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Agrobacterium-Mediated Genetic Transformation of *Populus deltoides* Marsh Clone G48 with gus and *npt-II* Genes

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Abstract

The present investigation had been carried out to standardize a protocol for *Agrobacterium*- mediated gene transfer in *Populus deltoides* Marsh. Clone G_{48} using petiole explants. Reproducibility of already standardized regeneration protocol for clone G_{48} had been evaluated. High frequency shoot regeneration (72%) from petiole explants was obtained on MS medium supplemented with 0.5 mg l^{-1} BAP, 0.2 mg l^{-1} IAA and 15 mg l^{-1} adenine sulphate. Root regeneration (100%) in *in vitro* developed shoots was obtained on MS medium supplemented with 0.1 mg l^{-1} IAA. Increasing concentrations of kanamycin (10-50 mg l^{-1}) were given to find out the minimum dose of kanamycin required for the selection of putative transformed cells during genetic transformation. It was observed that a doze of 50 mg l^{-1} kanamycin inhibited callus formation and shoot regeneration and the explants turned brown and started dieing. This concentration of kanamycin would be the most useful for selection of *npt-II* gene transformed petiole cells/tissues. Disarmed *Agrobacterium tumefaciens* LBA 4404 strain containing a reporter β-glucuronidase *(gus)* gene in binary vector pBI 121 along with kanamycin resistance gene *(npt-II)* was used for genetic transformation experiments. Only the transformed cells/shoots were able to grow on selective shoot regeneration medium containing 50 mg l^{-1} kanamycin, whereas control explants did not survive. Regenerated putative transgenic shoots were subjected to transient gene expression analysis using spectrophotometric *GUS* assay and were found positive. This genetic transformation protocol will provide a platform for genetic manipulation of *P. deltoides* clone G_{48} for incorporation of genes governing various silviculturally important traits in future.

Keywords

Populus deltoids; Clone G_{48}; Genetic transformation; Shoot regeneration; Antibiotic sensitivity; *GUS* assay

Introduction

Tree crop improvement by conventional breeding methods suffers serious constraints because of long reproductive cycles and complex genetic traits. The bottlenecks associated with these conventional techniques can be circumvented by genetic engineering technique in which gene(s) coding for novel traits can be incorporated into the genome of crop plants without affecting their genetic integrity. *Populus deltoides*, commonly known as 'Cottonwood', a native of North America, is generally found to grow above 28° N latitude and belongs to family *Salicaceae* and order *Salicales*. *P. deltoides* clone G48 has shown excellent performance in Uttar Pradesh, Punjab, Haryana and Maharashtra states since its introduction. *P. deltoides* clone G_{48} holds an excellent promise as a source of fibres for various grades of paper, fine paper, packing paper and newsprint. Its wood has been found very much suitable for the manufacture of artificial limbs, sports goods, construction materials, agriculture implements, poles and hurdles for drawing produce. The high productivity level of this species make itself ideal for plantation forestry and thus constitutes a varietable "saving bank" when harvested [1].

However, *P. deltoides* is severely attacked by a number of insect pests such as *Clostera cupreata, Clostera fulgurita, Apriona cinerera,* and *Ascotis species* and it is also prone to a number of fungal, bacterial and viral parasites such as *Botryodiplodia palmarum, Cladosporium humile, Phyllosticta adjuncta, Cercospora populina, Myrothecium roridum* etc. which cause tremendous yield loss. Secondly, high lignin content in this species makes the operational cost of delignification process quite expensive for pulp and paper industries. Development of reduced lignin content in woody tissues by genetic engineering technique has significant benefits by ensuring the environmental safety and reducing the operational costs of the delignification process [2]. With increasing demand for pulp and paper, reforestation of lowlands and phytoremediation of contaminated soil, fast growing poplar species are commercially important [3]. Genetic engineering offers one of the most potential tools for development of new cultivars with specific characteristics such as resistance to various insect-pests, diseases and improving wood quality. However, this technique requires a large number of totipotent cells that are capable of transformation via DNA addition, independent cell division and efficient regeneration of plants [4]. To carry out genetic manipulation at cell level, there is a need to standardize and develop an efficient *Agrobacterium*-mediated genetic transformation system using marker (*gus* and *npt-II*) genes. Further, the ability to genetically engineer *P. deltoides* will be particularly useful in view of the factors limiting genetic improvement such as the large size of mature plants and the long sexual generation times.

