Micro-propagation by adventitious organs

Caulogenesis

Rhizogenesis

Somatic embryogenesis
In vivo plant embryogenesis
Somatic Embryogenesis (s.e.) in Tissue culture

Somatic embryogenesis is a process whereby somatic cells differentiate into somatic embryos.

The first observation of somatic embryo formation in *Daucus carota* cell suspensions by Steward et al. (1958) and Reinert (1958).
Since its discovery the potential for s.e. has been shown to be characteristic of a wide range of tissue culture systems in plants and described in a large number of plant species.

S.e. can probably be achieved for all plant species provided that the appropriate explant, culture media and environmental conditions are employed.
During the course of evolution, many plant species have evolved different methods of asexual embryogenesis, including somatic embryogenesis, to overcome various environmental and genetic factors that prevent fertilization.

Somatic embryogenesis occurs to a limited extent under natural conditions, within ovules (e.g., *Paeonia*) and more rarely on leaves (e.g. *Asplenium* and *Kalanchoe*).
Somatic embryos resemble morphologically zygotic embryos

**Globular**

- Apical cells
- Basal cells
- Cotyledon
- Axis
- Protoderm
- Shoot apex
- Cotyledon
- Axis
- Root apex

**Stages:**
- Globular
- Heart
- torpedo
- Cotyledonar
S.e. stages in eggplant

- Globular
- Hearth
- Torpedo
- Germinated s.embryos
Seven day is the s.e. cycle in eggplants

• Cotyledon leaf explants at 7 days after of sowing were left to grow in a medium containing NAA.

In left inside panel, histological section of leaves after only 3 days on induction medium. In the middle, are visible somatic embryos and in the last histological section of seven days older explants in which are visible s.embryos and no usually structure of leaf.
Use of Somatic Embryogenesis

Somatic embryos are used as a model system in embryological studies.

The greatest interest of somatic embryos is centred in its practical application for large-scale vegetative propagation, particularly because of the possibility to scale up the propagation by using bioreactors.

In most cases, somatic embryos or embryogenic cultures can be cryopreserved, which makes it possible to establish gene banks.

Embryogenic cultures are also an attractive target for gene transformation.
Effects of auxin in s.e.

The process of somatic embryogenesis (s.e.) is often initiated in media containing high levels of auxins (especially 2,4-D), but embryos usually do not develop further until the auxin concentration is reduced.

In literature have been reported several exceptions to this general observation.

- In some cultures embryos developed in medium devoid of auxin. Embryogenesis may have been induced by endogenous hormones and reduction of its has permitted the formation of embryos.
- Higher level of 2,4D induces embryos formation in alfalfa while low callus
DIRECT EMBRYOGENESIS

Somatic embryos are often initiated directly upon explanted tissues.

The highest successful explant for direct embryogenesis is associated with, or immediately derived from, the female gametophyte.

The tendency for these tissues to give rise to adventitious somatic embryos is especially high in plants where sporophytic polyembryony occurs naturally, for example, some varieties of *Citrus* and other closely related genera.
The nucellus tissue of many plants has the capacity for direct embryogenesis *in vitro*

The high embryogenic competence of the nucellus is usually retained during subsequent cell generations in vitro, should the tissue be induced to form ‘callus’ (or cell suspensions).

It is not clear whether all cells of the nucellus are embryogenically committed.
Ovules, nucellar embryos, nucellus tissues and other somatic embryos are particularly liable to display direct embryogenesis.

In *Carica somatic* embryos originated from the inner integument of ovules (Litz and Conover, 1981a,b) and in carrot tissue of the mericarp seed coat can give rise to somatic embryos directly (Smith and Krikorian, 1988).
In Citrus

• In Citrus, somatic embryos are formed from the nucellus even in cultivars that are normally monoembryonic, whether the ovules have been fertilised or not.

