the Korean language) is one of the most favored and increasingly popular edible greens grown in Asian regions, including Korea (Moon et al., 1994). This wild perennial belongs to the Umbelliferae family. Because plants usually grow at high elevations and propagation by seed is not well established, several procedures for somatic embryogenesis have been reported (Son and Kim, 1999; Kim et al., 2001). However, little information is available about its anatomy or morphogenesis from callus of *P. brachycarpa*. To better exploit the nature of callus induction for clonal regeneration, it is necessary to study *in vitro*-grown EC and NEC at both tissue and cellular levels. The combined use of light and electron microscopy can provide a comprehensive understanding of the surface morphology and internal structures of such calli. Hence, we have now examined the anatomical differences between EC and NEC, and have compared their somatic embryogenic capacities.

MATERIALS AND METHODS

Plant Material

Seeds of *P. brachycarpa* were collected from wild species growing in Pyeongchang, Gangwon-do, Korea. Their petioles were rinsed with 70% ethanol for 5 s, sterilized with hydrogen peroxide for 20 min, and rinsed with sterilized distilled water five times. These sterilized tissues were cut into 0.5 cm sections with a sterilized razor blade on a clean bench. To induce callus formation, the explants were then transferred to an MS medium (Murashige and Skoog, 1962) containing 0.1 mg L^{-1} 2,4-dichlorophenoxyacetic acid, 3% sucrose, and 0.8% agar, and were incubated for two months at 25°C under a 16-h photoperiod (30 μmol m^{-2} s^{-1} photosynthetic photon flux; PPF). The embryogenic calli (EC) and non-embryogenic calli (NEC) that resulted were then prepared as specimens for microscopic evaluation.

Stereomicroscopy and Scanning Electron Microscopy

EC and NEC tissues (each 5 × 5 mm^2, approx. 3 mm thickness) were excised with a razor blade. Some of these specimens were directly examined under a stereomicroscope (S8 APO; Leica Microsystems GmbH, Germany). For scanning electron microscopy, the specimens were fixed for 3 h at 4°C in modified Karnovsky's fixative (Karnovsky, 1965) that consisted of 2% (v/v) glutaraldehyde and 2% (v/v) paraformaldehyde in 0.05 M sodium cacodylate buffer (pH 7.2). They were then washed with the same buffer thrice, for 10 min each time. The specimens were post-fixed with 1% (w/v) osmium tetroxide in the same buffer at 4°C for 2 h, and washed twice with distilled water. They were dehydrated in a graded ethanol series (30, 50, 70, 80, and 95%) and three times in 100% ethanol (10 min each). After being further treated with isoamyl acetate two times (10 min each), they were dried in a critical point drier (CPD 030; BAL-TEC, Liechtenstein) with liquid carbon dioxide as the transitional fluid. The specimens were mounted on metal stubs and made electrically conductive by coating them, under an argon atmosphere, with a thin layer (approx. 30 nm) of gold using a sputter-coater (JFC-1100E; JEOL, Japan). These coated specimens were then examined with a scanning electron microscope (JSM-5410LV; JEOL) operated at an accelerating voltage of 20 kV.

Light Microscopy and Transmission Electron Microscopy

EC and NEC samples (each 2 × 2 mm^2, approx. 1 mm thickness) were fixed and post-fixed as described for scanning electron microscopy. The post-fixed specimens were *en bloc*-stained with 0.5% (w/v) uranyl acetate at 4°C overnight, and dehydrated in an ethanol series (30, 50, 70, 80, and 95%), and three times in 100% ethanol (10 min each). After further treatment with propylene oxide as the transitional fluid (twice, 15 min each), the specimens were embedded in Spurr's resin (Spurr, 1969). For light microscopy, semi-thin sections (1 μm thickness) were made with a glass knife and an ultramicrotome (MT-X; RMC, USA). These thin sections were mounted on slide glasses and stained with 0.05% toluidine blue for 5 min. They were examined under a light microscope (Axiophot; Carl Zeiss, Germany). In addition, ultrathin sections (60 nm thickness) were made with a diamond knife, mounted on bare copper grids, and stained with 2% uranyl acetate and Reynolds' lead citrate (Reynolds, 1963), each for 7 min. They were examined with a transmission electron microscope (JEM-1010; JEOL) operated at an accelerating voltage of 80 kV.

