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Nodular Somatic Embryogenesis and Frond Regeneration in Duckweed, *Lemna gibba* G3

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Duckweed (*Lemna gibba*) is a useful model system for elucidating plant development, but the techniques needed for regenerating fronds from calli are not yet well established. This study examined the effects of auxin, sucrose, and gelling agents on callus and frond formation in *L. gibba* G3. After three weeks of culturing on a solid medium, two types of calli were observed: watery, pale-green, and undifferentiated; or white, compact calli that were organized into nodules and which resembled somatic embryogenic calli. Homogeneous callus lines were produced through selective subculture. To induce nodular calli, auxin (2,4-D) was absolutely required, with an effective concentration of 5 to 20 μM; induction was found to be possible with up to a maximum concentration of 4.4%. The calli were then maintained on a medium with a reduced 2,4-D concentration (1 μM), and were transferred every three weeks. Optimal callus induction and growth were obtained by using 3% sucrose with a combination of 0.15% Gelrite and 0.4% agar. Fronds, however, could be regenerated only on distilled water solidified with a combination of 0.4% agar and 0.15% Gelrite. On this medium, 87% of the callus explants regenerated into fronds after four weeks of culture. These new fronds were morphologically normal but small, approximately 15 to 20% of the size of stock fronds. Continued culture of these fronds in an SH medium produced normal duckweeds, and histological examination of the cultures revealed several distinct types of callus nodules. Nonetheless, because zygotic embryogenesis in *L. gibba* does not produce distinct bipolar structures, the developmental pathway of frond regeneration from these nodular cultures remains unknown.

Keywords: *Lemna gibba* G3, regenerated fronds, somatic embryogenic callus, zygotic embryogenesis

Lemna gibba is one of 32 duckweed species in the monocotyledonous family of *Lemnaceae*. Duckweeds are small, free-floating aquatic plants that multiply via asexual budding of their fronds. These plants are convenient subjects for biochemical and physiological studies because they proliferate rapidly on simple, liquid-nutrient solutions while under low light and without agitation (Landolt, 1986). When considered as the whole-plant equivalent of yeast, they can become a model system for understanding plant development. However, genetic and developmental studies of duckweed are limited, partially because the tools critical for investigating its molecular genetics and cell biology (e.g., gene transfer, DNA mapping, gene cloning) have not been widely extended to this family (Chang and Chiu, 1976; Chang and Hsing, 1978; Moon and Stomp, 1997).

Application of the gene-transfer technique in monocotyledonous plants had its beginnings with research on somatic embryogenic calli (Fromm et al., 1990; Vasil et al., 1992). This approach could now be used with duckweed if efficient methods were developed. Researchers have demonstrated that fronds can be regenerated from the calli of both *L. gibba* (Chang and Chiu, 1978) and *Lemna perpusilla* (Chang and Hsing, 1978). In addition, factors have been investigated for improving the efficiency of frond regeneration from calli (Moon and Stomp, 1997). The resulting method now makes it possible to produce a heterogeneous callus culture that contains both friable and compact calli. The color and morphology of the latter suggest that frond regeneration might be occurring via somatic embryogenesis. Here we report an optimal set of conditions for compact-callus proliferation and frond regeneration from duckweed strain *L. gibba* G3. We also describe the anatomy of frond regeneration at the histological level.

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MATERIALS AND METHODS

Materials and Culture Conditions

L. gibba G3 was provided by J. P. Slovin (USDA,

Beltsville, Maryland). Frond stock cultures were maintained on a liquid SH medium (Schenk and Hildebrandt, 1972) supplemented with 3% sucrose. Fully expanded fronds were harvested for the experiment two weeks after the cultures were transferred to a new medium. All media components, including naphthalene acetic acid (NAA) and benzylaminopurine (BAP), were combined and sterilized at 121°C for 20 min. Afterward, 25-mL aliquots of the cooled media were poured into 100 × 15 mm disposable plastic Petri dishes. GA_3 was filter-sterilized and added to the cooled media. All cultures were incubated at 23°C under a 16-h photoperiod (~40 μmol m^{-2} s^{-1}) from Gro-Lux fluorescent lights.