Poplar is considered as the prominent tree model for tissue culture, molecular mapping and transgenic technology. A significant progress in development and standardization of regeneration protocol in *Populus species* has already been achieved in our laboratory [1,5-9] in *P. deltoides*, Yadav et al. [10]; Thakur et al., [11]. However, only few studies had been carried out on genetic transformation of *P. deltoides* [12-15] and so far, there is no report describing the genetic transformation of G_{48} clone of this species using marker genes (*gus* and *npt-II*) and there is a strong need for such a protocol for G_{48} clonal improvement.

The present investigation had been aimed to validate the reproducibility of already developed regeneration protocol for *P. deltoides* clone G_{48}, to develop an antibiotic selection regime with kanamycin for selection of transformed cells during genetic transformation experiments and to standardize an efficient protocol for *Agrobacterium tumefaciens*-mediated genetic transformation of *P. deltoides* clone G48 using petiole explants through direct organogenesis.

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Materials and Methods

Plant material

The plant material (young and tender petioles; 10-12-day-old) were procured from the nursery of *P. deltoides* clone G_{48} established and maintained in the glasshouse of the department. The petioles were washed under running tap water for half an hour and then treated with 0.3% bavistin solution for 2-3 min followed by 2-3 times washing with sterilized distilled water. The petiole explants were then treated with 0.1% solution of mercuric chloride for 2-3 min and again washed with sterilized distilled water thrice to remove the traces of mercuric chloride.

Culture medium and culture conditions

Murashige and Skoog's [16] medium was supplemented with various concentrations and combinations of plant growth regulators viz. BAP, Kn, NAA, IAA and AdS (Table 1) to validate the reproducibility of already developed regeneration protocol in our laboratory [11]. All the media included 3% (w/v) sucrose, 100 mg/l (w/v) meso-inositol and 0.8% (w/v) agar as gelling agent. The pH of the medium was adjusted to 5.8 ± 0.02 with 0.1 N NaOH and 0.01N HCl prior to autoclaving at 121°C temperature and 15 lbs $inch^{-2}$ pressure for 20 min. The petiole explants were excised into 0.50-0.75 cm pieces and were cultured in flasks containing various concentrations and combinations of plant growth regulators. These cultures were incubated at 25 ± 2°C temperature under 16 hr photoperiod of 125 µmol $m^{-2}s^{-1}$ photon flux density provided by cool white fluorescent tubes (Philips, India) and 60% relative humidity and were observed for shoot bud induction. For every combination, six flasks with five explants each were inoculated. For shoot multiplication, the regenerated shoots were separated from each other and subcultured on MS medium containing same concentrations and combinations of growth regulators which were used for shoot regeneration. After obtaining a desirable number of shoots, the multiplied shoots were separated and transferred to MS medium containing 0.1 mg l^{-1} IAA and were evaluated for per cent root regeneration.

Kanamycin sensitivity in petiole tissues of *P. deltoides* clone G_{48}

Prior to transformation experiments, kanamycin sensitivity level of petiole explants was evaluated so as to determine the effective concentration for selection of transformed cells during genetic transformation experiments.

Preparation of selective shoot regeneration media

Kanamycin was dissolved in autoclaved distilled water and then stored in centrifuge tubes at -20°C before use. Selective shoot regeneration medium for kanamycin sensitivity experiment was prepared by adding kanamycin by filter sterilization through a 0.22 µm pore size (Millipore membrane filter) into pre-sterilized molten regeneration medium, under aseptic conditions. Different concentrations (10, 20, 30, 40 and 50 mg l^{-1}) of kanamycin were added to study the effect of antibiotic on the relative growth/fresh weight of the cultured explants.

Callus/adventitious shoot bud formation and measurement of relative growth

After surface sterilization, petiole explants were excised into small pieces and weighed on a mettler balance under aseptic conditions. The initial fresh weight of each explant was recorded. The explants were cultured on MS regeneration medium without (control) and with different concentrations of kanamycin (0, 10, 20, 30, 40 and 50 mg l^{-1}) in different petriplates. These cultures were incubated under culture room conditions as mentioned above. The cultured petiole explants were observed for adventitious shoot regeneration/callus formation and eventual changes in fresh weights. Morphological changes were observed in these tissues from 0 to 35 days in culture. Fresh weight of the explants/callus was measured at the interval of 7 days till 35 days. Relative growth (fresh weight) at 7 days interval was calculated.