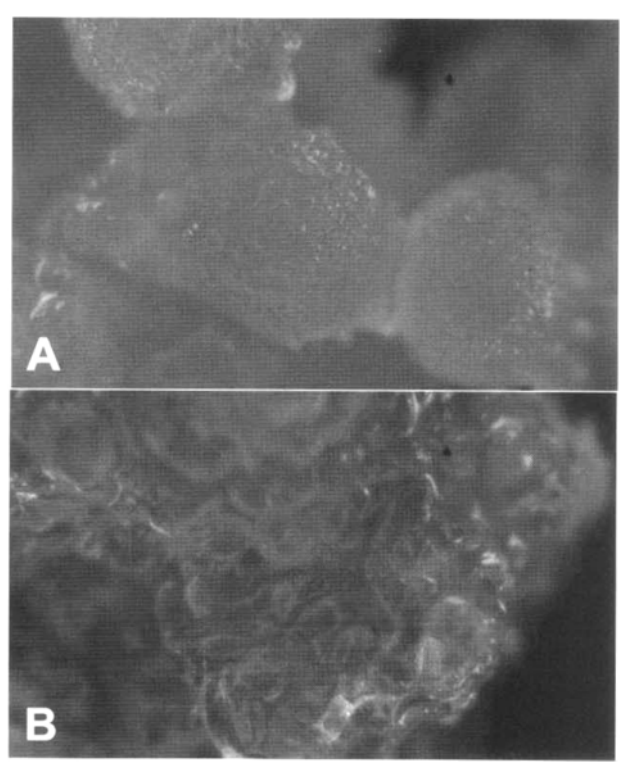

Figure 1. Stereomicrographs of calli induced from petioles of *P. brachycarpa*. (**A**) Embryonic calli (EC) cells. (**B**) Non-embryonic calli (NEC) cells.

Somatic Embryo Formation

To compare the regeneration capacities of these two types of calli, we assessed the formation of somatic embryos. EC and NEC tissues (approximately 0.1 g each) were filtered through a 60 mesh metal sieve over 250 mL conical beakers. The calli were then transferred to culture bottles containing a half-strength MS liquid medium (pH 5.8) supplemented with 3% sucrose, and were cultured in suspension (100 rpm) for four weeks. The liquid medium was replaced with fresh once a week. All cultures were incubated at 25°C under a 16-h photoperiod from fluorescent lights (50 μmol m^{-2} s^{-1} PPF). The numbers of normal, hyperhydric, shooted, and rooted somatic embryos were recorded after six weeks of culturing. Five replicates were taken for each treatment, and their data were pooled to calculate and compare mean numbers of embryos formed among regenerants per explant. Fisher's protected least significant difference (FLSD) tests were used with SAS PROC GLM (SAS Institute, USA).

RESULTS AND DISCUSSION

Stereomicroscopy and Scanning Electron Microscopy

Two types of initial callus tissue emerged concomitantly from *P. brachycarpa* explants after 14 d of culturing. These were embryogenic calli (EC; Fig. 1A) and non-embryogenic calli (NEC; Fig. 1B), which could be discerned based on differences in their fineness, transparency, color, and dimensions. EC were firm, rather opaque, and light yellow. They were observed on the edges of the explants, and usually formed small and compact clusters (approx. 300 μm diam.). NEC were friable, semitransparent, and yellow or gray. These