Callus Induction and Frond Regeneration

To test the effects of 2,4-D, gelling agents, and sucrose on callus induction and maintenance, experiments were conducted on MS basal media (Murashige and Skoog, 1962). Unless otherwise specified, the MS was supplemented with 10 μM NAA, 10 μM GA_3, 2 μM BAP, 3% sucrose, 5 μM 2,4-D, 1.5 g/L Gelrite (Sigma), and 4 g/L Bacto-agar (Difco). All experiments involved randomized complete-block designs that were replicated five times. For each replicate block (i.e., Petri dish), four fronds were placed ventral-side down on the medium (total of 20 observations per treatment). In the frond-regeneration experiment (six replications), five callus masses were plated per dish (total of 30 observations per treatment).

Four investigations were conducted. First, the effect of 2,4-D on callus induction was tested as a dose-response experiment, using concentrations of 0, 1, 5, 20, or 50 μM. Second, several gelling agents were analyzed: 2 or 4 g/L of Gelrite, 7 or 14 g/L of agar, as well as a combination of 1.5 g/L Gelrite with 4 g/L agar. Third, the effect of sucrose was examined, at concentrations of 0, 1, 3, or 8% (w/v). The fourth experiment, involving the effects of gelling agent on frond regeneration from calli, used only distilled water that was solidified without any phytohormone. The agents in this test included 0.7% Difco Bacto-agar, 0.7% Sigma agar, 0.7% washed Gibco phyto-agar, 0.6% unwashed Gibco phyto-agar, and the combination of 0.15% Gelrite with 0.4% Difco Bacto-agar.

Histology

For the histological studies, we fixed the nodular embryos in FAA (formalin, alcohol, and acetic acid), then dehydrated them in an ethanol-xylene series, and embedded them in paraffin wax at 52 to 54°C. The 10-μm-thick block sections were stained with safranin and counterstained with Fast Green.

Statistics

All the experiments were conducted four weeks after plating on the treatment media. To assess the level of callus induction, the final percentage of fronds that had proliferated from each callus was expressed as the induction frequency (Table 2, footnote b). For frond regeneration, the percentage of original callus pieces that regenerated at least one frond was indicated as the regeneration frequency (Table 2, footnote b). To determine treatment differences and the predicted optimal treatment, data were analyzed using SAS variance procedures PROCGLM and RSREG (SAS Institute, Cary, NC, USA). Tests of significance were conducted at $P < 0.05$, and means were compared using Duncan's multiple range test.

RESULTS

Callus Induction

Two types of calli were produced on MS media supplemented with various concentrations of 2,4-D. One type was characterized by a watery, undifferentiated callus; the other, by a more compact, white callus with a smooth surface and nodular or globular morphology. Both types originated from the meristematic region on the ventral side of the frond; each could be mechanically separated from the other. Through careful selection, we were able to produce cultures of primarily white calli. New nodules developed on the surfaces of older nodular structures of the white, compact calli. These were maintained on an MS medium with 1 μM 2,4-D, and were transferred to fresh media every three weeks to prevent callus browning and cessation of growth.

Induction of white calli was dependent on the presence of 2,4-D in the medium, although the frequency of induction did not differ significantly over the range of concentrations tested (5 to 20 μM; data not shown). Regression analysis gave a predicted optimal 2,4-D concentration of 24.7 μM. Approximately 4 to 5% of the fronds present at the end of the four-week subculture period produced white calli. Callus induction was apparent after two weeks of culture, and nodule proliferation was established within three weeks.

The type of gelling agent affected white-callus induction, with the combination of Gelrite and agar being a better promoter than either agar or Gelrite alone

Table 1. Effect of gelling agents on nodular callus induction in *L. gibba* G3.

Gelling agent[a]	% induction[bc]
2.0 g/L Gelrite	1.2% b
4.0 g/L Gelrite	0.7% b
7.0 g/L agar	1.9% b
14.0 g/L agar	1.8% b
1.5 g/L Gelrite +4.0 g/L agar	4.4% a

[a]Gelrite was purchased from Sigma; Bacto-agar, from Difco.
[b]Induction frequency was calculated as: (number of fronds that produced white, nodular calli during the four-week subculture period)/ (total number of fronds cultured) x 100.
[c]Means followed by the same letter were not significantly different using Duncan's critical range test at $P < 0.05$.
*The basic medium is an MS medium supplemented with 10 μM NAA, 10 μM GA_3, 2 μM BA, 3% sucrose, and 5 μM 2,4-D.

(Table 1). The frequency of induction also showed a broad response to sucrose. Of the four concentrations tested, neither 0% or 8% sucrose were able to support induction (data not shown). In contrast, frequencies resulting from 3% and 1% sucrose were 3.7% and 0.7%, respectively; a difference that was statistically significant.

