

Somatic embryogenesis and plant regeneration from cell suspension and tissue cultures of mature himalayan poplar (*Populus ciliata*)

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ABSTRACT

Somatic embryogenesis and plantlet formation were obtained from callus and cell suspension cultures of 40-year-old Himalayan Poplar (*Populus ciliata* Wall ex Royle). Callus and cell suspensions were obtained by transfer of inoculum of semiorganized leaf cultures, which were maintained on Murashige and Skoog (MS) medium supplemented with benzylaminopurine (BAP), to MS with 2,4-dichlorophenoxyacetic acid (2,4-D). Reduction of 2,4-D concentration during subsequent subculture of cell suspensions resulted in the formation of embryoids. These embryoids developed further only after being transferred to agar-based MS medium supplemented with BAP and naphthaleneacetic acid. Loss of embryogenic potential was observed in cell suspensions after 6 subcultures. However, callus cultures retained the embryogenic potential even after repeated subcultures for more than a year. Plantlets could be successfully hardened and grown in natural outdoor conditions.

Abbreviations : BAP: 6-benzylaminopurine; 2,4-D: 2,4-dichlorophenoxyacetic acid; NAA:1-naphthaleneacetic acid; MS: Murashige and Skoog (1962) medium.

Key Words : *Populus ciliata*, somatic embryogenesis, plant regeneration.

INTRODUCTION

A large number of tree species have been studied for developing methods of *in vitro* micropropagation (Bajaj 1986, Bonga and Durzan 1987). However, the explants used in most of these studies were seeds, seedlings or juvenile plant parts. Since the desirable characters in trees are identifiable only after a considerable period of time (at least half the rotation age and/or their maturity), these studies are not relevant to immediate use in tree improvement programmes. Success in developing propagation methods with explants from mature trees has been rather limited (Ahuja 1986, 1987; Fossard and Bourne 1976, Fossard 1978, Gupta et al. 1980, Gupta et al. 1981, LakshmiSita et al. 1980, Mehra and Cheema 1980).

The main objective of our investigation was to explore the possibility of somatic embryogenesis for large-scale clonal multiplication in tissue and

cell cultures of Himalayan poplar (*Populus ciliata* Wall ex Royle) using explants from selected, mature flowering trees. As embryos are discrete bipolar structures with integrated root-shoot axis, these can be used as artificial seeds for direct planting or efficient storage and delivery to the forest (Amirato 1983, Redenbaugh et al. 1986). *P. ciliata* is a tree of commercial interest and is used for a variety of purposes, e.g. match, pulp and paper. It is a large sized deciduous, unisexual tree which grows over the outer range of the Himalayas between 1200 to 3000 m. In a previous study (Mehra and Cheema 1980), we have demonstrated that it is possible to have continuous *in vitro* multiplication of mature trees of *P. ciliata* using the following type of explants:

- i) The twigs bearing axillary buds or the shoots possessing dormant apical buds can be induced to produce calli and redifferentiate shoots;
- ii) proliferation of apical buds can be stimulated to obtain multiple shoot formation; and
- iii) leaf disks taken from young leaves incubated in total darkness on modified Murashige and Skoog (1962) medium supplemented with BAP can give rise to a large number of shoots.

The shoots formed by any of the above methods can be rooted, suitably hardened and transferred to soil (Mehra and Cheema 1980).

The third type of cultures produced from young leaves can be maintained in semi-organized state (hereafter referred as 'leaf cultures') on MS-medium containing 2 mg/l BAP. Shoots have been regenerated by organogenesis from such tissues by transferring these to MS medium with BAP and NAA kept in light. The semi-organized cultures have been maintained in total darkness for eight years. In this paper, I report on the media and culture conditions which have induced somatic embryogenesis in these cultures.

MATERIALS AND METHODS

Semi-organized 'leaf cultures' were initiated from young leaves of female trees of *P. ciliata*. These trees, over forty years old, were growing in a forest near the Regional Fruit Research Station,

Mushobra, Simla, India. The details of the procedures used to initiate and maintain 'leaf cultures' have been reported earlier (Mehra and Cheema 1980). The cultures were maintained on MS medium containing 2 mg/l BAP at $25 \pm 3^\circ\text{C}$ in total darkness since 1979.

Callus and Cell Suspensions

Semi-organized 'leaf cultures' maintained on MS + 2 mg/l BAP in dark were transferred to MS + 2,4-D (0.2, 0.5 mg/l) media for callus initiation in the dark. The cell suspensions were initiated by transferring 2 to 3 gm (fresh weight) calli to about 40 ml liquid MS with 3% sucrose and 0.2 or 0.5 mg/l 2,4-D in 250 ml conical flasks. The cultures were maintained at 22°C in dark or at 25°C in continuous light on a gyratory shaker set at either 50 or 120 rpm. Cell suspensions were subcultured every two to four weeks depending on the growth of the cultures. The callus cultures were maintained on agar-based media and subcultured on respective media every four to six weeks to study growth and differentiation patterns in continuous light (4400 lux at the culture level) at $25 \pm 2^\circ\text{C}$. Five replicates for each treatment for suspensions and a minimum of 10 replicates for callus cultures were maintained.

