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Establishment of Embryogenic Cell Suspension Culture of Chilli [*Capsicum annum* L. var. accuminatum Fingerh] for Somatic Embryogenesis

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ABSTRACT. *Chilli [Capsicum annum L. var. accuminatum Fingerh] is one of the important spice crops of Sri Lanka. Embryogenic suspension cultures of chilli were developed with an objective to induce somatic embryogenesis Successful callus induction was obtained from both leaves and cotyledons of two weeks old seedlings in MS medium containing 1 ppm 2, 4 - D, after incubation in the dark for two to three weeks. A combination of Kinetin (0.2 ppm) and 2,4- D (1 ppm) promoted callus proliferation at a high rate. Cell suspension cultures were established using 2 g of four week old leaf and cotyledon calli in 20 ml of liquid MS medium with 1 ppm 2, 4- D in 100 ml Erlenmeyer flasks. Weekly sub culturing was performed. MS medium with 2, 4-D (1 ppm) stimulated embryogenesis on cotyledon callus after 12 weeks in culture. Embryogenic calli formed are pale yellow to brown, compact, organized and nodular in appearance. It comprised of small, richly cytoplasmic cells without large vacuoles. Both initiation of embryogenic cells and the subsequent development of these cells into embryoids occurred in the same MS (2, 4-D 1 ppm) medium. Within a period of five to seven days, 12 week old, 20 ml of embryogenic cell suspension produced 14 proembryoids After 7-14 days they developed into heart stage and to mature embryoids Plantlet development has not observed until now in the tested MS media containing activated charcoal, zeatin, IBA and GA3.*

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INTRODUCTION

Chilli (*Capsicum annum* L. var. accuminatum Fingerh) is one of the most important spice crops of Sri Lanka. It is cultivated extensively in the dry zone of Sri Lanka. However the production has not been sufficient to meet the local demand and a large number of production constraints have been identified. Diseases and pests are the major problems. Last decade a hitherto unknown disorder has been threatening the production, especially in the dry zone areas known as Chilli Narrow Leaf Disorder (CNLD).

'In vitro' culture has often been acknowledged as one of the more promising avenues in plant improvement. Embryogenic suspension culture can be used to produce true - to - type plants for both agronomic and research purposes. In this study embryogenic suspension cultures of chilli (MI2) were developed with an objective to induce somatic embryogenesis.

Somatic or asexual embryogenesis is the production of embryo - like structures from somatic cells. The somatic embryo is an independent bipolar structure and is not physically attached to the tissue of origin. Such embryos can further develop and germinate into plantlets through the events that correspond with the zygotic occurrences (Baker, 1994).

Since 1980, embryogenic callus cultures have been used to establish fast growing and finely dispersed suspension cultures in a few species including important crops such as wheat, maize, rice and sugarcane. Embryogenic calli have been described as compact, organized, nodular and generally non- friable (Hall, 1991). Such callus cultures have been used successfully to establish stable, highly dispersed and totipotent embryogenic cell suspension cultures. The non-embryogenic callus is soft and translucent, grows more rapidly but consist mostly of large and vacuolated cells (Vasil and Vasil, 1991). Early identification of embryogenic sectors, their selective subculture on fresh media every one to three weeks are critical for long term maintenance of the cultures and their embryogenic potential (Dixon, 1985). Somatic embryos and plantlets are often formed on the callus induction medium itself, as the effective amount of 2, 4-D in the medium is reduced gradually during culture (Ho and Vasil, 1983).

This paper describes attempts made to develop callus induction from seedling leaves and cotyledons, callus proliferation, embryogenic callus induction, its long term maintenance and subsequent somatic embryogenesis in chilli (*Capsicum annum* L. var. accuminatum Fingerh.).

MATERIALS AND METHODS

'In vitro' seedling establishment, callus induction, proliferation and maintenance

Chilli (MI2) seeds obtained from Agriculture Research Station, Maha Illuppallama were washed thoroughly with running tap water and dipped in 0.5% mercuric chloride for 10 mins. later rinsed thrice in distilled water. The material was then sterilized with 10% sodium hypochlorite. After sterilization excessive bleach was washed off with sterile distilled water under aseptic conditions in a Laminar flow cabinet. The sterilized seeds were sown aseptically on hormone free MS medium with 0.8% agar and kept under dark condition for 2-3 weeks for germination. Seedlings were kept in 16 hrs. photoperiod with 1000 lux illumination.