Frond Regeneration

We attempted to regenerate fronds on MS media, using various levels of phytohormones. However, a number of combinations proved incapable of inducing frond formation (data not shown). In fact, fronds could be regenerated only on distilled water solidified with a gelling agent, with formation commencing immediately upon transfer of the nodules to various gels. The combination of Gelrite and Difco Bacto-agar promoted the highest rate of frond regeneration (Table 2). Nodules that were white and opaque turned greenish and expanded further in the regeneration medium (Fig. 1, A, B, and C). Fronds were clearly evident 10 d after transfer (Fig. 1D). These regenerated fronds grew slowly and were about 15 to 20% the size of stock fronds maintained on liquid media. Once separated from their supporting nodules, however, the fronds proliferated readily when transferred to an SH medium. Roots arose from all of the regenerated fronds, being more easily observed with the naked eye in liquid rather than solid media.

Table 2. Somatic embryo germination and frond regeneration on different gelling media.

Gelling agent[a]	Germination frequency[b]
7 g/L Difco Bacto-agar	66.7% abc
7 g/L Sigma agar	73.3% ab
1.5 g/L Gelrite + 4 g/L Difco Bacto-agar	86.7% a
7 g/L Water-washed Gibco Phyto-agar	43.4% c
6 g/L Unwashed Gibco Phyto-agar	56.7% bc

[a]Germination frequency was calculated as: (number of somatic embryogenic callus pieces that regenerated at least one frond)/(total number of callus pieces cultured) x 100.
[b]Means followed by the same letters were not significantly different using Duncan's critical range test at <0.05.

Anatomical Analysis of Frond Regeneration

Histological studies of the compact white calli revealed complex anatomical structures, with the bulk of the internal tissue consisting of large, vacuolated cells. Within this tissue were areas characterized by smaller, more cytoplasmic cells (Fig. 2, A and B). These smaller cells were also associated with secondary nodules that formed at the surface of the bulk callus (Fig. 2C). We categorized the nodules into four groups: a) those with a few small cytoplasmic cells (Fig. 3A); b) those with small cytoplasmic cells on the surface layer (Fig. 3B); c) those that comprised primarily small cytoplasmic cells (Fig. 3C); and d) those in which the small cytoplasmic cells defined a polar structure (Fig. 3D). All four types were similar in that they had clearly differentiated layers of epidermal cells on all or most of their surfaces.

Microscopic examination showed that fronds regenerated from all four types of nodular structures. However, because we could not observed the classic bipolar structure that is characteristic of plant zygotic embryos, we might infer that frond regeneration in our system probably did not occur via an embryonic pathway, but instead by adventitious bud formation, as in the case of the daylily (Krikorian et al., 1988).

DISCUSSION

We demonstrated in this study that homogeneous lines of compact, white, nodular calli can be isolated, proliferated, and induced to regenerate fronds through careful selective subculture. What remains to be determined, however, is the particular developmental pathway taken by these cultures as they progress to frond formation. Two such pathways generally are characterized by compact, white, nodular calli. In the first, regeneration via somatic embryos arises from white, nodular structures or proembryos, as seen in a number of both monocotyledonous and dicotyledonous species (Vasil and Vasil, 1981; Hodges et al., 1986; Rout and Das, 1999). In the second pathway, it is adventitious shoot formation that is derived from the white, nodular