Embryo Development and Plant Regeneration

The calli maintained on MS + 2,4-D (0.2 or 0.5 mg/l) were either transferred to the same medium or to a variety of different media: MS basal medium, Half strength (salts only) MS basal medium with 2% sucrose or MS with BAP (0.4 mg/l) and NAA (0.2 mg/l). The last medium supports not only the induction, growth and differentiation of shoots from various explants and their calli but also induces roots from well-developed shoots three to six weeks after culture on the medium. For plant regeneration, cell suspensions which had undergone three to six passages on MS medium with 2,4-D and contained somatic embryos at various stages of development, were washed with MS basal medium and plated on the above-mentioned media.

Hardening and Transfer to Field Conditions

After an initial hardening procedure modified from Cheema and Sharma (1983), well-developed seedlings and rooted plants were transferred to soil. The procedure consisted of growing seedling/rooted plantlets on (1) half strength (salts only) MS without sucrose, and (2) sterilized soft coke substrate supplied with half-strength MS without sucrose. Plantlets were kept on these substrates for six to eight weeks. The plantlets with dormant bud-like structure (DBLS) characteristics were removed from agar media or from soft coke substrate and potted in red coarse sand which provided good drainage. Sand cultures were watered regularly on alternate days, to begin with, for a period of two weeks and thereafter daily. Sand cultures were maintained in a growth room (temp. 25°C , 16 h light/8 h dark cycle) for about three months. Plantlets having a height of 15-20 cm were transferred to soil in natural outdoor conditions in November, 1986.

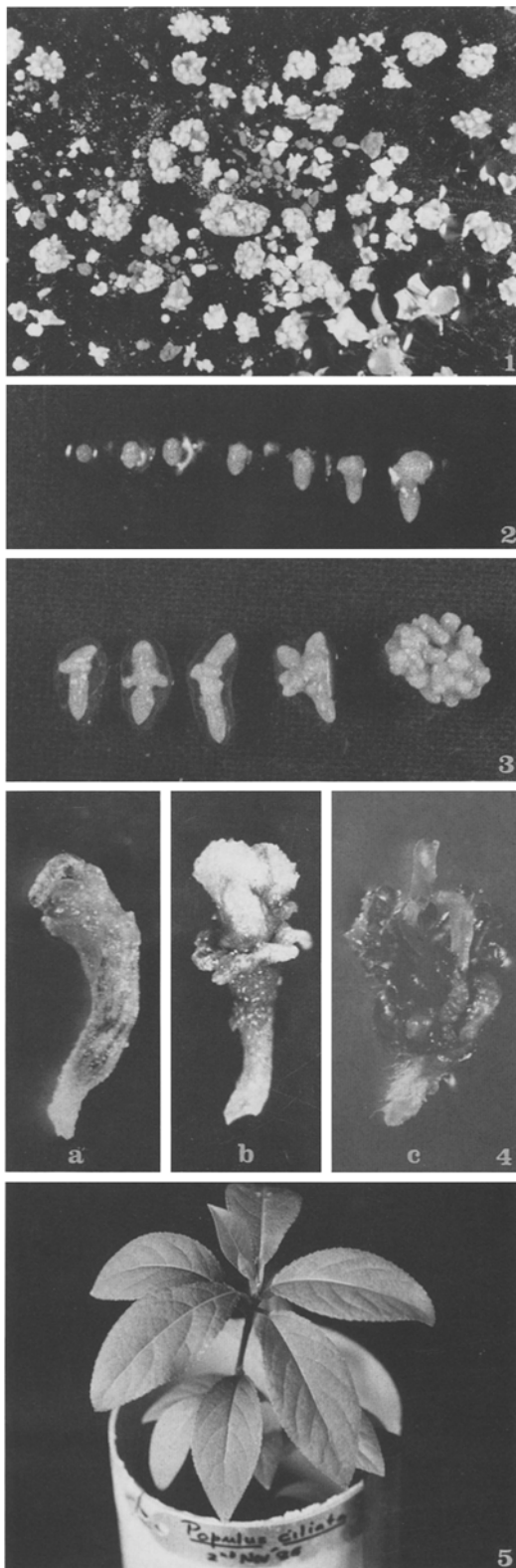
RESULTS AND DISCUSSION

Fast growing, friable, yellowish white calli were induced within a period of two weeks from inoculum of 'leaf cultures' on 2,4-D (0.2, 0.5