From two week old seedlings, leaf and cotyledon explants were excised aseptically and cultured on MS medium, supplemented with growth regulators kinetin and 2, 4-D. In this factorial design, 2, 4-D levels 0, 0.5, 1, 1.5 and 2 ppm and kinetin levels 0, 0.5, 1, 1.5 and 2 ppm were tested in 25 different treatment combinations for callus induction.

Callus obtained was further studied in a factorial experiment similar to above but supplemented with kinetin and 2, 4-D for callus proliferation. In this factorial design, same 2, 4-D levels tested in the above study were combined with kinetin levels of 0, 0.1, 0.2, 0.3 and 0.4 ppm. Calli were sub cultured in MS solidified proliferation medium (MSCPM) in every two weeks.

Establishment and maintenance of embryogenic cell suspension cultures

Two g. of four weeks old actively growing leaf and cotyledon calli taken from MSCPM were used to establish cell suspension culture in 20 ml liquid MS medium. Eight MS medium modifications with four different levels of 2, 4-D and four different combinations of 2, 4-D and kinetin were used to establish the medium for development of embryogenic potential. Different levels of 2, 4-D tested were 0.5, 1, 1.5 and 2 ppm; in the combinations of 2, 4-D and kinetin; the tested 2, 4-D level was 1 ppm; tested kinetin levels were 0, 0.1, 0.2 and 0.3 ppm. The Erlenmeyer flasks (100 ml) were placed on a gyrotary shaker at 100 rpm in 16 hrs. photoperiod of 1000 lux illumination. After 7-10 days the contents of the flask were transferred to two flasks, each with 20 ml of fresh medium. This was repeated 5-6 times at weekly intervals. At the end of this period a number of small groups of cells were seen in the cultures.

These small groups of cells were pipetted into a separate flask along with the medium. After allowing the cell clusters to settle for a few seconds, the medium from the top was discarded and replaced with an equal volume of fresh medium. This enrichment of the cultures with small groups of actively dividing cells was repeated once a week for 6 weeks in order to obtain a stable and well dispersed embryogenic cell suspension culture. The development of embryogenic callus was monitored using regular microscopic observations of suspension cells.

Somatic embryogenesis from established embryogenic cell suspension culture

For the subsequent development of embryogenic cells into embryoids, liquid and solidified MS media modifications with five different levels of 2, 4-D were tested. The tested levels of 2, 4-D were 0.2, 0.4, 0.6, 0.8 and 1 ppm For plantlet development solidified MS medium supplemented with 0.5% activated charcoal, sucrose (5%), zeatin, IBA and GA3 (each 0.1 mg /l) was tested.

Histological studies

Embryogenic calli were fixed in formalin acetic acid and then dehydrated in a series of ethanol solutions starting from 25%, 25-100% (each treatment for 2 hours and then in a series of treatments with absolute alcohol:xylene (3:1), abs. alcohol:xylene (1:1), abs. alcohol:xylene (1:3), pure xylene I, pure xylene II (each for 2 hours) before being embedded in paraffin heated to 50-55°C. Sections of 10 μm were prepared with a rocking microtome, mounted on glass slides and observed under microscope and plates were prepared using video microscopy. The same study was conducted to reveal the structures of embryoid developmental stages.

RESULTS AND DISCUSSION

Seedling establishment, callus induction and proliferation

Chilli seedlings were established in hormone free MS medium with 0.8% agar (Figure 1a). Successful callus induction was obtained on cotyledons (Figure 1b) and leaves (Figure 1c) of two weeks old seedling explants (as in

