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Plant Regeneration via Organogenesis and Somatic Embryogenesis

2.1 BACKGROUND AND BASICS

One can achieve plant regeneration in a test tube by using suitable culture media through two morphogenetic pathways. In general, there are two different modes of plant regeneration *in vitro* for propagation in practice, namely, organogenesis and somatic or non-zygotic embryogenesis. Demonstration of organogenesis was evident from the classical work in tobacco carried out by Skoog and Miller in 1957. Propagation of a wide variety of plant species through the techniques of cell, tissue and organ culture is popularly referred to as *in vitro* plant propagation or micropropagation. The major benefits of this method include rapid multiplication of superior clones within a short time and maintenance of the uniformity i.e. true to type. Practically, there are three different modes of *in vitro* plant regeneration in practice, namely, axillary shoot proliferation, organogenesis and somatic embryogenesis (Altman and Loberant, 1998).

Micropropagation is the only aspect of plant tissue culture, which has been convincingly documented with regard to its feasibility for mass scale propagation commercially. It is an extension of the traditional methods of plant propagation, wherein shoot tips, buds and nodal segments of desired elite plants are cultured

2.2 CALLUS INDUCTION, ORGANOGENESIS AND SOMATIC EMBRYOGENESIS

Callus and Callus Induction

Callus, a mass of undifferentiated plant cells, have the capacity to regenerate into a whole plant. Stems, leaves, roots, flowers, seeds or any other parts (preferably young explants) of plant species can be used to induce callus tissue, however the successful production of callus depends upon plant species and conditions provided. Dicotyledonous plants are rather amenable for callus tissue induction, as compared to monocotyledonous plants, whereas in woody plants growth of callus is generally slow. In culture, this dedifferentiated mass of cells can be maintained indefinitely, provided the callus is sub-cultured on the fresh medium.

***Protocol for callus induction and maintenance using explants from H. muticus* L.**

- Follow the similar protocol for germination of seeds *in vitro* as described in the Chapter 1.
- Collect the aseptically germinated seedlings with fully expanded cotyledons of *H. muticus* L. after ten days of growth *in vitro*. Remove each seedling, and place one at a time on a sterile petri dish and prepare by trimming the explants using a sterile scalpel blade.
- Alternatively, remove a seedling, cut and remove the root portion and place or inoculate it on to MS basal agar medium for further growth. The *in vitro* grown plants derived from the seedlings can also be used as a source of explants for callus induction and other experiments.
- Sub-culture these plants onto fresh MS medium on a monthly basis to maintain them as master cultures.
- Excise the two cotyledons from the seedlings and cut each into equal halves. Culture them abaxial side up or upside down, on MS basal agar medium supplemented with NAA (1.0 mg/l), 2,4-D (0.2 mg/l) and BA (0.5 mg/l) for callus induction.
- Hypocotyl sections from the decapitated seedlings can also be used in the above medium for callus initiation.
- Seal the culture vessels with parafilm and incubate them in culture room conditions (as described in Chapter 1) for 4 weeks.
- Observe regularly hypocotyl vs. cotyledon explants for callus induction and record the growth patterns.
- Excise small pieces of callus of about 0.3-0.5 g fresh weight, and sub-culture on fresh (above mentioned) callus induction medium every month to maintain a callus stock.

Organogenesis

Organogenesis, both directly from explants or indirectly from callus cultures, relies on the inherent plasticity of plant tissues.This diffrenetiation of plant organs i.e. roots and shoots from callus or tissue (organogenesis) is an important achievement in plant tissue culture. It is one of the widely used methods employed for *in vitro* plant regeneration. Plant growth regulator regimes can be used to manipulate the morphogenetic response of plants under *in vitro* cultures.

Protocol for induction of shoots from callus cultures of H. muticus L.

- Select an actively growing healthy callus culture stock obtained from the previous protocols.
- Place one to two pieces of callus of about 0.5 g fresh weight in each culture vessel preferably test tubes or disposable plastic Petri dishes (90-100 mm) containing 10-15 ml of MS agar media supplemented with 3% (w/v) sucrose, meso-inositol (100 mg/l), NAA (0.5mg/l) and BA (0.25 or 0.5 mg/l).
- Seal the culture vessels with parafilm and incubate in culture room conditions for 4 weeks. Observe the cultures after a week regularly for morphogenetic response.
- Observe the organogenesis response in the callus cultures in culture room condition in light.
- Observe the emergence of shoots from callus surface after four weeks of growth using the dissection or stereo-microscope.
- Subculture the callus once again onto fresh regeneration media of the same composition and incubate for another 4 weeks if the cultures show little morphogenetic response.
- Remove the shoots carefully from callus growing on above MS regeneration medium and transfer them to simple MS or half strength MS basal medium for rooting.
- Observe rooting response after four weeks of growth of shoots in the above rooting medium.