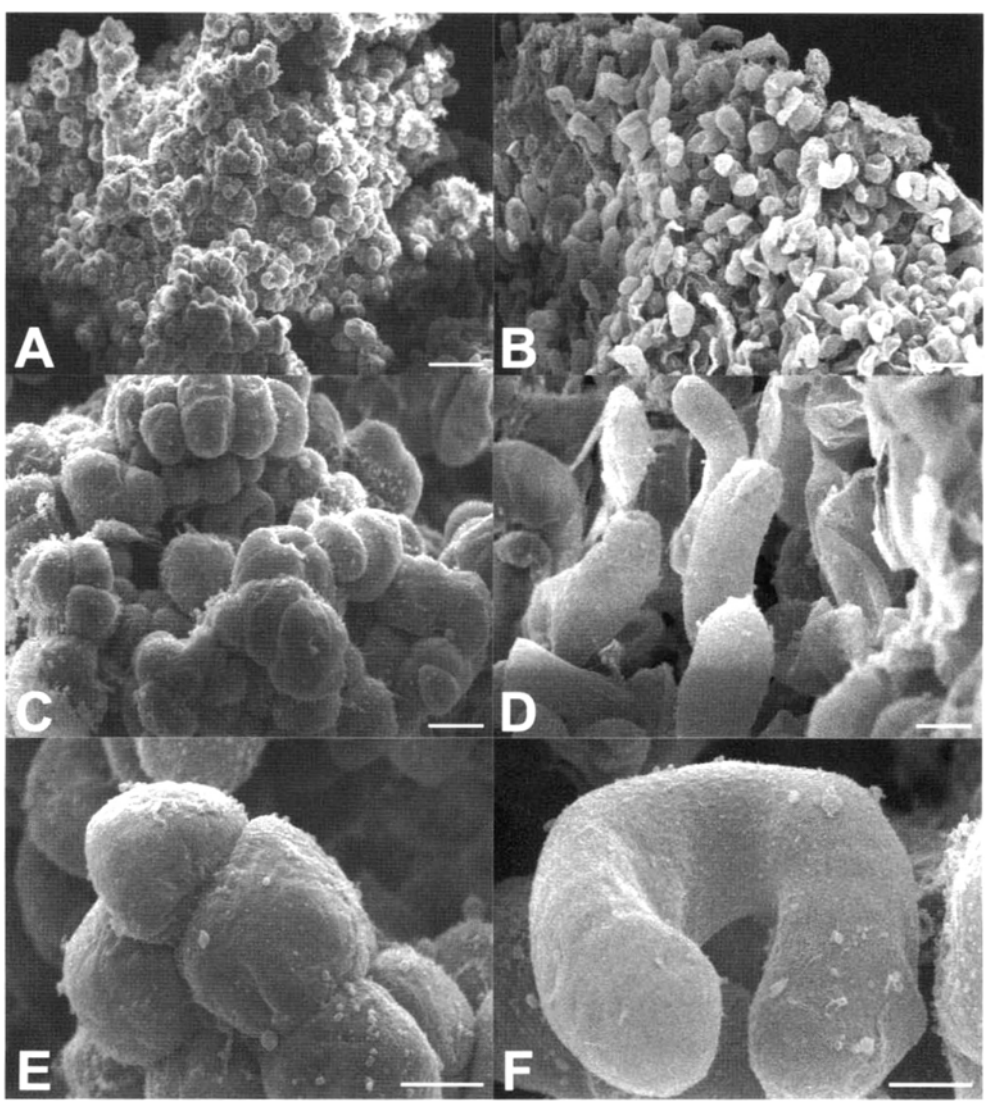

Figure 2. Scanning electron micrographs of calli induced from petioles of *P. brachycarpa*. (**A**) EC composed of individual spherical cells that form clusters. (**B**) NEC comprising elongated and loosely held cells. Bars = 100 μm. (**C**) EC cells at higher magnification are rather regular in size. (**D**) NEC cells, at higher magnification, that are tubular and irregularly protruding to varying heights. Bars = 20 μm. (**E**) EC cells, at higher magnification, held tightly together and forming clusters. (**F**) NEC cells, at higher magnification, with some being dissociated and u-shaped. Bars = 10 μm.

formed bigger and more loosely held clusters (approx. 500 μm diam.).

Scanning electron microscopy (SEM) revealed morphological differences, with EC being composed of individual compact and spherical cells (Fig. 2A) that were rather regular in size and approximately 20 μm long (Fig. 2C). They were tightly held together and appeared to organize globular embryos in small clusters (Fig. 2E). By comparison, NEC showed elongated and loosely held cells on the surface (Fig. 2B), with tubular cells that were approximately 50 μm long and which protruded irregularly to varying heights along the cell aggregates (Fig. 2D). Dissociated u-shaped cells were also found on the surfaces of NEC (Fig. 2F).

