4. INITIATION OF CALLUS AND SUSPENSION CULTURES OF *ECHINACEA*

The methods outlined in this section describe callus cultures initiation, preparation of cell suspension, addition of precursors and elicitors to cultured *Echinacea purpurea* and the extraction and quantification of polyphenols.

Methods

a. Initiation of callus from plant

Callus induction is necessary, as the first step, in many tissue culture experiments. Callus is produced when the initial response of the tissues to a wound is followed by the external addition of growth regulators in an aseptic medium in order to maintain the rapid cell division response and sustain it indefinitely. Calli can be obtained from almost any part of the plant; here the root was chosen to be the explant. Phytohormones, such as cytokinin and auxin, are necessary ingredients in any plant cell culture environment. Generally speaking, an equal proportion of auxin to cytokinin hormones in solution will impact only cell proliferation and result in the formation of callus masses. Here we used BA as a cytokinin and NAA as an auxin for callus induction. Stock solution of 1 mg/ml BA was prepared by adding 100 mg of the plant growth regulator to a 100 ml volumetric flask then 2-5 ml of 1 N HCl solution was added drop wisely to dissolve the powder while 1 mg/ml NAA stock solution was prepared by adding 2-5ml absolute ethanol to dissolve 100 mg of the powdered hormone, once they are completely dissolved, the volume was adjusted to 100 ml with double distilled water; the stock solution was stored at 2-8°C.

Echinacea purpurea seeds were washed with abundant tap water to eliminate dust and then surface sterilize by immersion in 70% ethanol for 5 min followed by 20 minutes in 20% (v/v) Clorox® bleach containing two drops of Tween-20[84]. The surface-sterilized seeds were subjected to UV light for 1 hour in the laminar flow cabinet. Flame sterilization of the forceps was performed by dipping them in ethanol and holding into the spirit lamp. The seeds were thoroughly washed 3 times using sterile distilled water, blotted dry on sterile filter paper, then sown on the MS basal medium supplemented with 30 gm/L sucrose, 0.8 % agar at pH 5.8 in glass jars (15seeds/ jar) the jars' necks were Flamed and the caps were replaced tightly then incubated at 25 ± 2 ° C under photo-period (16-h light/8-h dark; daylight fluorescent tubes; 50 µmol/m^2/s) **(Figure 5)**.

Callus Initiation cultures has been described according to the method described by Koroch et al. with modifications[85]. After four weeks of culture, germinated seeds were used as a source of root explants. For callus induction, roots were excised from 4 weeks-old plantlets **(Figure 5)**, scored and placed on MS-based medium supplemented with 1.5 mg/L BA, 0.5 mg/L

NAA, 30 g/L sucrose and semi solidified with 0.8% (w/v) agar, at pH 5.8 in 9 cm glass petri dishes. Dishes were sealed with parafilm and incubated at 25 ± 2 ° C under photo-period (16-h light/8-h dark; daylight fluorescent tubes; 50 µmol/m^2/s).

Explants showed cell divisions at the cut ends **(Figure 5)**, and gradually extending over the entire surface of the explant, leading to the formation of an irregular mass callus. Dark yellowish green callus tissue appeared on the explants in the course of 2–4 weeks **(Figure 5)**. After 4 weeks, the callus was separated from the parent explant and subcultured to fresh medium of the same composition **(Error! Not a valid bookmark self-reference.)**. The calli were periodically subcultured on fresh media for 6 months till obtaining the desired calli quantities.

b. Initiation of suspension culture from callus

To initiate mother cell cultures[86], pieces of established undifferentiated, friable calli were subdivided into small sections and transferred from the surface of solid agar in a 250 ml Erlenmeyer flasks **(Figure 5)** containing 100 ml MS liquid medium supplemented with 1.5 mg/L BA, 0.5 mg/L NAA, 30g/L sucrose, at pH 5.8 and incubated on an orbital shaker (120 rpm), at 25 ± 2 ° C under photo-period (16-h light/8-h dark; daylight fluorescent tubes; 50 µmol/m^2/s). After 21 days mother cell suspension cultures **(Figure 5)** were used for routine cultures. Sterile serological pipettes (with a wide tip) were used to transfer 10 ml packed cell volume (PCV) to Erlenmeyer flasks containing 90 ml autoclaved media. Periodic addition of fresh media and draining out the exhausted media ensures maintenance of suspension cultures under steady state of growth for long periods. Cell suspension cultures were subcultured for several weeks to expand the total biomass to be collected.

Figure 5: **A**: 2 months-old plantlets, **B**: 3weeks-old plantlets, **C, D**: Dark yellowish green callus tissue appeared on the explants in the course of 2–4 weeks, **E, F and G**:sub-culture of callus on MS solid media supplemented with 1.5 mg/L BA and 0.5 mg NAA ; callus left to grow for 4 weeks, **H**: callus transferred to liquid MS media supplemented with 1.5 mg/L BA and 0.5 mg NAA, **I**: 4 weeks-old suspension culture, **J**: 3 months-old suspension culture **K and L**: homogenized mother cell suspension cultures prepared for further experimentation