Plant regeneration and genetic transformation studies in petiole tissue of Himalayan poplar (*Populus ciliata* Wall.)

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Plant regeneration and genetic transformation techniques have been developed in petiole tissue of Himalayan poplar (*Populus ciliata*). High frequency shoot regeneration via direct organogenesis was obtained on MS medium supplemented with 1.50 mg/l Kn and 0.10 mg/l IAA using petiole explant. High percentage root regeneration in *in vitro* developed shoots was obtained on MS medium supplemented with 0.10 mg/l IAA. Himalayan poplar plantlets were able to regenerate within two months. Genetic transformation studies were carried out using disarmed *Agrobacterium tumefaciens* strain LBA 4404 containing *gus* gene (β-glucuronidase) in binary vector pBI 121 along with *npt-II* (neomycin phosphotransferase-II) gene. After cocultivation, the transformed cells were selected on the selective medium containing 50 mg/l kanamycin. Successful genetic transformation in the transformed calluses from petiole explant was confirmed by β-glucuronidase enzyme assay.

Keywords: Direct organogenesis, genetic transformation, β-glucuronidase, plant regeneration, *Populus ciliata*.

POPLARS occupy a unique and important position in the rural economy of India. Among the indigenous poplars, Himalayan poplar, i.e. *Populus ciliata* is a large, deciduous, dioecious and fast-growing tree of temperate and subtemperate regions of the Himalayas. Its wood is used for making general purpose plywood, packing cases, crates, support doors, matches, artificial limbs, fine paper and newsprint. However, Himalayan poplar is severely affected by a large number of insect pests, which lead to a considerable yield loss. Secondly, high lignin content in this species makes the operational costs of de-lignification process quite expensive in pulp and paper industries. Being an economically important crop, application of plant tissue culture and plant genetic engineering in Himalayan poplar cultivation is of special value, to obtain improved or desired traits like disease and insect resistance and development of reduced lignin content.

Efforts devoted to the use of explants of mature trees of proven worth for propagation through tissue culture technique have been limited to the work of only a few investigators[1–6]. Several procedures for transforming *Populus* species using *Agrobacterium tumefaciens* have been described[7–9]. However, before undertaking this approach, it is important to know whether the somatic cells of *P. ciliata* are able to regenerate in such a way as to give rise to whole plantlet and the conditions required for such plant regeneration and to standardize the technique of *Agrobacterium*-mediated gene transfer in Himalayan poplar cells with marker/reporter genes.

The work described here was initiated to establish a protocol for high frequency plant regeneration of Himalayan poplar (male plant) using petiole explant, and genetic transformation and expression of neomycin phosphotransferase-II (*npt-II*) and β-glucuronidase (*gus*) genes in the transformed calluses using the binary vector (pBI 121) in *A. tumefaciens* LBA 4404.

The plant material (*P. ciliata* Wall., male plant, petiole explants) was procured from the nursery maintained in our department. The nursery of Himalayan poplar was raised and maintained in a glasshouse in the department. Young and tender petioles were used as explant for plant regeneration and genetic transformation studies in Himalayan poplar.

To optimize the culture medium for high frequency shoot regeneration, petiole explants were excised from the plants. MS salts (macro and micro), vitamins supplemented with 100 mg/l meso-inositol, 3% sucrose and 0.8% agar–agar were used as basal medium[10]. Petioles were surface-sterilized and then cultured in flasks containing MS medium supplemented with various combinations and concentrations of plant growth regulators such as BAP and NAA, BAP and IAA, Kn and NAA, Kn and IAA (mg/l) respectively (Table 1). All the cultures were kept in a culture room at 26 ± 2°C with a 16 h photoperiod. The regenerated shoots were separated and individual shoots were transferred to the MS medium containing various concentrations of different auxins, IAA and IBA, for root induction and elongation to get a complete plantlet (Table 2). The regenerated Himalayan poplar plantlets were acclimatized.

