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Factors influencing initiation of embryogenic cultures in *Pinus kesiya* Royle ex Gord.

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Seeds, 5-6 week old, secondary needles and apical domes of *Pinus kesiya* were collected during January-February, March-June and May-July, respectively. The explants were primed before initiation of culture. The embryogenic cultures were obtained on mMS medium from zygotic embryos (79.6%), dissected out from imbibed seeds (for 24 hrs at 4°C), on MS medium from secondary needles (88.6%) and on ½DCR medium from 0.2-0.5 mm thick pre-cultured (on ½DCR medium, containing 2% sucrose, 0.4% activated charcoal, at 4°C for 72 h) apical dome sections (92.6%). All media were supplemented with 2% sucrose, 1000 mg l^{-1} casein-hydrolysate, 1000 mg l^{-1} myo-inositol and 500 mg l^{-1} L-glutamine, adjusted to *p*H 5.5. For initiation of embryogenic cultures, 5 mg l^{-1} each of 2,4-D and NAA along with 2.5 mg l^{-1} BAP were incorporated to media for both zygotic embryos and apical dome sections, while 3.0 mg l^{-1} of 2,4-D and NAA each along with 1.0 mg l^{-1} BAP were incorporated for secondary needles. The proembryonal masses and proembryos were developed from embryogenic cultures of zygotic embryos and secondary needles, and cold treated (at 4°C for 24 h) cultures of apical dome sections by culturing them on their basal media containing $1/10^{th}$ level of growth regulators followed by growth regulator free media. Further, the cotyledonary embryos were formed on their respective basal media containing 4% sucrose and 4 mg l^{-1} ABA.

Keywords: activated charcoal treatment, MS medium, *Pinus kesiya*, seed imbibition period, somatic embryogenesis

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Introduction

Somatic embryogenesis in conifers was first documented from immature zygotic embryos of *Picea abies*[1,2]. Since then several reports have been published on successful regeneration of conifer plantlets *via* somatic embryogenesis using different explants, like mature and immature zygotic embryos, cotyledons from germinated seeds and needles from seedlings, female gametophytes, etc[3-9]. A key factor for successful initiation of embryogenic cultures has been the right choice of explant. In addition, the different physico-chemical factors may also influence the embryogenesis in conifers.

Present paper describes the factors controlling induction of somatic embryogenesis in *Pinus kesiya* Royle ex Gord., an economically important tree species of North East India, from various explant sources, such as mature zygotic embryos, secondary needles and apical dome sections from mature trees.

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Materials and Methods

Plant Materials

Seeds were collected during late January to March from mature cones. They were extracted by air-drying and stored in polythene bags for 3-4 years at 4°C before use. About 20 seeds were placed equidistant in an open 90 mm diam Petridish containing moist cotton pad and allowed to germinate in normal day light. The young seedlings (5-6 week old), after release of the first batch of secondary needles, from both field and glasshouse were placed in water containing 100 mg l^{-1} polyvinyl pyrrolidone (PVP). The shoot tips (~2-3 cm) were collected (prior to emergence of needles) round the year from mature 'plus' trees marked by Department of Forests, Government of Meghalaya. The tips were transferred to 200 mg l^{-1} PVP solution. PVP, an anti-oxidant was used to prevent oxidation of phenolic compounds.

Media

For initiation of embryogenic cultures, various media like MS[10], mMS[9], DCR[11] in full and half strength were used. The influence of casein-hydrolysate (CH, 0-1500 mg l^{-1}), myo-inositol (0-1500 mg l^{-1}), L-glutamine (0-1000 mg l^{-1}), PVP (200 mg l^{-1}) and sucrose (0-3%) as organic carbon source in the medium was investigated. For the culture of zygotic embryo and apical dome sections, 2,4-D and NAA (0-10 mg l^{-1}) singly or in combination along with BAP (2.5 mg l^{-1}) were used in the media, whereas for secondary needles the concentrations of 2,4-D and NAA were 0-5 mg l^{-1}, and BAP 1.0 mg l^{-1}. A range of *p*H (4.25-7.5) was used to study its effect on initiation of embryogenic cultures. Chemicals, such as ABA, L-glutamine and myo-inositol, were filter sterilized and incorporated into medium after autoclaving.

Sterilization and Priming of Plant Materials

(*a*)—*Zygotic Embryos:* Stored seeds were surface cleansed with sodium hypochlorite (0.2% available chlorine) for 5 min and washed under running tap water for 15 min. The seeds were further treated with 6% (v/v) hydrogen peroxide for 10 min and washed 4-5 times with sterilized distilled water. Thereafter the sterilized seeds were imbibed on moist filter paper at 4°C for 0-96 hrs.