Protocol for induction of shoots from leaf explants of H. muticus L.

- Excise and collect healthy leaves from *in vitro* grown plants of *H. muticus* L. described earlier as master cultures.
- Cut the leaves into required uniform sizes (0.3 cm approx) and inoculate them into culture vessels preferably test tubes or disposable plastic petri dishes containing 10-15 ml of MS agar media supplemented with 3% (w/v) sucrose, meso-inositol (100 mg/l), Kn or BA (0.05-0.06 mg/l).

- Remove the shoots carefully from cotyledonary node explants growing on above MS medium and transfer them to MS or ½ strength MS basal medium supplemented with IBA (1.0 mg/l) and 0.3% (w/v) PVP for rooting.
- Observe rooting response after four weeks of growth of shoots in the above rooting medium. **(Fig. 5)**

Somatic Embryogenesis

In somatic embryogenesis, embryo-like structures analogous to zygotic embryo are formed either directly from the tissue without callus formation or develop indirectly from the callus. Generally, indirect somatic embryogenesis has been commonly reported in plant in comparison to the direct embryogenesis.

Protocols for callus induction and maintenance using explants from Aconitum heterophyllum Wall., a Himalayan high altitude medicinal plant.

- Collect the plant materials of *A. heterophyllum Wall.* from its natural habitat and allow them to grow in controlled growth chamber at 15 to 20°C. Collect shoot buds, nodal explants and shoot base with axillary meristems.
- Surface-clean the above explants with detergent teepol and wash it several times to remove dust particles.
- Further, the explants may be sterilized with 0.1% (w/v) bavistin for 5 minutes followed by 5-6 washes with sterile distilled water to avoid inherent fungal infection if any.
- Subsequently, surface sterilize with 0.1% (w/v) $HgCl_2$ for 4-5 minutes followed by 8-10 washes with sterile distilled water.
- Incubate the surface sterilized explants on to MS basal agar medium with lower concentration of BA (0.25 mg/l) for further growth. The *in vitro* grown plants derived from these explants can be used as a source of explants for callus induction and other experiments.
- Sub-culture these plants onto fresh MS medium supplemented with BA (0.25 mg/l) every month to maintain as master cultures.
- Excise the leaf and petiole explants from these *in vitro* grown plants and cut into equal halves each.
- Culture them on MS basal agar medium supplemented with 2,4-D (1.0 mg/l), Kn (0.5 mg/l) and 10% (v/v) coconut water (CW) for callus induction.
- Seal the culture vessels with parafilm and incubate them in culture room conditions for 4 weeks.

- Excise small pieces of induced callus (~0.5 g fresh weight), and subculture onto MS medium supplemented with NAA (1.0 mg/l) every month to maintain a callus stock and is referred to as Sequence-I.
- Alternatively, induce callus on MS basal agar medium supplemented with NAA (5.0 mg/l), BA (1.0 mg/l) and maintained on MS medium supplemented with NAA (1.0 mg/l) and is referred to as Sequence-II.
- Transfer the callus developed through Sequence-I, Sequence-II to MS medium supplemented with NAA (0.1 mg/l) and BA (1.0 mg/l) for embryogenic callus initiation and somatic embryogenesis.
- Transfer the cotyledonary stage somatic embryos to MS medium supplemented with NAA (2.0 mg/l) for their conversion to plants.
- Alternatively, dip cotyledonary stage somatic embryos in IBA (1.0 mg/ml) for 5 minutes and culture in MS basal medium till complete plant conversion. **(Fig. 6)**

Protocols for callus induction and somatic embryogenesis using mature zygotic embryo (MZE) explants of T. chebula Retz., a medicinal tree.

Surface sterilize, excised mature zygotic embryos following the steps mentioned below:

Step I: Surface sterilize the excised mature zygotic embryos with 0.1% (w/v) fungicide bavistin solution for 20 minutes, and rinse thoroughly with sterile distilled water for 5-6 times, giving 3 minutes for each wash.

Step II: Disinfect or surface sterilize the excised MZE with 0.1% (w/v) mercuric chloride for 5-6 minutes. Decant off the mercuric chloride solution and rinse MZE explants thoroughly with sterile distilled water for 6-8 times to remove the remaining traces of mercuric chloride giving 3 minutes for each wash.

Detailed protocol is as follows:

- Transfer or inoculate MZE explants on to MS medium supplemented with 2,4-D (2.0 mg/l) and KN (0.1 mg/l) or 2,4-D (2 mg/l) and KN (0.25 mg/l) for embryogenic callus induction.
- Incubate the cultures at 25 ± 2°C, 80% relative humidity in dark for three to four weeks.
- Transfer the MS + 2,4-D (2 mg/l) and KN (0.1 mg/l) media derived embryogenic callus to MS basal medium supplemented with 50 g/l sucrose for somatic embryo induction.

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