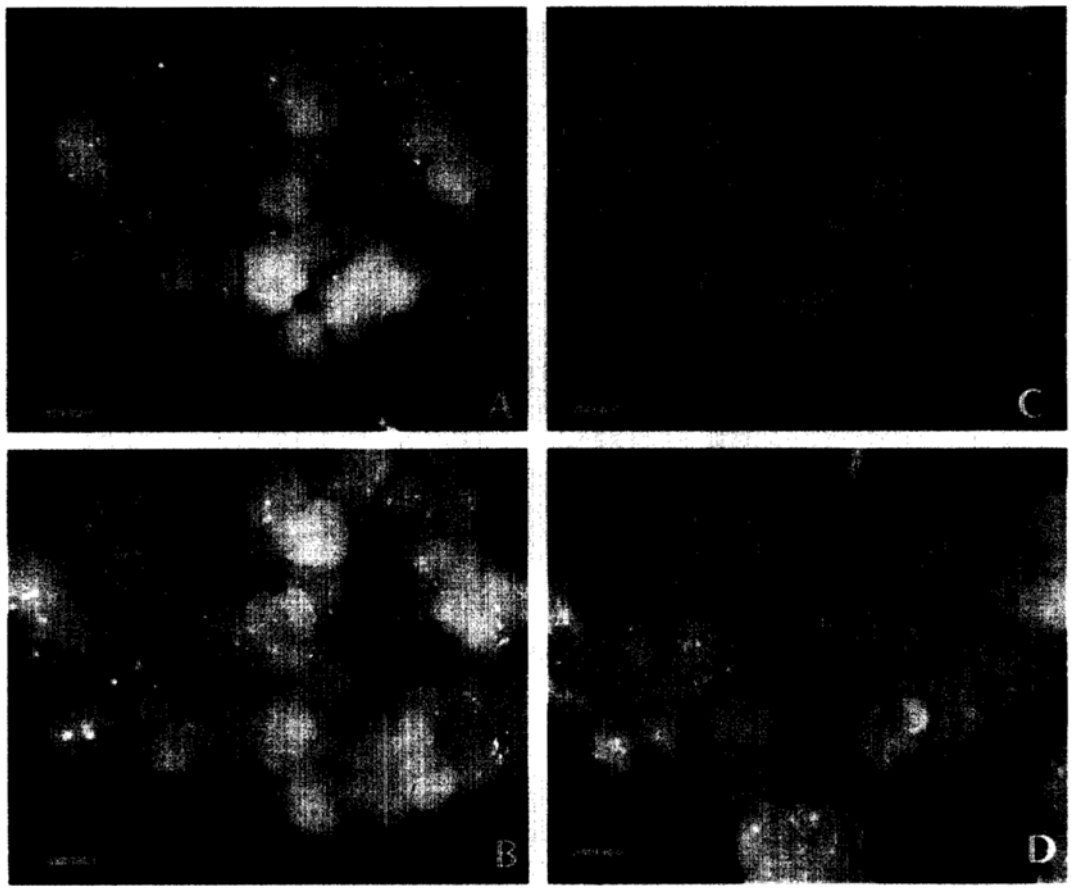

Figure 1. Frond regeneration from nodular somatic embryos. White and opaque nodules turned greenish and expanded further after 3 d (A), 1 week (B), and 3 weeks (C) in the regeneration media. Frond regenerated from nodule culture (D). Scale bars = 100 μm.

structures, as in daylilies (Krikorian and Kann, 1979, 1981). Further experiments will be needed to distinguish between these two pathways.

In our experiments with *L. gibba* G3, auxin was absolutely required for induction of white, compact calli. This requirement is shared by many somatic embryogenic-callus systems (Pierik, 1987). We found that the range of auxin concentrations capable of duckweed-callus induction was quite broad, a result that is also true for ryegrass (Creemers-Molenaar and Beerepoot, 1992) and rice (Zimmy and Lorz, 1986). In virtually all the plant species studied, including daylily, the further differentiation of somatic embryos is facilitated by the absence of or very low concentrations of auxin. Likewise, our duckweed system resulted in frond regeneration in an auxin-free medium.

Somatic embryogenic calli are most commonly established from flower or embryo explants (Pierik, 1987). For example, the daylily nodular system was produced from immature floral parts (Krikorian and Kann, 1979). In the case of duckweed, however, our original explants were the vegetative fronds. Flowering of *L. gibba* G3 has been observed under the culture conditions used for maintaining stock fronds (unpublished data). There, the rudimentary floral structures are reduced in size, and are not visible until rather advanced in their development. It is possible that those rudimentary structures, present at low frequency in our fronds, were actually the source of the callus cells. This hypothesis is intriguing because less than 5% of the fronds produced calli, even though they were presumably of the same genotype, and were cultured under identical conditions. The most obvious explanation for this observation is that some epigenetic difference existed among the explants; the infrequent presence of floral structures could illustrate that difference. We are currently testing this hypothesis by examining callus induction from floral organs.