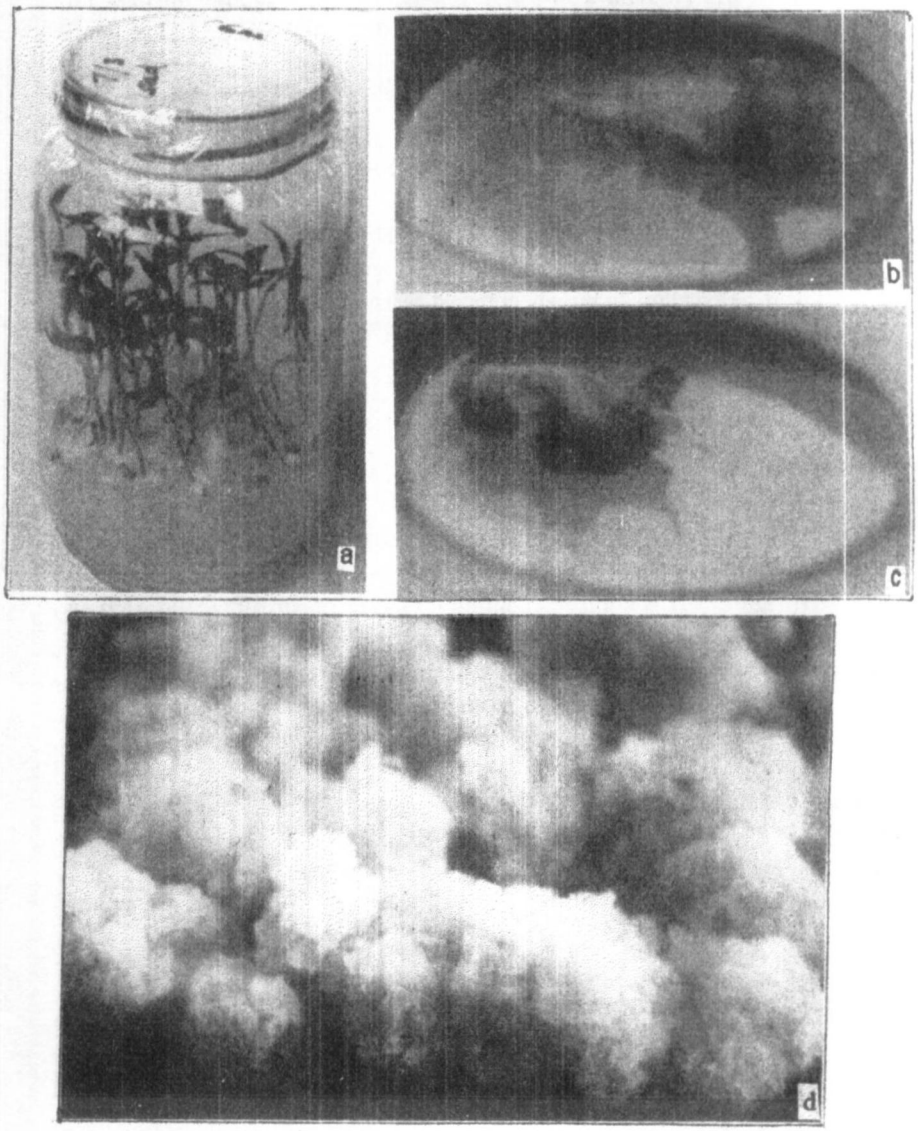

Figure 1. **Seedling establishment, callus induction proliferation of *Capsicum annum*. a) Chilli seedlings established in hormone free MS medium; b and c) Callus induction of cotyledon, leaf explant in MS medium with 1 ppm 2, 4-D; d) Callus proliferation in MS medium with kinetin (0.2 ppm) and 2, 4-D (1 ppm).**

Figure 1a) in MS medium with 1 ppm 2, 4-D after incubation in the dark for two to three weeks. A combination of kinetin (0.2 ppm) and 2, 4-D (1 ppm) promoted callus proliferation (Figure 1 d).

Establishment of embryogenic cell suspension culture

MS medium with 1 ppm 2, 4-D stimulated embryogenesis on cotyledon callus after 12 weeks in culture (Figure 2a). Embryogenic calli formed were pale yellow to brown, compact and nodular in appearance (Figure 2b). It comprised of small, richly cytoplasmic cells without large vacuoles (Figure 2c).

Histological study

After 12 weeks of culture the callus tissue consisted mostly of small, round and friable nodules. The cross section of embryogenic callus (Figure 2b) showed (Figure 2d) a compact continuous layer of smaller cells, rich in cytoplasm, underneath a layer of large partly disrupted superficial cells. Towards the center of the nodules, cells become larger and vacuolated. In the parenchymatous tissue of nodules, numerous meristematic centers, oriented towards the surface, were observed. They resembled root or shoot primordia. The development of embryoids was frequently observed in histological sections. Globular stage was often seen in the same specimen (Figure 3b). The embryoids were found at or near the surface of the callus, but their location could not be determined precisely, since they tend to be released by sectioning of the tissue.