It has been suggested that only those cells destined to become zygotic proembryos can become somatic proembryos or give rise to embryogenic callus (Sabharwal, 1963); somatic embryos have been shown to arise particularly from the micropylar end of Citrus nucellus.
Embryogenesis from microspores or anther culture

- Somatic embryos can be initiated directly from microspores.
- Usually it is necessary to culture the microspores within anthers, but occasionally it has been possible to induce embryogenesis from isolated microspores.
- Anther culture can result in callus formation; the callus may then give rise to plants through indirect embryogenesis or adventitious shoot formation.
Zygotic embryos as explant for direct embryogenesis

Adventitious embryos are commonly formed *in vitro* directly upon the zygotic embryos of monocotyledons, dicotyledons and gymnosperms, upon parts of young seedlings (especially hypocotyls and cotyledons) and upon somatic embryos at various stages of development (especially if their growth has been arrested).
What is a Protocorm?

The seeds of orchids contain a small embryo (0.1 mm diameter), without any endosperm storage tissue.

Upon germination, the embryo enlarges to form a small, corm-like structure, called a protocorm, which possesses a quiescent shoot and root meristem at opposite poles.
Protocorm-like bodies (PLBs) formation in orchids

During *in vitro* culture of different types of orchid organs and tissues, bodies are formed and appear to be identical with seedling protocorms growing into plantlets.

These somatic protocorms can appear to be dissimilar to seedling protocorms, and many workers on orchid propagation, have used terms such as ‘protocorm-like bodies’ (PLBs) to describe them.
Practical uses in propagation

From a quantitative point of view, indirect embryogenesis does provide an efficient method of micropropagation; the same is not true of direct embryogenesis when it is unaccompanied by the proliferation of embryogenic tissue.

Although plants can be regenerated from embryos directly initiated *in vitro*, and may be present in sufficient numbers for limited plant production in breeding programmes, the numbers of primary embryos per explant will usually be inadequate for large scale cloning.
Abnormal embryos and plantlets

A proportion of the seedlings developing from somatic embryos can also be atypical: abnormalities include the possession of multiple or malformed cotyledons, more than one shoot or root axis, and the presence of secondary adventive embryos.

Embryos with three cotyledons have been observed to give rise to well-formed plantlets (Smith and Krikorian, 1990).

Abnormal somatic embryos do however produce secondary embryos, which are usually of normal morphology.
Abnormal embryos

- Trumpet-like
- One cotyledon
- Root
Abnormal embryos and plantlets

Unfortunately embryogenesis in both callus and suspension cultures is seldom synchronous so that embryoids at different stages of development are usually present in a Stage II culture from the onset.

This presents a major drawback for plant propagation which could otherwise be very rapid, especially from suspensions.
Current applications

Few plant species are at present propagated on a large scale via embryogenesis *in vitro*. This method of morphogenesis does however offer advantages which suggest that it will be used increasingly for plant cloning in the future:

Table 2
*In vitro* somatic embryogenesis of major ornamental pot plants

<table>
<thead>
<tr>
<th>Species/Cultivars</th>
<th>Culture response</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Begonia gracilis</em></td>
<td>emc, gse, pt</td>
<td>Castillo and Smith</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(1997)</td>
</tr>
<tr>
<td><em>Dendranthema grandiflora</em> cv. Yellow Spider</td>
<td>emc, gse, pt</td>
<td>Sauvadet et al. (1990)</td>
</tr>
<tr>
<td><em>Dendranthema grandiflora</em></td>
<td>emc, gse, pt</td>
<td>May and Trigiano (1991)</td>
</tr>
<tr>
<td><em>Dendranthema grandiflora</em> cv. Yellow Spider</td>
<td>emc, gse, pt</td>
<td>Pavingerova et al. (1994)</td>
</tr>
<tr>
<td><em>Dendranthema grandiflora</em></td>
<td>emc, gse, pt</td>
<td>Tanaka et al. (2000)</td>
</tr>
<tr>
<td><em>Cyclamen persicum</em></td>
<td>ecs, gse, pt</td>
<td>Hohe et al., 2001; Schwenkel (2001)</td>
</tr>
<tr>
<td><em>Cyclamen persicum</em></td>
<td>emc, gse, pt</td>
<td>Pueschel et al. (2003)</td>
</tr>
<tr>
<td><em>Euphorbia pulcherrima</em> cv. Angelika</td>
<td>emc, gse, pt</td>
<td>Osiemack et al. (1999)</td>
</tr>
<tr>
<td><em>Rosa hybrida</em> cvs. Domingo, Vickey Brown, Tanja, Azteca</td>
<td>emc, gse</td>
<td>de Wit et al. (1990)</td>
</tr>
<tr>
<td><em>Rosa hybrida</em> cv. Landora</td>
<td>emc, gse</td>
<td>Rout et al. (1991)</td>
</tr>
<tr>
<td><em>Rosa rugosa</em></td>
<td>emc, gse, pt</td>
<td>Kunitake et al. (1993)</td>
</tr>
<tr>
<td><em>Rosa</em> sp. cvs. Baccara, Mercedes, Ronto, Soray</td>
<td>emc, gse, pt</td>
<td>Kintzios et al. (1999)</td>
</tr>
<tr>
<td><em>Rosa hybrida, Rosa chinensis minima</em></td>
<td>emc, gse, pt</td>
<td>Li et al. (2002a)</td>
</tr>
<tr>
<td><em>R. hybrida</em> cv. Sumpath</td>
<td>emc, gse, pt</td>
<td>Kim et al. (2003a)</td>
</tr>
<tr>
<td><em>Saintpaulia ionantha</em> cv. Benjamin</td>
<td>emc, gse, pt</td>
<td>Murch et al. (2003)</td>
</tr>
</tbody>
</table>