The tissue that proliferated in the first two weeks of frond culture was a mixture of undifferentiated, muci-

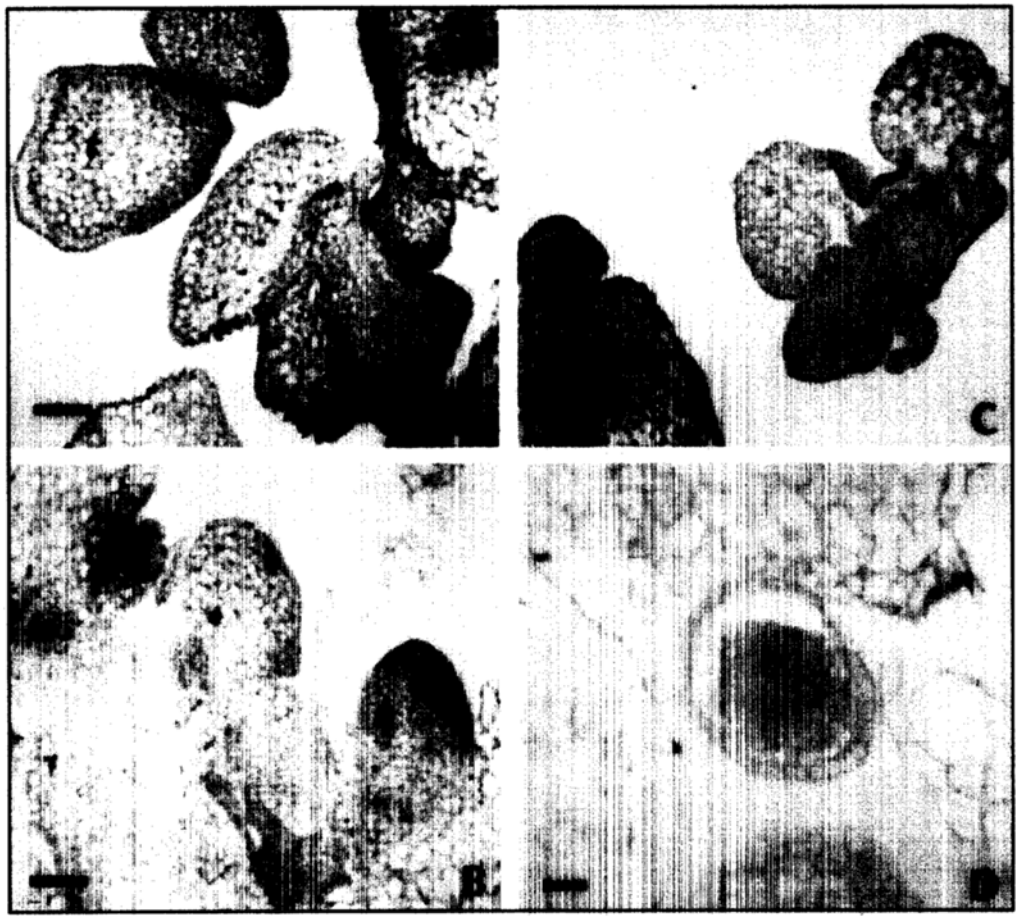

Figure 2. Histology of *L. gibba* G3 nodular tissue. Tissue consists of loose, unorganized, and large vacuolated cells (A and B) inside, and smaller, cytoplasmic cells (C) at its surface. D shows the polar structure of a nodular somatic embryo. Scale bars = 100 μm.

laginous calli and white, compact, nodular calli. By selectively sub-culturing the latter, we were able to produce homogeneous calli. Initial reports of somatic embryogenic calli in monocots have included this description of similarly mixed calli and morphology (Lu et al., 1989; Creemers-Molenaar and Beerepoot, 1992). The more homogeneous, friable, undifferentiated calli, which were capable of embryo regeneration, were produced after further culture manipulations that were designed to produce suspension cultures (Shillito et al., 1989).

In the daylily, culture on a solidified medium initially produces compact, nodular callus cultures capable of shoot regeneration. Under a variety of culture conditions, green, bipolar structures are obtained (Krikorian and Kann, 1979). Although it was initially supposed that those structures were proembryos, numerous manipulations of the conditions failed to promote complete embryo development. Further work showed that suspension cultures, comprising compact and white nodules, could be achieved by substituting coconut water for kinetin (Krikorian and Kann, 1981). These small nodules were labeled "nubbins" to distinguish them from the nodules of somatic embryogenic calli (Krikorian et al., 1988). Regeneration from the nubbins was assumed to be adventitious shoot formation because no macroscopic roots were observed, and because earlier cultural manipulations failed to produce the bipolar structure indicative of somatic embryos. However, photographs during nubbin development clearly showed globular proembryos and macroscopic bipolar forms (Krikorian and Kann, 1981). These photographs are quite similar to those of somatic embryogenesis in pearl millet (Vasil and Vasil, 1981). It is conceivable that the nubbin system of daylily is actually unrecognized somatic embryogenesis. If so, it is highly probable that frond regeneration in our duckweed system also occurred through somatic embryogenesis.

