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Establishment of an embryogenic suspension culture of *Pinus kesiya* (Khasi pine) from various explants

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The embryogenic cultures were obtained from mature zygotic embryos (79.6%) and apical dome sections (92.6%) (pretreated with 0.4% activated charcoal at 4°C for 24 hrs) on mMS and ½DCR media, respectively containing 5 mg l^{-1} 2,4-D, NAA in combination along with 1 mg l^{-1} BAP, while, from secondary needles (88.6%) it was on MS medium containing 3 mg l^{-1} each of 2,4-D and NAA in conjunction with 1 mg l^{-1} BAP. Using 150-200 mg embryogenic tissues per 15 ml medium at 120 rpm raised embryogenic suspension cultures. But the embryogenic cultures from apical dome sections were cold treated at 4°C for 24 hrs before transferring to suspension culture. The cultures were subcultured at 6-7 days interval and were diluted at 1:4 ratio. The embryonal suspensor masses developed from the resulting elongated single cells within 3-4 passages in respective basal medium with $1/10^{th}$ growth regulators of initiation medium. The proembryonal head formed in growth regulators free medium at 100 rpm. The embryonal head formed in basal medium containing 4% sucrose and 4 mg l^{-1} ABA in combination.

Keywords: activated charcoal pre-treatment, cold-treatment, embryogenic suspension culture, *pinus kesiya*, somatic embryogenesis

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Introduction

Somatic embryogenesis in conifers was first documented from immature zygotic embryos of *Picea abies*[1-3]. Currently there are about 100 million hectares of industrial tree plantations worldwide, of which 70-80% comprises conifers[4]. For mass clonal propagation of conifers somatic embryogenesis can play a vital role. Since 1985 several papers have been published on successful regeneration of conifer plantlets via somatic embryogenesis both on semi-solid medium and in suspension cultures[5-20].

Pinus kesiya Royle ex Gord. is an economically important early successional tree species, which is predominant in the subtropics (800-2000 m altitude) of North-East India, Myanmar and Philippines. It is the major source of timber in Khasi hills of Meghalaya (India) where it covers about 30% of the total forest area. Some genotypes have tremendous biomass potential and oleo-resin prospects. The Pine forest of the region is getting denudated due to 'Slash and burn' cultivation, ruthless commercial exploitation and unplanned developmental activities. Therefore, there is an urgent need for large-scale afforestation programme using cloning material. In this paper the authors report establishment of embryogenic suspension culture from zygotic embryos, secondary needles and apical dome sections from mature 'plus' trees of *P. kesiya* (identified by Department of Forestry, Government of Meghalaya).

Materials and Methods

Collection and Priming of Explants

The mature seeds were collected during January-February from mature cones and stored at 4°C in sealed polythene bags till used. The secondary needles were harvested from 5-6-week-old seedlings, during April-May, while 2-3 cm apical domes were collected from 'plus' trees prior to emergence of needles during May-July of every year. Both secondary needles and apical domes immediately after harvesting were placed in anti-oxidant solution (200 mg l^{-1} PVP) to prevent exudation of phenolic compounds.

Initiation of Culture on Semisolid Phase

The sterilized seeds were imbibed for 24 hrs at 4°C while, the apical domes were cut into 0.5 mm thick

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transverse sections and pretreated on ½DCR medium[21] containing 2% sucrose, 0.4% activated charcoal (AC) at 4°C for 72 hrs in the dark before culturing them on growth regulators rich medium for priming the explants. Mature zygotic embryos from imbibed seeds, secondary needles and pretreated apical dome sections were cultured on modified MS [half strength MS salts[22] containing 550 mg l^{-1} NH_4NO_3 and 4460 mg l^{-1} KNO_3 (mMS)], MS and ½DCR media, respectively containing 0-3% sucrose, 500 mg l^{-1} L-glutamine, 1000 mg l^{-1} casein-hydrolysate and myo-inositol, 0-10 mg l^{-1} 2,4-D and NAA singly or in combination and 0-5 mg l^{-1} BAP. Difco-bacto agar (0.7%) was used as gelling agent before autoclaving. Chemicals like ABA, L-glutamine and myo-inositol were filter sterilized and incorporated in the media. In each tube (size-25 × 100 mm) 15 ml medium was poured and for each treatment 50 explants were cultured. The cultures were maintained in the dark at 25±2°C and experiments were repeated thrice. The resulting embryogenic cultures were subcultured biweekly on optimum growth condition for 2 passages. Thereafter, the embryogenic cultures from apical dome sections were cold-treated at 4°C for 0-24 hrs.