Disarmed *A. tumefaciens* LBA 4404 strain containing a β-glucuronidase (*gus*) gene in binary vector pBI 121 system along with a kanamycin resistance (*npt-II*) gene for selection in both bacteria and plant was used for co-cultivation experiment (Figure 1).

After surface-sterilization, petioles were cut into small pieces of 0.5–1.0 cm size and inoculated on the shoot regeneration medium (on which high frequency shoot regeneration was obtained) for 48 h. Fresh cultures of *Agrobacterium* were centrifuged for 10 min at 5000 rpm and the supernatent was discarded to get a pellet. This pellet was resuspended in MS liquid medium to get a concentration of 10^8 cells/ml. This bacterial suspension and pre-cultured petiole explants on regeneration medium were used for the co-cultivation experiment. Petiole explants were immersed into bacterial suspension for 15–20 s and then were blotted dry on pre-sterilized filter paper and inoculated on the respective medium for co-cultivation. After co-cultivation for 48 h, petiole explants were transferred to the fresh selective regeneration medium containing kanamycin (50 mg/l) and

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Table 1. Shoot regeneration frequency of Himalayan poplar from petiole explants

Medium composition	Average no. of shoots formed per explant	Per cent shoot regeneration
MS basal medium + 1.50 mg/l BAP + 0.10 mg/l NAA	0.21	22.50 (28.14)
MS basal medium + 1.50 mg/l BAP + 0.50 mg/l NAA	4.097	60.00 (51.12)
MS basal medium + 0.50 mg/l BAP + 0.20 mg/l IAA	3.70	76.00 (64.46)
MS basal medium + 1.50 mg/l BAP + 0.50 mg/l IAA	1.120	32.00 (34.24)
MS basal medium + 0.50 mg/l Kn + 0.20 mg/l NAA	3.135	67.17 (55.44)
MS basal medium + 1.50 mg/l Kn + 0.10 mg/l NAA	0.426	17.98 (24.99)
MS basal medium + 1.50 mg/l Kn + 0.10 mg/l IAA	5.150	79.74 (63.43)
MS basal medium + 1.50 mg/l Kn + 0.20 mg/l IAA	1.075	31.87 (34.00)
$CD_{0.05}$	1.236	18.25 (13.55)

Numbers in brackets are arc sine transformed values.

Table 2. Root regeneration in *in vitro* developed shoots from petiole explants

	Concentration (mg/l)			
Treatment	0.05	0.10	0.20	Mean
IBA	61.50 (51.67)	66.66 (54.83)	58.36 (49.85)	62.17 (52.12)
IAA	75.00 (60.29)	100.00 (90.00)	83.30 (66.28)	86.10 (72.19)
$CD_{0.05}$				6.6818 (4.1581)

Numbers in brackets are arc sine transformed values.

Figure 1. Structure of expression vector: T-DNA region of pBI 121, containing (i) transcriptional fusion of NOS promoter with the coding region of *npt-II* and NOS terminator and (ii) transcriptional fusion of CaMV 35S promoter with the coding region of *gus* and NOS terminator. LB, Left border of T-DNA; RB, Right border of T-DNA; 35S, CaMV 35S promoter; NOS-ter, Nopaline synthase terminator; NOS-pro, Nopaline synthase promoter; *npt-II*, Neomycin phosphotransferase-II; *gus*, β-glucuronidase.

cefotaxime (500 mg/l) for the selection of transformed cells and to inhibit further agrobacterial growth. Petiole explants were subsequently subcultured to fresh selective regeneration medium in order to check excessive bacterial growth.