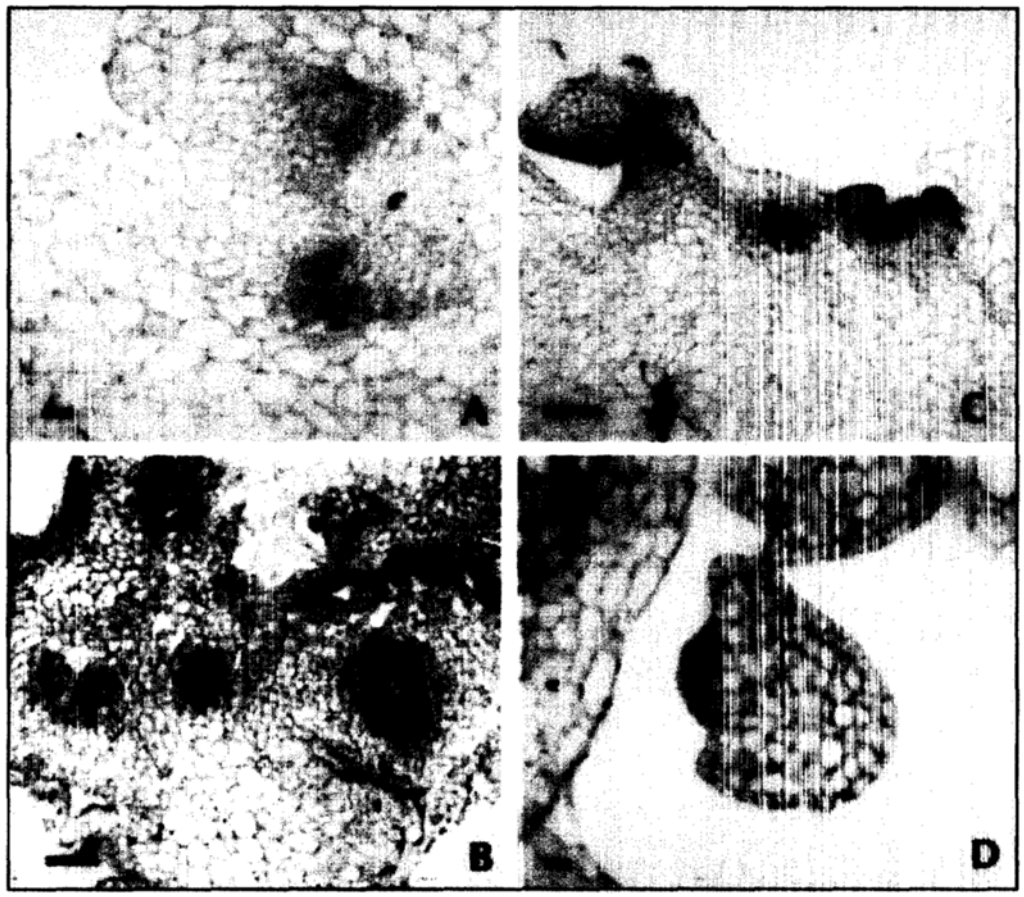

Figure 3. Cross-sectioned nodular somatic embryos. The secondary nodules formed at the surface of the bulk callus could be divided into four categories: those with few, small, cytoplasmic cells (A), those with small, cytoplasmic cells on the surface l ayer (B), those that comprised primarily small cytoplasmic cells (C), and those in which the small cytoplasmic cells defined a polar structure (D). Scale bars = 100 μm.

Here we have described the results when using an optimized method, i.e., suitable media type, phytohormones, and culturing conditions, for *L. gibba* G3. Compared with the strategy reported by Chang and Chiu (1978), the current study involved a two-week period for frond regeneration versus the eight weeks required in the previous research. The developmental anatomy of callus structure and frond regeneration should now clearly establish the sequence of events leading to frond formation. Our macroscopic examination of the regeneration from compact duckweed calli did not reveal any bipolar structures. However, bipolarity is not readily apparent in the zygotic embryos of *Lemna* because no radicals are formed (Blodgett, 1923a, 1923b; Maheshwari and Kapil, 1963).

This is the first report of somatic embryogenesis for any duckweed species. Further experiments are necessary to determine with certainty the developmental pathway for frond regeneration from calli. Regardless of that pathway, however, this tissue culture system can provide one possible route to genetically engineering duckweed.

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