mg/l) medium. These friable calli were used to induce cell suspensions in the same medium as used for induction of callus. High levels of 2,4-D (0.5 mg/l) yielded fine suspension of single cells and small cell clusters. At lower concentration of 2,4-D (0.2 mg/l) cell suspensions were composed of relatively large cell clumps. The cells in both types of culture media were richly cytoplasmic and contained abundant starch grains. Suspensions maintained in continuous light showed fast growth and doubling of initial cell mass in 7-10 days. It was rather difficult to maintain such cultures in good condition due to rapid build-up of anthocyanin pigment and subsequent browning. On continuous growth in light, cells changed from isodiametric form, characteristic of dark grown cells, to elongated form. Consequently, all the experiments for embryogenesis were performed with dark-grown cell suspensions.

The reduction in levels of 2,4-D from 0.5 mg/l to 0.2 mg/l in suspensions induced the growth and development of somatic embryos. Embryogenesis was also observed by third subculture, in the cell suspensions which were maintained throughout on MS + 0.2 mg/l 2,4-D. Different stages of embryogenic organizations, ranging from minute to globular to torpedo stage were observed to occur in the same medium. Secondary embryogenesis from the hypocotyl and cotyledon regions often led to the formation of multiple embryo clumps (Fig. 1, 2, 3). Further growth and development into mature somatic embryos required such structures to be plated on agar-based medium. Complete deletion of auxin (2,4-D) from the medium did not sustain further growth and development of embryos. On the contrary, it led to the rooting of some embryos, followed by general senescence of cultures. Transfer of embryogenic cell suspensions to MS medium with 2,4-D, on agar, led to the recalling of embryos. The transfer of embryogenic mass to a medium containing BAP (0.4 mg/l) + NAA (0.2 mg/l) was essential for the further development of embryogenic mass. A typical, well-developed normal embryo showed bipolar structure, hypocotyl tapering into a root and two cotyledons at the opposite end (Fig. 4a). A variety of developmental patterns such as precocious germination, arrested shoot development, arrested root development, secondary embryogenesis and good seedling development were observed on this medium (Fig. 4b-c). After six subcultures of cell suspensions that were maintained on MS + 2,4-D containing media, there was a loss of embryogenic potential. Such cultures showed a very large number of elongated, highly vacuolated cells with very few starch grains, the presence of which characterized initial phase of cell suspensions.

Contrary to the nature of cell suspensions at this stage, callus cultures that were maintained on agar based MS + 0.5 or 0.2 mg/l, 2,4-D under 24 h light consisted of actively growing, richly cytoplasmic, distinct embryogenic masses. Embryogenic stages ranging from minute to late globular and early torpedo stages with distinct, discretely closed radicular ends were observed. Complete removal of 2,4-D only favoured root growth of embryos followed by senescence. The transfer of these calli after four to five weeks growth on MS with 2,4-D to MS with BAP (0.4 mg/l) and NAA (0.2 mg/l) led to greening and further development of embryos in different stages of development within a period of fifteen days. By four to six weeks all such cultures showed a few (8-10) well-formed embryos, many (50-60) teratomatous and large numbers



(hundreds) of globular embryos per culture. Subculture of such embryogenic mass led to three types of development : single well formed embryos, multiple embryogenic mass by secondary embryogenesis and plant regeneration and rooting (Fig. 6). Contrary to the behaviour of cell suspensions, which lost embryogenic potential after six subcultures, callus tissues kept on agar-based medium with 2,4-D have retained their embryogenic potential for more than a year as tested by frequent transfer to MS medium with BAP and NAA.