Abbreviation: emc = embryogenic callus, ecs = embryogenic cell suspension, gse = germination of somatic embryos, pt = plantlet development.
Current applications

• In some monocotyledons (e.g. cereals, date palm and oil palm) it provides a method of micropropagation where shoot culture has not been successful (but note however that in some attempts to clone oil palms through embryogenesis, the resulting plants have been very variable);

• Providing embryogenic cell suspensions can be established, plantlets can theoretically be produced in large numbers and at much lower cost because plantlets do not have to be handled and subcultured individually;

• Somatic embryos probably provide the only way for tissue culture methods of plant propagation to be economically deployed on extensively planted field crops and forest trees.
Fig. 1. *In vitro* somatic embryogenesis of *Euphorbia pulcherrima*. (A) Isolated somatic embryos of *E. pulcherrima* (bar = 0.1 cm). (B) Germination of somatic embryos (bar = 0.25 cm). (C) Somatic embryos derived plantlets acclimatised in the greenhouse (bar = 0.5 cm). (D) Flowering of somatic embryo-derived plants (bar = 2.5 cm).
Direct somatic embryogenesis and plant regeneration from leaf explants of *Phalaenopsis amabilis*

Abstract

Leaf explants of *Phalaenopsis amabilis* var. formosa formed clusters of somatic embryos directly from epidermal cells without an intervening callus within 20 - 30 d when cultured on 1/2-strength modified Murashige and Skoog medium supplemented with 0.1, 1 and 3 mg dm\(^{-3}\) TDZ.

Repetitive production of embryos involved secondary embryogenesis could be obtained by culturing segments of embryogenic masses on TDZ-containing media.

Plantlet conversion from embryos was successfully achieved on regulator-free growth medium.
Introduction

Phalaenopsis (Orchidaceae), has highly economical value in flower markets in the world.

Therefore, lots of in vitro culture protocols have been developed in this genus.

However, only two among them described somatic embryogenesis. In this communication, we describe a simple and suitable protocol for induction of direct somatic embryogenesis and secondary somatic embryogenesis in Phalaenopsis.
Materials and methods

Plants and culture conditions

- **Green capsules** were collected from pot plants of *Phalaenopsis amabilis* Shimadzu var. *formosa* after self-pollination for three months. The capsules were immersed in 70 % alcohol for 30 s, and followed by agitation for 15 min in a solution of 2 % sodium hypochlorite and 0.05 % *Tween (1:1 v/v)*.

- **Seeds** from these capsules were sown on modified Murashige and Skoog (1962; MS) basal medium containing halh-strength macro- and micro-elements supplemented with [mg dm-3]: myo-inositol (100), niacin (0.5), pyridoxine HCl (0.5), thiamine HCl (0.1), glycine (2.0), peptone (1 000), NaH2PO4 (170), sucrose (20 000), and *Gelrite (2 200)*.