Light Microscopy and Transmission Electron Microscopy

Bright-field light microscopy of these EC presented dense aggregates of compact cells with small intercellular spaces (Fig. 3A). In cross section, these cells were about 20 μm long, and were mainly characterized by dense cytoplasm. Seemingly granule-like cytoplasmic components were usually observed in the cells. In contrast, highly vacuolated cells were common in NEC (Fig. 3B). As was also apparent via SEM, individual cells of NEC were tubular or u-shaped, and approximately 50 μm long. They had widened intercellular spaces and did not form clusters, unlike the cells of EC.

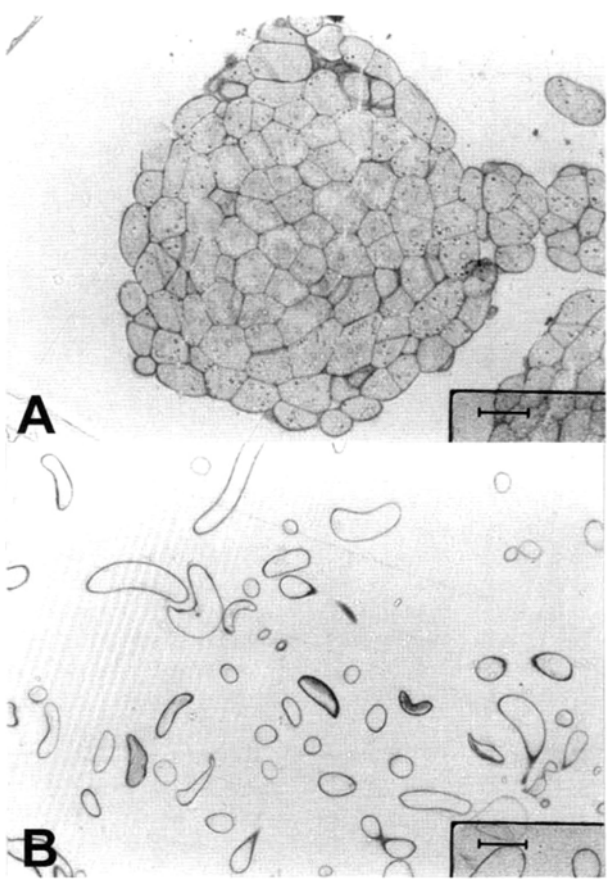

Figure 3. Bright-field light micrographs of calli induced from petioles of *P. brachycarpa*. (**A**) EC cells forming compact aggregates with dense cytoplasm. (**B**) NEC cells that are highly vacuolated and almost devoid of cytoplasmic components. Bars = 20 μm.

Except for central vacuoles, few if any typical cytoplasmic components were detected. Our observations are consistent with the differences in calli formation reported from rubber trees, in which the EC consist of large clusters as well as cells that are more cohesive than in the NEC (Charbit et al., 2004). These results suggest the importance of cell cohesion and intercellular communications in the acquisition of embryogenic competence.

Transmission electron microscopy of EC revealed typical eukaryotic cytoplasmic components. These included nuclei, mitochondria, and vacuoles in the cytoplasm, all enclosed by electron-transparent cell walls (Fig. 4A). It is also possible that the granule-like cytoplasmic components shown in light microscopy were protein bodies, if one uses the criteria of electron density and size (approx. 2 μm in length here). These particular organelles were frequently found along the cell walls; this proximity to the plasma membrane is strikingly similar to that found with protein bodies in the vacuoles of maize EC cells (Samaj et al., 1999). Likewise, mitochondria, usually being of smaller diameter than the protein bodies, were observed in the cytoplasm. Those cells, which appeared to be well-organized and metabolically active based on the numerous ribosomes within the cytoplasm, were in close contact with adjacent cells through cell-to-cell attachments.

In comparison, NEC cells were two or three times larger and highly vacuolated (Fig. 4B). These cells, delimited by walls, had widened intercellular spaces. This implies that the cells had undergone remarkable enlargement and become highly vacuolated, which could have accounted for their friable and semi-transparent characteristics. In addition, the NEC cells seemed to be almost devoid of other cellular contents except nuclei and plastids. The latter had no inner membrane systems or starch grains, suggesting possible deterioration (Fig. 4C). A single nucleus was observed in each ultrathin section, revealing a nucleolus and heterochromatins positioned at the center of the nucleoplasm (Fig. 4D). Similar findings have been reported in *Aesculus hippocastanum* (Profumo et al., 1987).