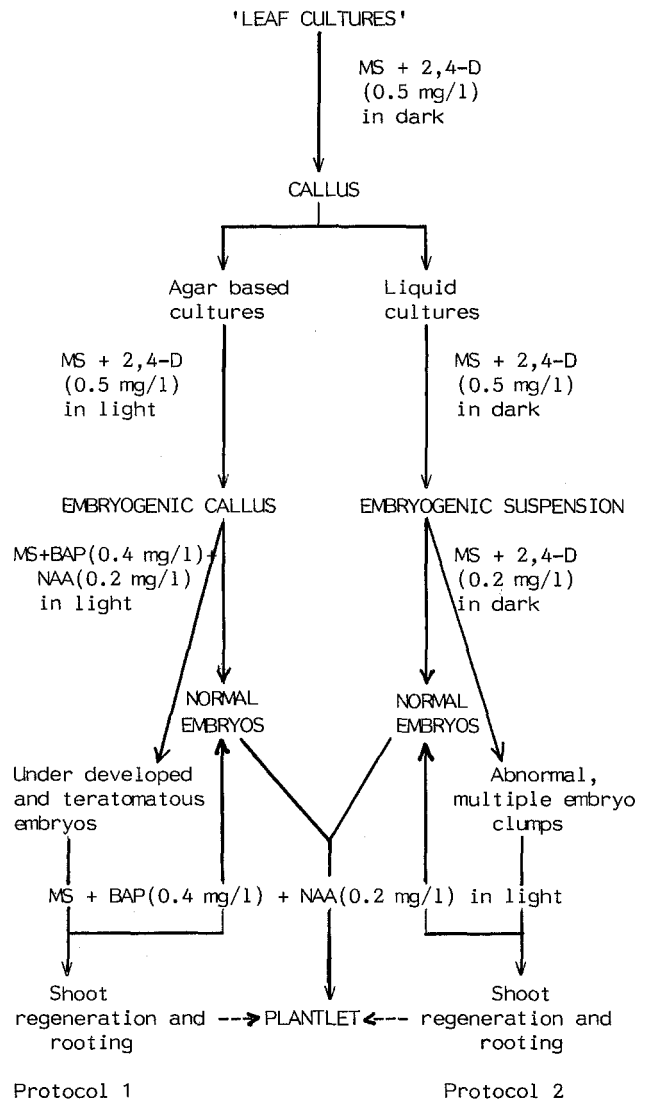


Fig. 6. Summary of the two protocols used for induction of somatic embryogenesis and plant regeneration from tissue and cell suspension cultures of *P. ciliata*.

Figs. 1-5. Somatic embryogenesis and plantlet formation in *P. ciliata*. Fig. 1. Composition of embryogenic cell suspension maintained on MS + 2,4-D (0.2mg/l) Fig.2. Different stages of embryo development in cell suspension(X2.25)Fig. 3. Secondary embryogenesis leading to embryogenic clump formation in cell suspension (X3.75)Fig. 4a-c. Developmental patterns of embryos plated on agar based MS + BAP (0.4 mg/l) + NAA (0.2 mg/l) medium(X5.25)4a. Typical well developed embryo. 4b. Precociously germinating embryo showing secondary embryogenesis from hypocotyl regions. 4c. Secondary embryogenesis leading to a group of green embryos. Fig.5. Plantlet raised from a somatic embryo (X0.3)

The two protocols, one utilizing agar based cultures and the other liquid culture for regeneration of plants through somatic embryogenesis are summarized in Fig. 6. The embryogenic cultures in protocol 1 retain the embryogenic potential over long period of time as compared to cell suspension in protocol 2. Therefore, high frequency regeneration of plants in agar based cultures, makes the large scale cloning of *P. ciliata* through somatic embryogenesis a distinct possibility. However, this would require further studies aimed at maintaining sustained embryogenic potential in cell suspensions and control of abnormal developmental patterns in both the protocols.

All the embryo-derived plants could be successfully hardened. This was indicated by the induction of dormant bud-like structures. These hardened plants could be transferred to field soil in natural outdoor conditions. The plantlets showed juvenile leaves (Fig. 5) typical of seedlings derived from sexual progeny. A total of 15 such plantlets were successfully grown in field soil in pots, five of which have grown for more than a year in the field. The performance of these will be compared with plants raised from cuttings.

In most of the plants the basic protocol for obtaining somatic embryos involves the induction of callus growth in an auxin-enriched medium and somatic embryogenesis upon transfer of such calli to a medium free of auxin (Tisserat et al. 1979, Ammirato 1983). In some cases, such as the present one, the requirement for somatic embryogenesis is slightly different: it involves initiating and maintaining a morphogenetic system of leaves in dark on BAP-containing medium, inducing the callus with an auxin (2,4-D), followed by transfer to a medium with a different auxin (NAA) and a cytokinin (BAP). The requirement of such specific conditions like initial dark incubation to induce growth of specific morphogenetic group of cells from different plants and the inhibitory effect of light is known for several species e.g. coffee (Sondahl et al. 1977; poplar (Mehra & Cheema 1980, 1985) and species of gramineae (Vasil 1985).

Reports on somatic embryogenesis from explants of mature forest trees are very few. The notable examples where plants could be taken to soil are *Eugenia* species (Litz 1984), and sandalwood (LakshmiSita et al. 1980). In recent reviews on tissue cultures of aspens and poplars (Ahuja 1986, 1987) there was no report on somatic embryogenesis in the genus *Populus*. The present studies show that it is possible to regenerate plants from in vitro established longterm cultures of mature

trees on a defined medium by both organogenesis and somatic embryogenesis using appropriate media and sequential treatments with different growth substances. It also demonstrates that embryo derived plants could be successfully hardened and transferred to soil and grown in field.

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