Spectrophotometric determination of β-glucuronidase activity in the plant tissues/calluses (transformed and non-transformed/control) was conducted according to Herman and Depicker[11]. Reaction mixture in a total volume of 1 ml contained 1 mM *p*-nitrophenyl β-D-glucuronide and suitable volume of enzyme extract. The reaction was incubated at 37°C for 60 min and was stopped using stop buffer. The absorbance was measured at 415 nm using the time point 0 as blank. Molar extinction coefficient of *p*-nitrophenol is 14,000; thus an absorbance of 0.014 represents 1 nmol of *p*-nitrophenol liberated (product of the reaction).

Each treatment consisted of at least 30 explants and each experiment was repeated thrice. Data recorded for the different parameters were subjected to Completely Randomized Design[12].

Young and tender petioles were used for plant regeneration studies. After one week of inoculation, the size of the petioles had increased and little callus formation was seen at the cut ends of the explants. Shoot buds and shoots started originating via direct organogenesis from the tissue around the cut edges after 14–15 days. The average number of shoots formed per explant was recorded. It was highest on MS medium supplemented with 1.50 mg/l Kn and 0.10 mg/l IAA (Table 1, Figure 2 *a*). Shoot elongation occurred on the same medium (Figure 2 *b*).

Elongated shoots (2–3 cm length) were excised and cultured on MS medium supplemented with various concentrations of different auxins, i.e. IAA and IBA. The period for induction of roots was variable among various concentrations of different auxins, but it was generally 10–15 days in culture. The number of shoots forming roots was highest (Table 2, Figure 2 *c*) on MS medium containing 0.10 mg/l IAA (100%). Himalayan poplar plantlets were able to regenerate within two months and young plantlets were transferred to pots containing a mixture of sand and soil (1 : 1) and acclimatized.

Among the various plant growth regulators tried in MS medium for shoot regeneration, it has been found that all