Somatic Embryo Formation

More than 500 somatic embryos were formed from EC (Table 1), with most being either hyperhydric (77%) or normal somatic embryos (15%) that had not yet formed leaves or roots (Fig. 5). However, some embryos (approximately 20) did indeed produce leaves and roots. Whereas normal somatic embryos are thought to be suitable materials for transplant production via embryogenesis, rooted and/or shooted somatic embryos are considered inadequate because it is difficult to separate individual embryos, which are often hyperhydric, from their population in culture vessels or bioreactors. In the current study, several somatic embryos formed from suspension cultures. Frequently hyperhydric somatic embryos would require more time for regeneration and be more vulnerable to dehydration stress than normal somatic embryos (Debergh et al., 1992). Research is ongoing in efforts to minimize the occurrence of hyperhydric somatic embryos, and to synchronize their developmental stages by controlling the physical and chemical environment (Albarran et al., 2005).

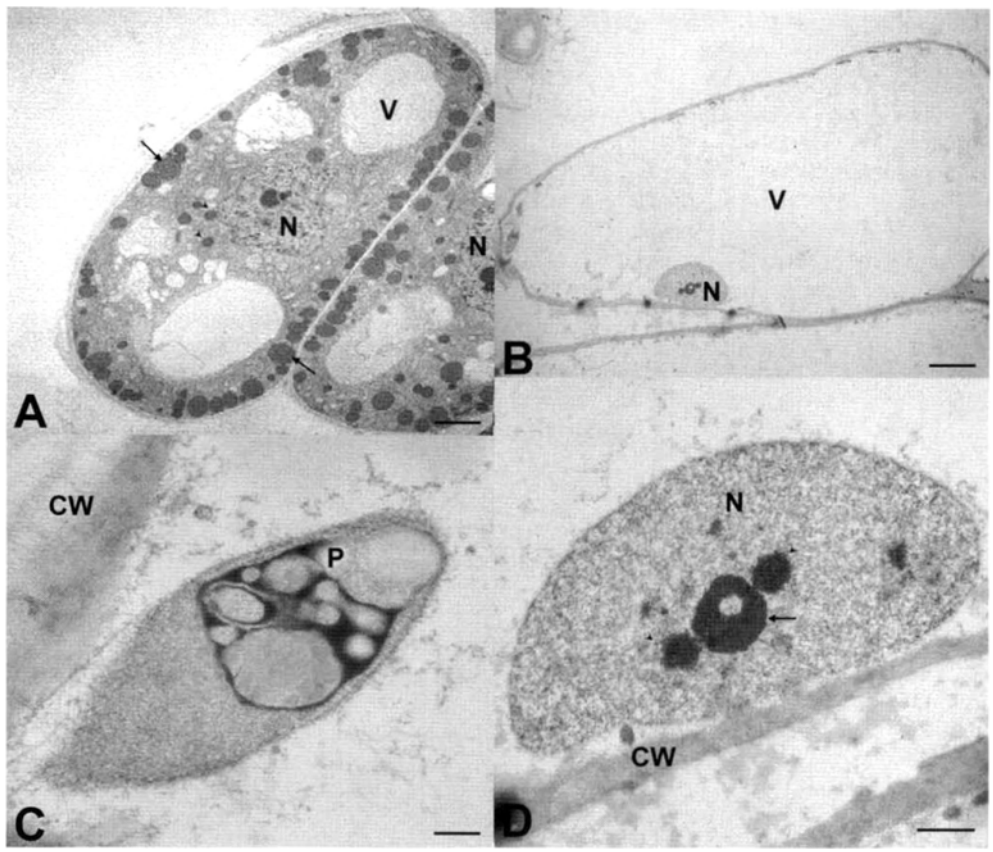

Figure 4. Transmission electron micrographs of calli induced from petioles of *P. brachycarpa*. (**A**) EC cells that contain eukaryotic cytoplasmic components, e.g., mitochondria (arrowheads) and protein bodies (arrows). N = nucleus. V = vacuole. Bar = 2 μm. (**B**) NEC cells. Note a central vacuole (**V**) occupying majority of cytoplasm and a nucleus (**N**) positioned near plasma membrane in cytoplasm. Bar = 5 μm. (**C**) NEC plastids (**P**) that appear to be devoid of inner membrane systems and starch grains. CW = cell wall. Bar = 0.5 μm. (**D**) NEC nucleus (N). Note a nucleolus (at arrow) and heterochromatins (arrowheads) in nucleoplasm. Bar = 1 μm.