(*b*)—*Secondary Needles:* The healthy needles were washed under running tap water, followed by 0.25% (w/v) mercuric chloride for 3-5 min and then washed 4-5 times with sterilized distilled water.

(*c*)—*Apical Dome Sections:* Young needles, scales, etc were removed from shoot tips and apical domes measuring ~1.0 cm were cut and washed under running tap water. The apical domes were then treated with 0.25% (w/v) mercuric chloride for 5 min and washed several times with sterilized distilled water. The sterilized apical domes were cut into thin transverse sections (0.2-1.0 mm thick) and precultured on ½DCR medium containing 2% sucrose and 0-1% activated charcoal (AC) at 4 and 25°C for 0-168 hrs in the dark.

Tissue culture

Two embryos, dissected out from imbibed seeds, were cultured per culture tube (25×150 mm), while 5 sterilized needles were cultured per Petridish (90 mm diam) and 2 pre-cultured apical dome sections per culture tube. In each case 50 explants were cultured per treatment and experiments were repeated thrice.

The cultures were grown in dark and light (20 and 1900 lux), at 12 hr photoperiod at 25±2°C. The embryogenic cultures obtained from zygotic embryos and secondary needles (from optimum growth conditions) were subcultured for 2-3 passages, while cultures from apical dome sections were subcultured for 2 passages and then cold-treated at 4°C for 0-72 hrs. All cultures were subcultured at a 2-week interval until mentioned otherwise. The embryogenic cultures obtained from zygotic embryos, secondary needles and apical dome sections were then transferred on MS, mMS and ½DCR medium, respectively containing 2% sucrose and reduced growth regulators ($1/10^{th}$ level of initiation medium). They were maintained for 2-3 passages for the formation of proembryonic masses (PEMs) and proembryos. Thereafter, the cultures were maintained on their respective basal media free from growth regulators and subcultured twice. The resulting proembryos were maintained for 3-4 passages on basal medium containing varying concentrations of sucrose (0-5%) and ABA (0-8 mg l^{-1}) singly or in combination for cotyledonary embryo formation.

Results and Discussion

Collection of Plant Materials

In the present investigation, the seeds collected during late January to late February produced optimum embryogenic cultures, while those collected during March exhibited poor embryogenic response (data not presented). This might be because mature seeds of *P. kesiya* are dehisced during January-February and embryos are fresh and healthy during this period. Secondary needles (5-6 week old) collected during March-June were found ideal for embryogenic cultures as compared to those collected in other parts of the year (data not presented). During this period, a favourable temperature and rainfall for seed germination were also experienced. From June onwards the secondary needles became harder and showed poor initiation of embryogenic cultures. However, apical domes collected during May-July, before the emergence of the needles, produced better embryogenic cultures. If the apical domes were collected after emergence of the needles, the response declined sharply.

Priming of Explants

Prior to dissecting out of embryos, 24 hr imbibition of *P. kesiya* seeds on moist filter paper at 4°C resulted in 79.6% embryogenic cultures, whereas unimbibed seeds showed a poor response of about 20% (Fig. 1). Further increase in imbibition period recorded a gradual decline in initiation of embryogenic cultures. Pre-cotyledonary to pre-germinating embryos were found suitable for initiation of embryogenic cultures in *Larix deciduas*[12] and *Pinus palustris*[13], while 4 hr imbibition was reported to be optimum in *Picea glauca*[14].

The pre-culture treatment of apical dome sections at 4°C, on ½DCR medium containing 0.4% AC, was helpful in induction of embryogenic cultures (Fig. 2); ~2-fold increase in the induction of embryogenic cultures was recorded as compared to control. However, the response of explants cultured at 25°C was much lower and resulted in browning of cultures. The lower concentrations of AC in the medium resulted harder calli, while the higher concentrations proved inhibitory for embryogenic response and the cultures subsequently degenerated. Even incubation of such cultures at lower temperature could not make any difference. However, addition of 1% AC to the culture medium triggered the embryogenic culture formation of *P. strobes* in an earlier study[15]. The thickness of apical dome sections was also found to be a crucial factor. Thinner the apical dome sections (0.2-0.5 mm), better was the embryogenic response. Section thickness >0.5 mm resulted in poor response and formation of hard calli. Increase in duration of AC treatment of cultures up to 72 hrs recorded a marked increase in embryogenic culture formation, which subsequently declined at longer incubations (Fig. 3). The pre-cultured apical dome sections at 4°C exhibited better response than the ones cultured at 25°C. A low temperature treatment of explants in the medium containing AC might have helped in adsorption of phenolic compounds and other inhibitors. Studies on polyembryogenesis in *P. menziesii* also showed that AC in the medium helped in absorbing endogenous ABA and other inhibitory metabolic by-products[16].