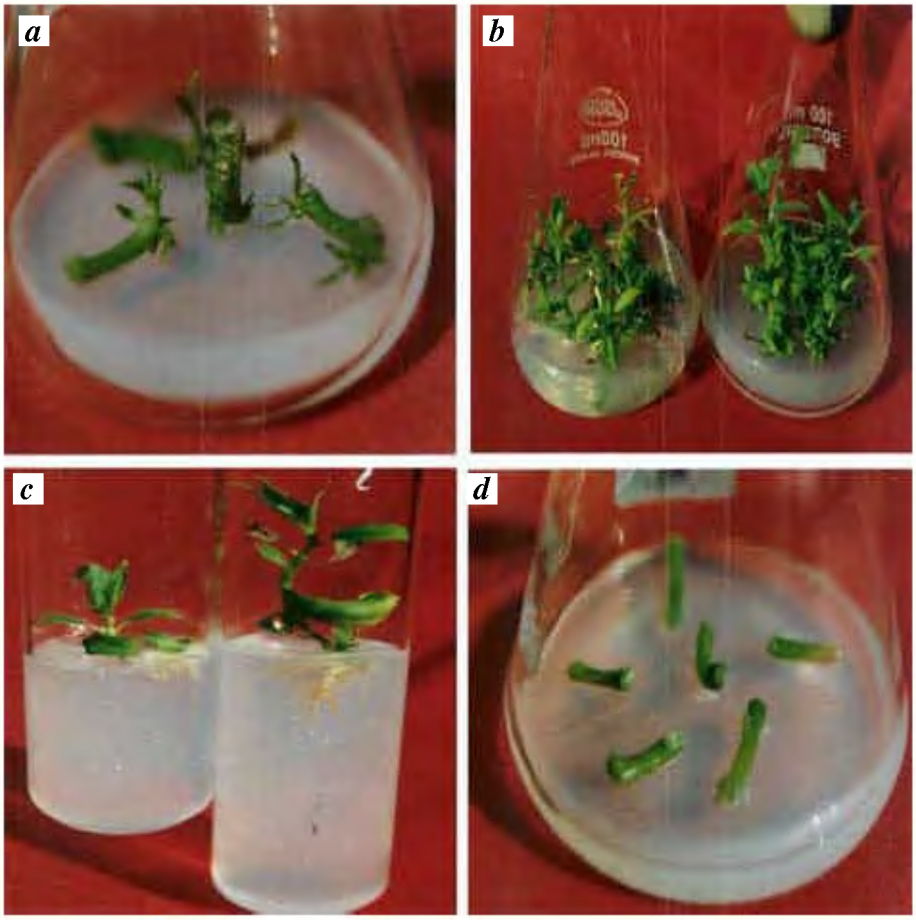

Figure 2. Plant regeneration and genetic transformation studies in Himlayan poplar tissue (*Populus ciliata* Wall.). ***a***, Regenerated shoots obtained from petiole explants. ***b***, Elongation of regenerated shoots. ***c***, Root regeneration after 10 days in culture from shoots regenerated from petiole explants. ***d***, Callus formation in petiole explants on selective medium containing kanamycin (50 mg/l) after two weeks of co-cultivation.

combinations of BAP and Kn with NAA and IAA were able to form shoots from petiole explant, but Kn in combination with IAA in nutrient MS medium was more effective in shoot induction than any other combination studied (Table 1). The highest percentage of root regeneration was observed in MS medium containing 0.10 mg/l IAA (Table 2). Han *et al.*[13] reported shoot regeneration from petiole explant in *Populus trichocarpa* × *P. deltoides* hybrids in WP medium supplemented with 5 µm zeatin and root regeneration in WP medium supplemented with 10 µm IBA. Jafari *et al.*[14] reported a high frequency of plant regeneration from petiole explants of four poplar genotypes, i.e. *Populus nigra* forms Osli 96 × Lassi 7, Ugod, Osli 96 and 462-5 on MS medium supplemented with 2.5 mg/l BA and 0.2 mg/l NAA through callusing. Plant regeneration studies in *P. ciliata* (female plant) were also carried out by Mehra and Cheema[15], and Cheema[16], in which they reported regeneration from immature leaf lamina discs and cell suspension. But they could not get any regeneration from male plant. Shen *et al.*[17] reported callus induction of *Populus alba* cv. 'Pyramidalis', *P. euphratica* and *P. maximowiczii* × *P. plantierensis* in leaf explant on MS medium containing BAP, NAA and 2,4-D and regenerated adventitious shoots after subculturing the calluses on MS medium containing BAP in combination with NAA or GA_3. On the other hand, in our experiments, we were successful in obtaining direct organogenesis without callus phase, with high frequency shoot regeneration (Figure 2 *a*). The regeneration system developed will serve as a pre-requisite for genetic transformation studies.

Young and tender petiole segments were pre-cultured on the shoot regeneration medium for 48 h, then co-cultivated with *A. tumefaciens* strain containing the binary vector pBI 121 along with the disarmed helper Ti-plasmid for 48 h and then transferred to the fresh selective shoot regeneration medium containing antibiotics. Callus formation was seen after 15–16 days at the cut edges of the petiole segments and also at the wound site, where the tissue was damaged during inoculation (Figure 2 *d*). The non-transformed (control) tissue did not survive on the selective

medium containing kanamycin (50 mg/l). The developing putative transformed calluses from the petiole segments were tested for β-glucuronidase enzyme assay.

β-glucuronidase enzyme assay was carried out to confirm the transfer of *gus* gene from *Agrobacterium* into the genome of cells of Himalayan poplar. Chimeric *gus* gene was expressed in transformed calluses of Himalayan poplar (Table 3).

To date, a number of reporter and marker genes have been used in studies of gene expression in higher plants[18]. The two most useful reporter genes have been the bacterial genes, chloramphenicol acetyl transferase (*CAT*) and neomycin phosphotransferase-II (*npt-II*), which encode enzymes with specificities not normally found in plant tissues[19,20]. However, both *CAT* and *npt-II* are relatively difficult, tedious and expensive to assay[21,22]. Another reporter gene, i.e. the firefly luciferase gene has been used as a marker in transgenic plants, but the enzyme is labile and difficult to assay with accuracy[23].