- **Plant growth regulators** were added prior to autoclaving. The pH of the media was adjusted to 5.2 with 1 M KOH or HCl prior to autoclaving for 15 min at 121 °C.
Materials and methods

- **Culture growing condition**: Leaf explants were incubated in 90 × 15 mm Petri dishes under a 16-h photoperiod at irradiance of 28 - 36 μmol m-2 s-1 (daylight fluorescent tubes FL-30D/29, 40 W, China Electric Co., Taipei, Taiwan) and temperature of 26 ± 1 °C.

- **Subculture and explants**: The subculture period was 2 months. After 180 d of culture, these seeds developed into plants with 3 - 5 leaves and 2 - 4 roots. These seedlings were used as donor plants.

- **Induction of direct embryo formation from leaf explants**: Leaf tip segments (about 1 cm in length) taken from the donor plants were used to test the effects of naphthaleneacetic acid (NAA; 0, 0.1, 1 mg dm-3) and thidiazuron (TDZ; 0, 0.1, 1, 3 mg dm-3) on direct somatic embryogenesis. Eight replicates (dishes) each with four leaf explants were used for each treatment.
Materials and methods

- **How to score data:** The percentage of explants forming somatic embryos was recorded. The number of embryos formed from each responding explant was counted under a stereomicroscope *(SZH, Olympus, Tokyo, Japan)* at the protocorm stage (45 d of culture). Treatment means were compared by following Duncan's Multiple Range Test *(Duncan 1955)*.

- **Induction of embryo formation from leaf-derived nodular masses and secondary embryogenesis:** Pieces of nodular masses (about 0.1 g) were used as explants to test the effect of TDZ *(0, 0.01, 0.1, 1, 3 mg dm^-3)* on secondary somatic embryogenesis. Eight replicates (tubes) each with one explant were used for each treatment. The proliferation rate of nodular masses was measured as final fresh mass divided by initial fresh mass. The number of embryos formed from each responding explant was counted at the protocorm stage (45 d of culture).
Materials and methods

- **Histology of direct somatic embryogenesis from leaf explants:** Tissues for histological observations were fixed in FAA (95 % ethyl alcohol + glacial acetic acid + formaldehyde + water, 10:1:2:7), dehydrated in a tertiarybutyl-alcohol series, embedded in paraffin wax, sectioned at 10 μm thickness and stained with 0.5 % safranin-O and 0.1 % fast green (Jensen 1962)
Direct somatic embryogenesis from leaf explants

Table 1. Effects of NAA and TDZ on direct embryo formation from leaf explants of *Phalaenopsis amabilis*. The frequency of embryo-forming explants and the mean number of embryos per explant were scored after 45 d of culture. Means of 32 replicates (explants) with the same letters are not significantly different at $P \leq 0.05$ (Duncan 1955).

<table>
<thead>
<tr>
<th>NAA  [mg dm$^{-3}$]</th>
<th>TDZ  [mg dm$^{-3}$]</th>
<th>Embryogenesis [%]</th>
<th>Number of embryos [explant$^{-1}$]</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0.0g</td>
<td>0.0g</td>
</tr>
<tr>
<td>0</td>
<td>0.1</td>
<td>62.5c</td>
<td>6.6d</td>
</tr>
<tr>
<td>0</td>
<td>1.0</td>
<td>71.9ab</td>
<td>7.5cd</td>
</tr>
<tr>
<td>0</td>
<td>3.0</td>
<td>93.8a</td>
<td>19.4a</td>
</tr>
<tr>
<td>0.1</td>
<td>0</td>
<td>0.0g</td>
<td>0.0g</td>
</tr>
<tr>
<td>0.1</td>
<td>0.1</td>
<td>25.0ef</td>
<td>1.3fg</td>
</tr>
<tr>
<td>0.1</td>
<td>1.0</td>
<td>43.8d</td>
<td>3.5e</td>
</tr>
<tr>
<td>0.1</td>
<td>3.0</td>
<td>87.5a</td>
<td>13.3b</td>
</tr>
<tr>
<td>1.0</td>
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<td>0.0g</td>
<td>0.0g</td>
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<tr>
<td>1.0</td>
<td>0.1</td>
<td>12.5fg</td>
<td>0.7fg</td>
</tr>
<tr>
<td>1.0</td>
<td>1.0</td>
<td>37.5de</td>
<td>2.4ef</td>
</tr>
<tr>
<td>1.0</td>
<td>3.0</td>
<td>81.3ab</td>
<td>9.1c</td>
</tr>
</tbody>
</table>
Plant regeneration through direct somatic embryogenesis from leaf explants of *P. amabilis*