Culture Media and Conditions for Initiation of Embryogenic Culture

The effect of different media and inorganic and organic nitrogen sources on somatic embryogenesis in conifers has been studied extensively[12,17]. Amongst

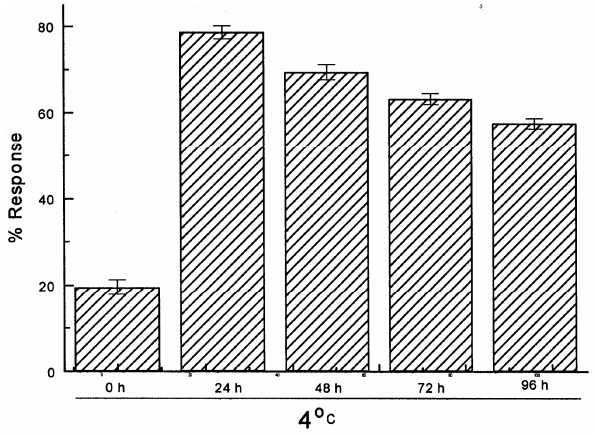

Fig. 1—Effect of seed imbibition period on induction of embryogenic cultures from zygotic embryos

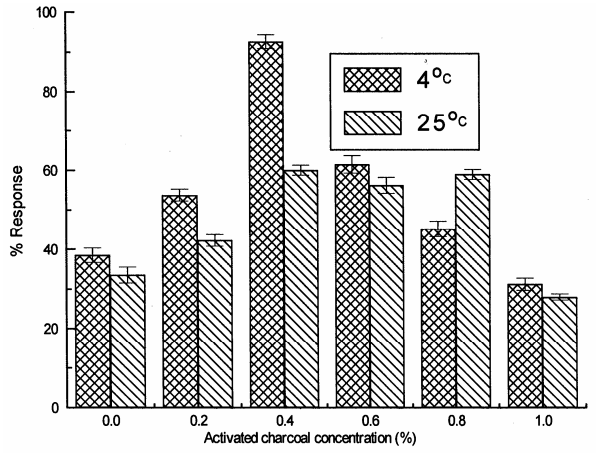

Fig. 2—Effect of activated charcoal and temperature as priming factors on induction of embryonic cultures from apical dome sections

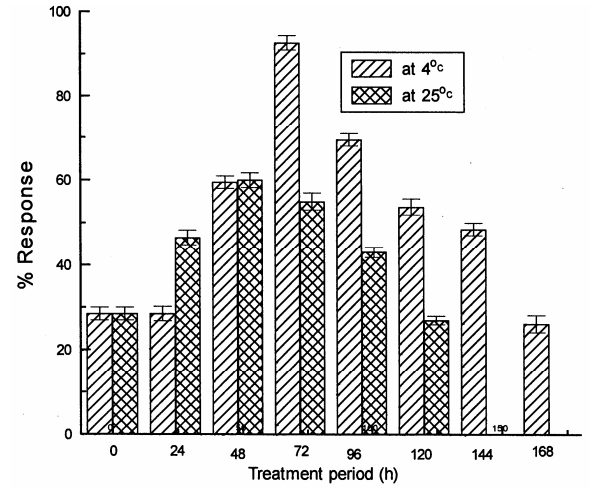

Fig. 3—Effect of pretreatment duration and temperature with activated charcoal (0.4%) on induction of embryonic cultures from apical dome sections

the different media tested for *P. kesiya* somatic embryogenesis, mMS medium for zygotic embryos (79.6%), MS medium for secondary needles (88.6%) and ½DCR medium for apical dome sections (92.6%) containing 2% sucrose were found suitable for initiation of embryogenic cultures (Fig. 4). In the present investigation, *p*H 5.5 of the media was the optimum for the induction of embryogenic cultures from zygotic embryos (Fig. 5). The response exhibited by secondary needles and apical dome sections were also similar (data not presented). However, *p*H 6.0 of the medium was earlier reported optimum for the initiation of embryogenic cultures of *P. palustris*[13]. Further, All the three explants of *P. kesiya* required higher concentrations of CH (1000 mg l^{-1}) and L-glutamine (500 mg l^{-1}) besides inorganic nitrogen sources for initiation of embryogenic cultures. The findings are in agreement with the other studies on *Picea abies*[18] and *P. glauca*[17].