β-glucuronidase is a superior reporter gene system for plant transformation studies. It is easily and sensitively assayed spectrophotometrically, fluorometrically *in vitro* and can also be assayed histochemically to localize *GUS* activity in plant cells and tissues. *GUS* is stable and tissue extracts continue to show high level of *GUS* activity after prolonged storage[24].

The gene expression was reliably measured from a small transformed callus of Himalayan poplar. Phenotypic and enzymatic data indicate that the chimeric *npt-II* and *gus* genes were expressed in Himalayan poplar cells (callus). Expression of chimeric *npt*-II and *gus* genes has also been reported in the transformed tissue of walnut[25], peach[26], cotton[27], watermelon[28] and cabbage[29]. Genetic transformation technology in combination with advances in plant regeneration studies in this species for the present data on high frequency plant regeneration, may eventually lead to the recovery of transgenic plants of Himalayan poplar. Genetic transformation studies in *Populus* species have also been carried out to introduce marker, reporter and desirable genes[30–35]. Dai *et al.*[36] also reported genetic transformation of two hybrid poplar clones (*P. canescens* × *P. grandidentata* and *P. tremuloides* × *P. davidiana*) with *gus* and *npt-II* genes, and confirmed the success of their experiments by *GUS* assays, PCR and Southern blot analysis. However, no work has been done on genetic transformation studies of *P. ciliata*, except for the present investigation.

Table 3. β-glucuronidase activity (nmol *p*-nitrophenol liberated/h/g fr wt) in control and transformed tissue (callus) of Himalayan poplar

Sample	Enzyme activity (nmol *p*-nitrophenol liberated/h/g)
Control tissue (non-transformed)	0.00
Transformed callus	1357 ± 2.37*

0.014 OD (Absorption) = 1 nmol *p*-nitrophenol liberated
*Standard error.

This is a report of successful recovery of high frequency plant regeneration via direct organogenesis and genetic transformation in petiole tissue of male plant of *P. ciliata* with *npt-II* and gus genes. Attempts are being made to regenerate plantlets from transformed calluses and analyse them for expression of foreign gene. The protocol standardized for plant regeneration and genetic transformation is being used in our laboratory to manipulate *Populus* spp. by introducing antisense gene of *CAD* (cinnamyl alcohol dehydrogenase) to develop plants with low lignin content for reducing the cost of paper manufacturing and environmental pollution.

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Comparative studies on species richness, diversity and composition of oak forests in Nainital district, Uttaranchal

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Species richness, diversity and composition of herb species in oak forests, viz. Banj oak (*Quercus leucotrichophora*) A. Camus, Tilonj oak (*Quercus floribunda*) Rehder and Kharsu oak (*Quercus semecarpifolia*) Smith were evaluated. The total number of species, genera and families observed for Kharsu oak forest was higher than Banj and Tilonj oak forests. Only a few species were dominant in all study sites. Asteraceae and Lamiaceae were found to be the dominant families in all the forest types. Regarding ecological structure and composition, the study revealed that Banj and Tilonj oak forests were less complex in comparison to Kharsu oak forest.

Keywords: Composition, diversity, oak forests, species richness.

HIGH biodiversity favours ecological stability, whereas accelerating species loss could lead to collapse of the ecosystem. Human domination of earth's ecosystems, which is markedly reducing the diversity of species with many habitats worldwide, is accelerating species extinction[1].

Biotic disturbances generally have caused substantial reduction in forest cover, which has led to serious ecological disasters such as soil erosion, loss of fertility and violent floods in the adjacent plains[2].

The forest vegetation of Himalaya has been of major interest to ecologists since long. Osmaston[3] worked out the forest flora of Kumaun Himalaya, and Dudgen and Kenyor[4]

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