Somatic Embryogenesis from Established Embryogenic Cell Suspension Culture

Although Vasil and Vasil (1991) reported the need for much lower levels of 2, 4 - D for embryo maturation, it was found that both, initiation of embryogenic cells and subsequent development of these cells into embryoids occurred in the same MS (1 ppm 2, 4 - D) medium. Within a period of five to seven days, 12 weeks old embryogenic cells in the 20 ml. suspension produced 14 pro embryoids (Figure 3a and b) and after 7 - 14 days they developed into heart stage (Figure 3c) and then to mature embryoids. (Figure 3: d, e, f, and g) Plantlet development was not observed with the tested MS media.

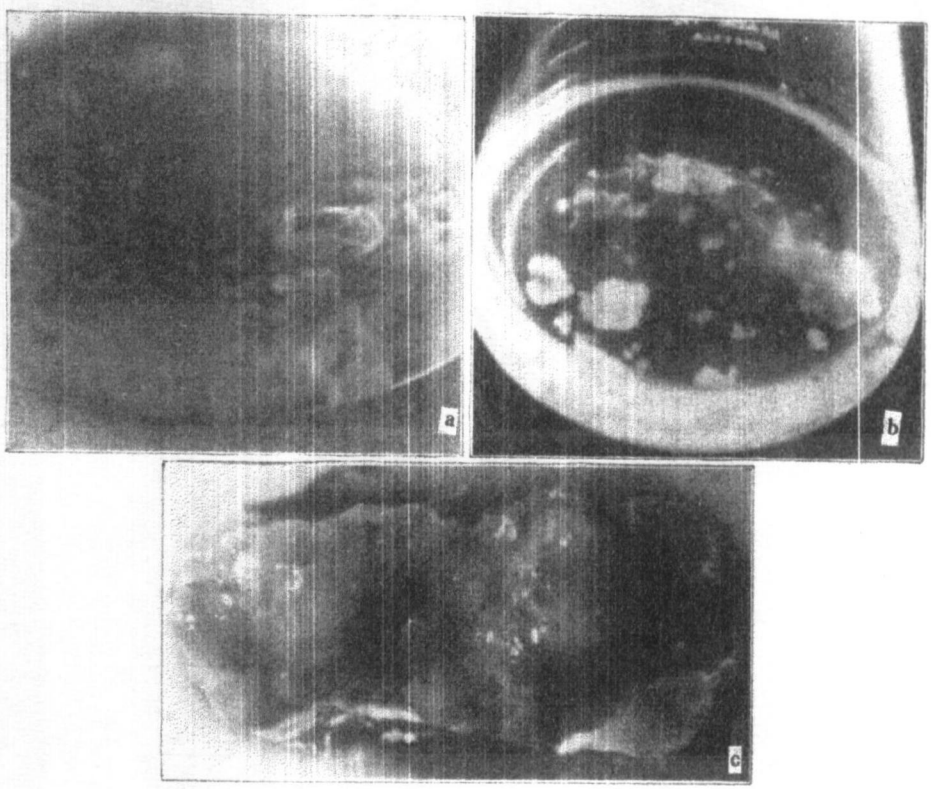

Figure 2(a-c). **Establishment of embryogenic cell suspension culture.**
a and b) Embryoids maturing after 13 and 15 weeks from 12 weeks old established embryogenic cell suspension culture in liquid MS with 1 ppm 2, 4-D x 2;
c) Established embryogenic callus in liquid medium with 1 ppm 2, 4-D x 10.