Initiation of Suspension Culture

About 50-400 mg embryogenic cultures from above culture conditions were pressed gently with a scalpel blade and transferred to 50 ml conical flask containing 15 ml of respective initiation medium and shaken at 120 rpm on an 'Orbital shaker' (B Braun, model-Certomat U) in the dark at 25±2°C unless mentioned otherwise. The cultures were subcultured at 6-7 day interval for 3-4 passages and were diluted at 1:4 ratio at every subculture.

Maintenance of Cultures

The cultures with embryonal suspension masses (ESMs) elongated single cells from above culture conditions were transferred to respective basal medium containing reduced growth regulators (0, $1/5^{th}$ and $1/10^{th}$ of initiation medium) for 4-5 passages. Thereafter, the cultures were grown in growth regulator free medium for 3-4 passages at 100 rpm.

Somatic Embryo Development

The cultures with ESMs were transferred in respective basal medium supplemented with 0-5% sucrose, 0-8 mg l^{-1} ABA singly or in combination for 6-7 passages.

Results and Discussion

Within two weeks of culture two different types of calli developed. Zygotic embryos and secondary needles formed (i) white, soft and translucent embryogenic (79.6 and 88.6%, respectively) (Figs 1a and b) and (ii) hard and green non-embryogenic cultures. While, from apical dome sections pretreated

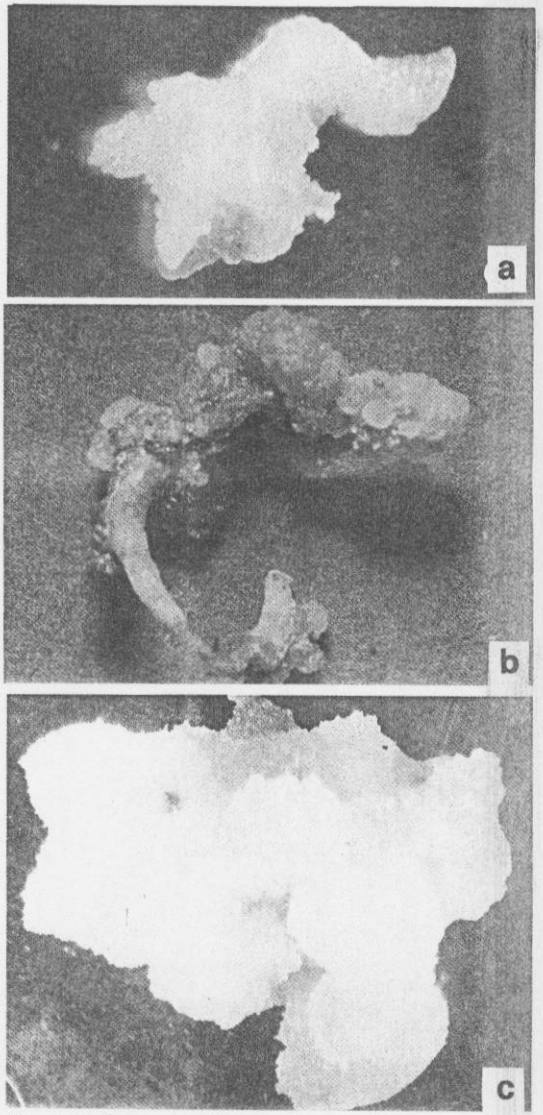

Fig. 1—Embryogenic calli formed from different explants on initiation medium, a. from zygotic embryo, b. secondary needles and c. apical dome sections

at 4°C with 0.4% AC formed (i) white, soft and translucent embryogenic (92.6%) (Fig. 1c) and (ii) white but hard non-embryogenic cultures (data not presented). The better embryogenic culture formation from zygotic embryo and apical dome sections were obtained with mMS and ½DCR media, respectively containing 5 mg l^{-1} each of 2,4-D and NAA along with 2.5 mg l^{-1} BAP while from secondary needles with MS medium containing 3.0 mg l^{-1} each of 2,4-D and NAA along with 1.0 mg l^{-1} BAP. It was observed that apical dome sections primed on activated charcoal rich medium resulted in softer calli when cultured on growth regulators rich medium. It is believed that, during AC pre-treatment the phenolic compounds, growth regulators and other inhibitors adsorbed into the AC, which resulted into soft callus. The promotary effect of AC on the initiation of polyembryogenesis has been documented. Webb and Flinn added 1% AC in the initiation medium which triggered embryogenic culture formation in *P. strobus*[23] while, Gupta *et al* used AC in embryogenic initiation medium for absorbing endogenous ABA and other inhibitory metabolic by-products[9].