Although, 2,4-D and NAA were the preferred auxins for *P. kesiya* like other conifers[3,6,16,19-21], NAA singly was more effective than 2,4-D for all the explants. Further, 2,4-D and NAA at 5.0 mg l^{-1} each were highly effective for initiation of embryogenic cultures from zygotic embryos[9] and apical dome sections but in case of secondary needles, they were effective at 3 mg l^{-1} each[8]. It was also observed that incorporation of BAP 2.5 mg l^{-1} for both zygotic embryos and apical dome sections, and 1.0 mg l^{-1} for secondary needles, along with auxins (NAA and 2,4-D) in the media were stimulatory for initiation of cultures. This observation supports the other reports on conifers[5,7,12,13]. However, a combination of 3.0 mg l^{-1} 2,4-D and 0.5 mg L-1 BA was reported better for initiation of embryogenic cultures in *P. taeda*[6].

Induction of embryogenic cultures in conifers has mostly been done in the dark[3,22], except in case of Norway spruce where initiation of embryogenic culture was equally well under light as well as dark[19]. However, 20 hr photoperiod was reported inhibitory for induction of embryogenic cultures and lowered the induction frequencies in Norway spruce[22]. In case of *P. kesiya*, light was found to be inhibitory for initiation of embryogenic cultures, which produced more non-embryogenic cultures. Dark was preferred but diffused light formed moderate embryogenic cultures, except apical dome sections where completely non-embryogenic culture were formed.

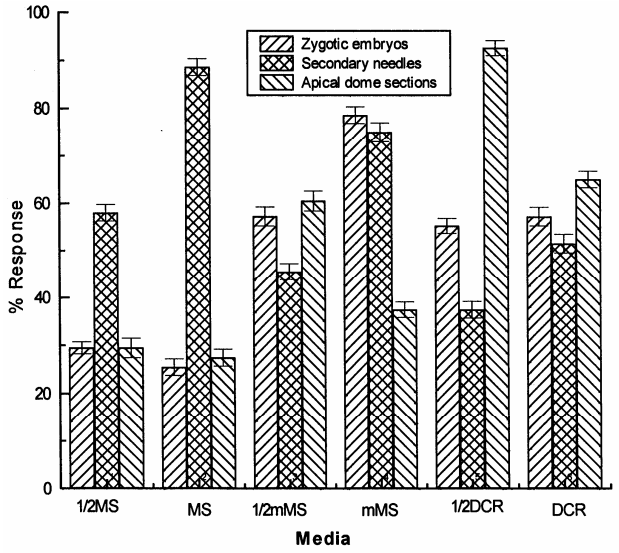

Fig. 4—Effect of media composition on initiation of embryonic cultures from different explants

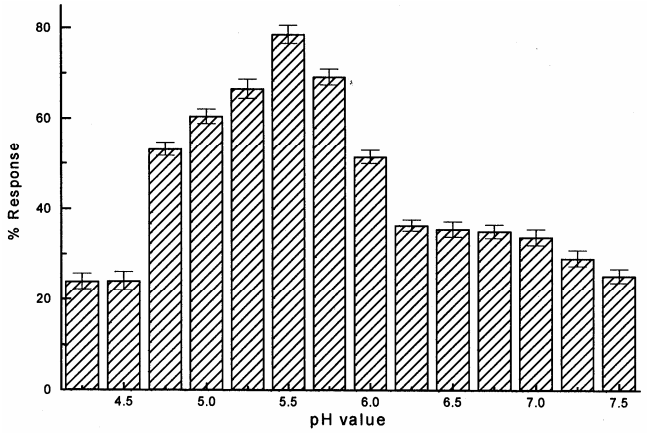

Fig. 5—Effect of mMS medium *p*H on initiation of embryonic cultures from zygotic embryos

Embryo Development

PEMs developed in cultures after 2-3 transfers of the embryogenic cultures on their respective basal media, containing $1/10^{th}$ level of growth regulators of initiation medium. If left on the initiation medium, they ceased proliferating and turned brown. The cultures raised from apical dome sections were not very soft and required a cold treatment at 4°C for 24 hrs for converting into softer and translucent cultures. The above-mentioned cultures developed into proembryos during 2 passages in basal medium free from growth regulators. The resulted proembryos formed cotyledonary embryos after 3-4 passages on basal medium enriched with higher concentration of sucrose (4%) and ABA (4.0 mg^{-1}).

The different factors that control the initiation of somatic embryogenic cultures in *P. kesiya* have been worked out in the present investigation. This information would be useful for investigations of somatic embryogenesis in conifers.

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