A) somatic embryos formed from a leaf explant after 20 d of culture (bar = 700 μm);

B) leaf-derived embryos enlarged and elongated after 30 d of culture (bar = 750 μm);

C) the embryos turned green under light and developed into young somatic protocorm after 45 d of culture (bar = 850 μm);

D) somatic embryos formed from subculture leaf-derived nodular masses (bar = 950 μm);

E) the embryos developed shoots and some formed secondary embryos (bar = 1.2 mm);

F) leaf-derived embryos formed shoots (bar = 2 mm);

G) a plantlet converted from the leaf-derived embryos (bar = 7.2 mm)
Results and discussion

Histology of direct somatic embryogenesis

Fig. 2. Histology of direct somatic embryogenesis from leaf explants of Phalaenopsis amabilis: A - small and densely stained meristematic cells were originated from the epidermal layer of a leaf explant (bar = 200 μm); B - a somatic embryo (SE) contained small embryonic cells was form from the epidermal layer of a leaf explant (bar = 150 μm); C - several bulges of meristematic cells and a SE from from epidermal cell layers of a leaf explant (bar = 600 μm); D - small and densely stained cells were originated from both of the adaxial side (Ad) and the abaxial side (Ab) of a leaf explant (bar = 450 μm); E - bulges of meristematic cells on the leaf explant (bar = 450 μm); F - somatic embryos formed from meristematic cells (bar = 150 μm).
Results and discussion

Repetitive production of leaf-derived embryos and secondary embryogenesis

Table 2. Effect of TDZ on embryo formation from leaf-derived embryogenic masses of *Phalaenopsis amabilis*. Data were scored after 45 d in culture for the proliferation rate (final fresh mass divided by initial fresh mass) and the mean number of embryos. Means of 8 replicates are not significantly different at $P \leq 0.05$ (Duncan 1955).

<table>
<thead>
<tr>
<th>TDZ [mg dm$^{-3}$]</th>
<th>Proliferation rate</th>
<th>Number of embryos [explant$^{-1}$]</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>necrosis</td>
<td>-</td>
</tr>
<tr>
<td>0.01</td>
<td>1.9 c</td>
<td>3.1c</td>
</tr>
<tr>
<td>0.10</td>
<td>2.2 c</td>
<td>3.9c</td>
</tr>
<tr>
<td>1.00</td>
<td>3.1 b</td>
<td>10.8</td>
</tr>
<tr>
<td>3.00</td>
<td>5.4 a</td>
<td>13.8</td>
</tr>
</tbody>
</table>
Results and discussion

Plant regeneration

Whole leaf cultures with embryos/protocorms were transferred onto hormone-free medium and kept under a 16-h photoperiod.

Under this condition, protocorms continued developing and furtherformed shoot (Fig. 1F). *Plantlets were obtained after 6 – 8 weeks of culture* (Fig. 1G). After subculturing every 6 weeks for three times, the plantlets developed 5 - 6 leaves and 3 - 4 roots. These plants were potted in sphagnum moss for acclimatization in greenhouse. After 6 months, the plants performed normal and the survival rate was 100 %. There were no obvious differences in morphology between TDZ-induced and TDZ + NAA-induced plants.
Figure 1. Schematic diagram of *in vitro* embryonic developmental pathways.
Stresses

Exogenous PGRs

ARF7, ARF19, PRC1

Polymerase, LBD29, KYP/SUVH4

Response to auxin and brassinosteroid stimulus, Jasmonic acid metabolic

Cellulose biosynthetic and metabolic activity

Change in Cell wall and acquire Dedifferentiation Potential

LEC2, LEC1

Increase endogenous auxin levels

CLF

WUS

SERK1

Chromatin remodeling
Jasmonic acid signalling, auxin signalling
Brassinosteroid homeostasis/signaling, auxin signalling, GTPase signalling, salicylic acid signalling

Somatic embryos

Development

Commitment to embryogenic cells

Ethylene signaling, abscisic acid signaling, G2 cell cycle regulation, methylation, cell division and microtubule organization

STM, WUS, CDKA;1, PRZ1

Auxin

Expression of Totipotency