The embryogenic cultures from apical dome sections required a cold treatment at 4°C for 24 hrs before transfer to liquid medium. In the present study, for initiation of suspension cultures, lower inoculum size (150-200 mg/15 ml medium) was found to be suitable at 120 rpm. By decreasing the inoculum (<150 mg/15 ml medium) a poor culture growth was recorded, which may be due to inadequate culture density. Higher inoculum at the initial stage slowed the proliferation of cultures, which may be due to over-crowding of cells. Finer *et al* reported that embryogenic cultures of *P. strobus* proliferated well with low culture density[24] while, Krogstrup reported that culture density was crucial and determined the quality of early stage embryos in suspension cultures in *P. sitchensis*[25]. In the present investigation, the cultures were proliferated by subculturing at 6-7 days interval for 3-4 passages and cultures were diluted at 1:4 ratio at every subculture. During this period cultures yielded elongated embryogenic cells which in turn started cleaving (Fig. 2a) after 2-3 passages in culture. It was necessary to free the single cells from mother tissues at the early stage failing which degeneration of cultures resulted. The ESMs (Figs 2b & c) formed from cleaving cells when they were maintained on respective basal medium with 1/10th growth regulators to that of initiation medium and

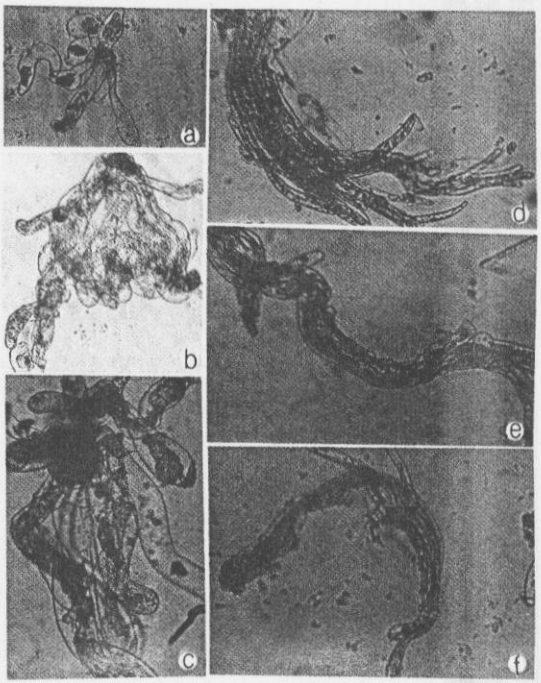

Fig. 2—Different stages involved in somatic embryogenesis of *P. kesiya* in suspension culture; a, elongated single cell started cleaving; b and c, embryonal suspensor masses; d, embryonal cell masses showing accumulation; e, somatic embryo with proembryonal head & f, somatic embryo with embryonal head.

maintained for 3-4 passages. The ESMs started accumulation (Fig. 2d) in the medium free of growth regulators and at low agitation where cultures formed proembryonal head. The distinct embryonal head formed (Figs 2e & f) after 5-6 passages when cultures were maintained in respective basal medium containing 4% sucrose and 4 mg l^{-1} ABA in combination. ABA has been used for cotyledonary embryo maturation in many plant species[26] including conifers[18,27]. In the present study, it was observed that neither sucrose nor ABA singly could promote embryonal head formation but 4% sucrose and 4 mg l^{-1} ABA in combination was found effective. Tremblay and Tremblay reported that in Black spruce 4-6% sucrose was very effective for maturation of somatic embryos[28]. Carrier *et al* studied the effect of exogenous sucrose on maturation and germination of somatic embryo of interior spruce and reported that embryos placed on the medium with added sucrose developed roots and epicotyls and increased their

fresh biomass by about 13-fold[29]. Vagner *et al* reported that with increase in exogenous ABA levels (5-40 μM), an increase in somatic embryo maturation in *P. abies* both in semisolid and liquid media. In the absence of the exogenous ABA, practically no embryos were formed[16].

In the present study, the initiation, development and maturation of *P. kesiya* somatic embryos have been achieved. This opens up a great potential for large-scale propagation of this species, which is facing rapid denudation.

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