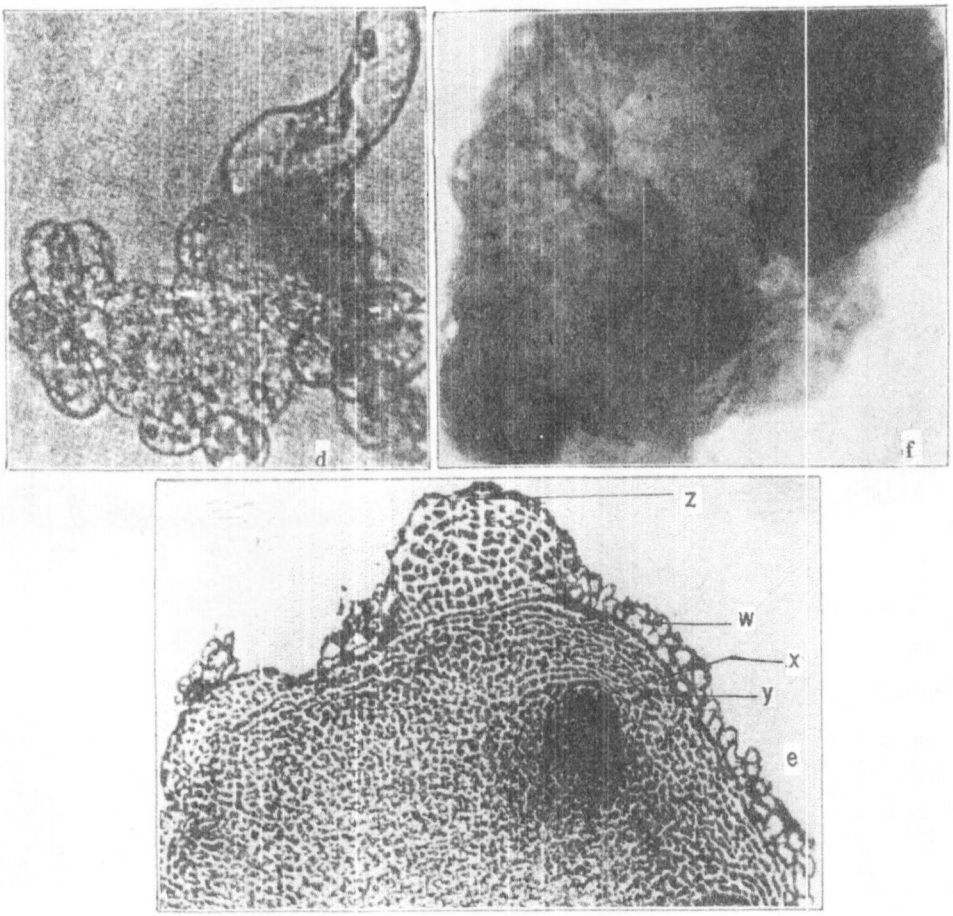

Figure 2 (d-f). **Establishment of embryogenic cell suspension culture.**
d) Group of embryogenic cells (small and richly cytoplasmic) from the cell suspension culture x 40;
e) Cross section of an embryogenic callus grown on 1 ppm 2, 4-D containing medium, (w) large superficial cells, (x) continuous tissue of small cytoplasmic cells, (y) meristematic centers and (z) peripheral spheric outgrowths;
f) Established embryogenic callus showing groups of embryogenic cells x 40.