Table 1. Somatic embryo formation of *P. brachycarpa* after 8 weeks of culture, according to callus type.

Callus type	Number of somatic embryos			
	Normal somatic embryos	Hyperhydric somatic embryos	Shooted somatic embryos	Rooted somatic embryos
EC	81.6 a[z]	412.0 a	19.0 a	21.0 a
NEC	0.0 b	0.0 b	2.8 b	2.2 b

[z]Mean separation within columns by FLSD test ($P \leq 0.05$).

Meanwhile, our NEC tissues produced only a few shooted or rooted somatic embryos, their rare occurrence being attributed to the scarcity of embryonic-origin cells. Furthermore, very small quantities of extremely hyperhydric plantlets were obtained (Fig. 5). Such abnormal growth is useless in micropropagation systems that employ embryogenesis. Our callus sectors probably contained both EC and NEC, but populations of the former were apparently diminished due to overgrowth of the latter during sub-culturing. It is interesting that two different types occurred on these culture media because all had been supplemented with the same nutritional and hormonal substances. In fact, Sharp et al. (1980) have demonstrated two means for somatic embryogenesis: 1) direct, in which minimal proliferation of unorganized tissue precedes embryo formation; and 2) indirect, where the calli proliferate profusely before embryo formation. Therefore, if one is to utilize indirect techniques to successfully develop a transplant production system, it is essential that one classify and proliferate embryogenic calli with the greatest potential for somatic embryo formation.

The results of our current experiments suggest that we adopt classification criteria for EC and NEC based on anatomical characteristics and somatic embryo formability. These can be verified visually, using color, density, and shape. This protocol is necessary because it is critical that the EC sector be rescued at an early culture stage if we are to establish an efficient regeneration system. Moreover, research that elucidates the signal transduction pathways for callus induction and acknowledges the importance of knowing their regeneration capacities will enhance our understanding into the nature of somatic embryogenesis in plants.

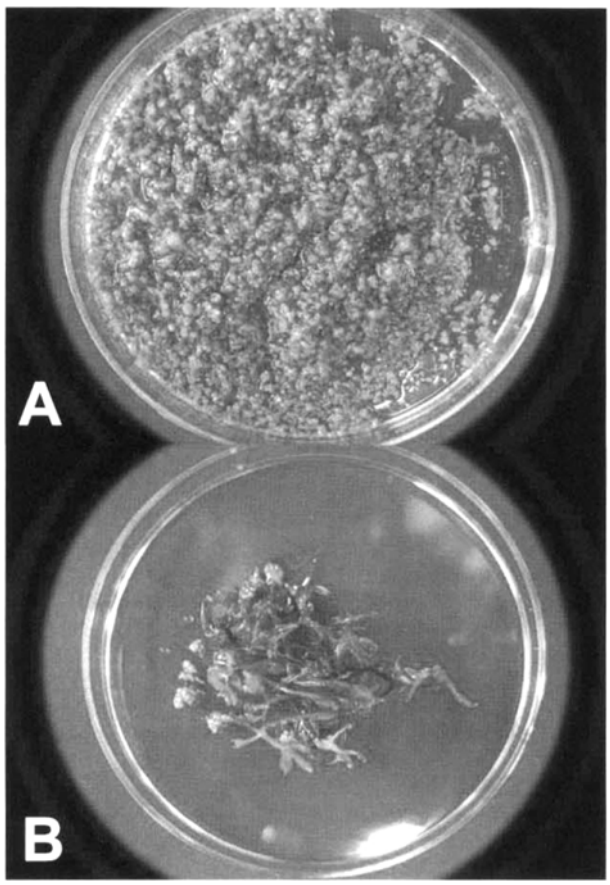

Figure 5. Somatic embryos of *P. brachycarpa* formed from EC (**A**) and NEC (**B**).

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