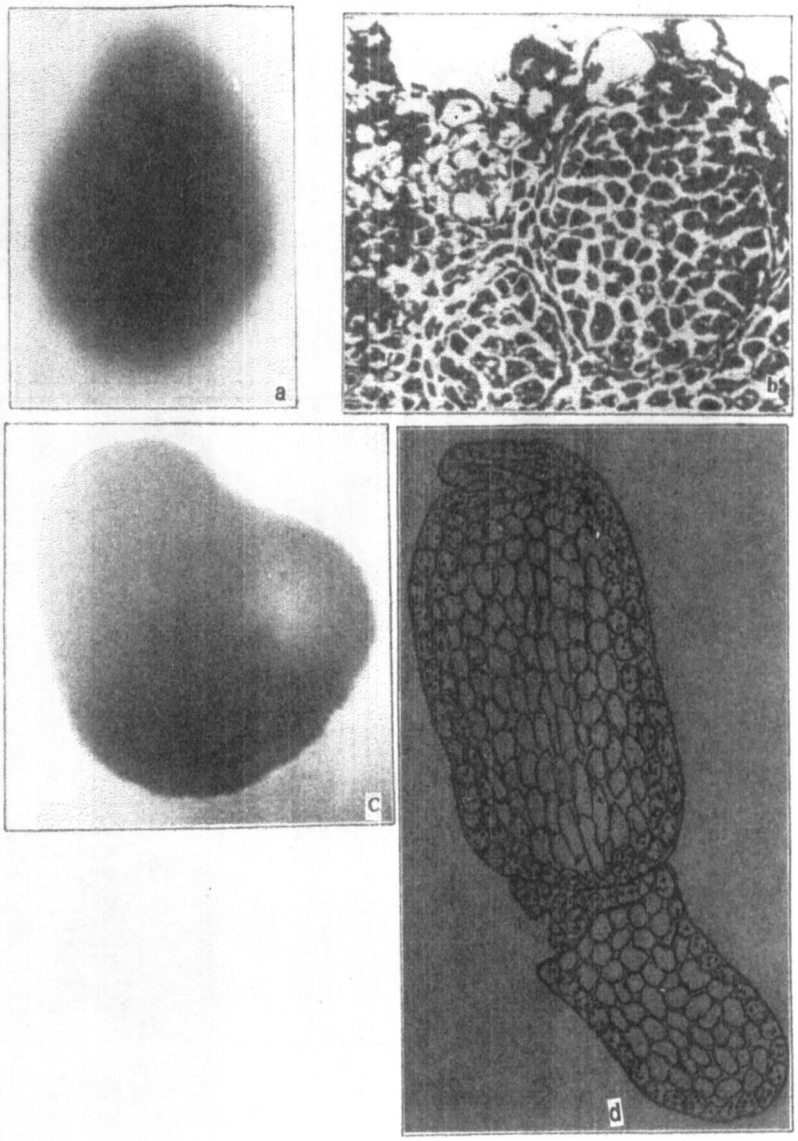

Figure 3(a-d). **Somatic embryogenesis from established embryogenic cell suspension culture.**
a) Globular embryoid x 40;
b) Sections of two globular embryoids x 40;
c) Heart - shaped embryoids x 40;
d) Section of Torpedo - shaped embryoid x 60.

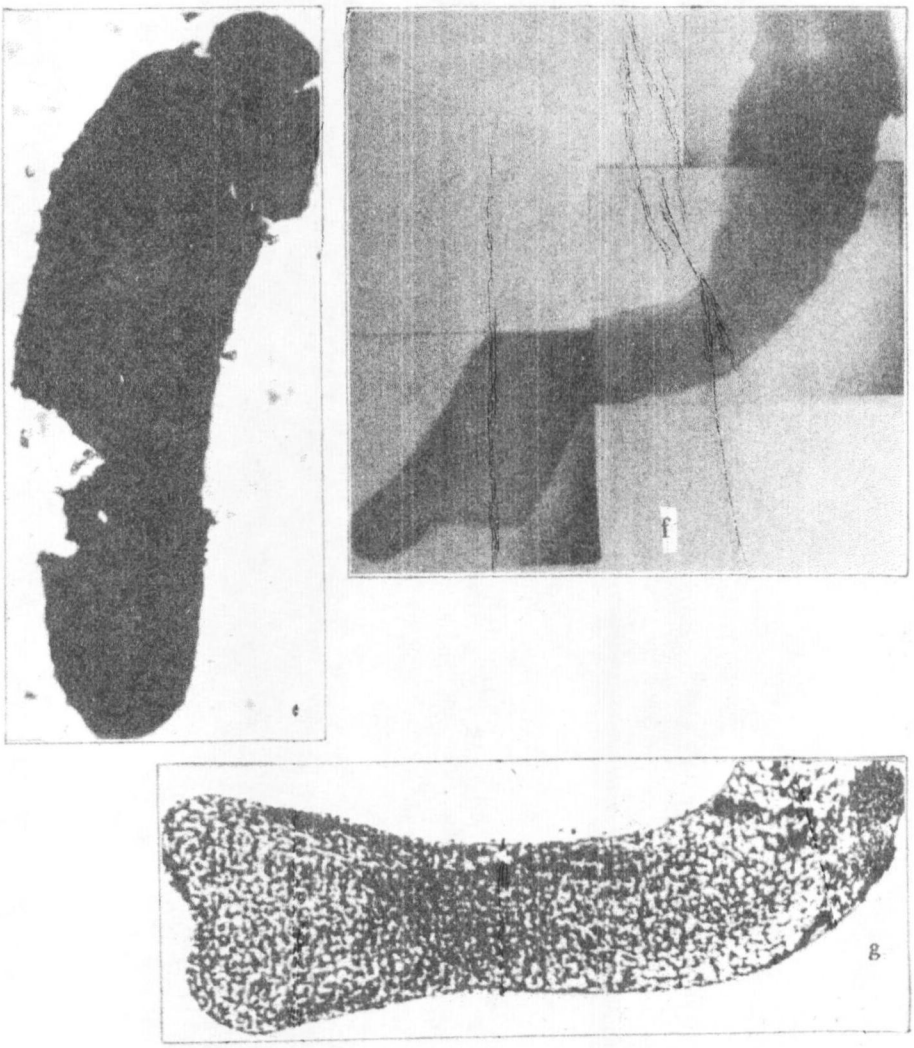

Figure 3(e-g). **Somatic embryogenesis from established embryogenic cell suspension culture.**
e) Torpedo - shaped embryoid x 40;
f) Late Torpedo - shaped embryoid x 40;
g) Section of late Torpedo - shaped embryoid x 40.

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