Genetic transformation in *P. deltoides* clone G_{48}

Binary vector and *Agrobacterium* strain: Disarmed *Agrobacterium tumefaciens* LBA 4404 strain containing a reporter/marker β-glucuronidase (*gus*) gene in binary vector pBI 121 along with kanamycin resistance gene neomycin phosphotransferase-II *(npt-II)* for selection in both bacteria and plant was used for co-cultivation experiments. The coding sequence of *gus* was connected to 35S promoter of cauliflower mosaic virus (CaMV35S) and terminator from nopaline synthase gene (NOS). The coding sequence of *npt-II* was connected to nopaline synthase gene promoter and terminator sequences as shown in Figure 1. The *Agrobacterium* strain LBA 4404 was obtained from Dr D.P.S. Verma, Plant Biotechnology Centre, Ohio State University, Kansas Columbus (USA) (through personal communication) and was maintained by sub-culturing the bacterial colonies on YMB medium containing 50 mg l^{-1} kanamycin. To achieve proper growth of Agrobacterial cells, the subcultured plates were incubated at 28°C under dark conditions for 1-2 days and then were placed at low temperature (4 ± 2°C) for storage.

Pre-culturing of petiole explants: Petiole explants were cut into small pieces of 0.5-1.0 cm size and inoculated onto regeneration medium (MS medium supplemented with 0.5 mg l^{-1} BAP, 0.2 mg l^{-1} IAA and 15 mg l^{-1} adenine sulphate) and incubated in culture room for a period of 48 hrs. With 50 explants in each treatment, the transformation experiment was repeated thrice, following completely randomized design.

Co-cultivation and infection: Fresh cultures of *Agrobacterium* strain were prepared by inoculating a loopful of culture in 10 ml liquid

Table 1: Effect of various combinations and concentrations of BAP, Kn, NAA, IAA and adenine sulphate on callus formation/shoot regeneration from petiole explant of *Populus deltoides* Marsh. clone G_{48}.

S. No.	Treatment/ medium	Average number of shoots formed per explant	Percent shoot regeneration
1.	MS (full strength) basal medium + 0.5 mg l^{-1} BAP + 0.5 mg l^{-1} NAA +15 mg l^{-1} AdS	1.20	40
2.	MS (full strength) basal medium + 0.5 mg l^{-1} BAP + 0.2 mg l^{-1} IAA +15 mg l^{-1} AdS	3.16	72
3.	MS (full strength) basal medium + 1.5 mg l^{-1} BAP + 0.1 mg l^{-1} IAA +15 mg l^{-1} AdS	2.04	52
4.	MS (full strength) basal medium + 2.0 mg l^{-1} BAP + 0.1 mg l^{-1} IAA +15 mg l^{-1} AdS	1.52	48
5.	MS (full strength) basal medium + 1.5 mg l^{-1} Kn + 0.5 mg l^{-1} IAA +15 mg l^{-1} AdS	0.80	22
6.	$C.D._{0.05}$	0.40	17

YMB medium containing 50 mg l^{-1} kanamycin (filter sterilized). The cultures were kept overnight in orbital shaking incubator at 26°C at 150 rpm. Then the agrobacterial cells were pelleted by centrifugation at 10,000 rpm for 5 minutes and re-suspended into liquid YMB medium containing 50 mg l^{-1} kanamycin for getting an optimum concentraton of 10^8 bacterial cells per ml for effective infection on the explants. Petiole explants after a period of pre-culturing for 48 hrs were infected with *A. tumefaciens* suspension for 1-5 minutes in petri dishes. After infection, the explants tissues were blotted with sterile filter paper to remove the excess of agrobacterial cells and inoculated on the respective antibiotic free co-cultivation medium. The petriplates were then sealed with parafilm and the co-cultivated explants were incubated at 26 ± 2°C under dark conditions for 48 hours for successful gene transfer.

Selection of transformed cells and regeneration of transformed shoots: For the preparation of selective shoot regeneration medium, kanamycin and cefotaxime were added at the concentrations of 50 mg l^{-1} and 500 mg l^{-1}, respectively by filter sterilization. The whole assembly of the filter sterilization unit and the syringe were sterilized before using and the antibiotics were added by filter sterilization under laminar flow cabinet to maintain aseptic conditions. After co-cultivation, petiole explants were washed 3-4 times with sterilized distilled water containing 500 mg l^{-1} cefotaxime to eliminate the bacterial growth, blot-dried on sterile filter paper and then subcultured onto selective regeneration medium. The cultures were subcultured frequently onto the fresh selective regeneration medium after blotting whenever any agrobacterial growth reappears and were incubated for six weeks for further growth and multiplication of transformed shoots.

Biochemical *GUS* assay

Spectrophotometric determination of *GUS* activity in the leaf tissues (transformed and non-transformed) was conducted according to Herman and Depicker [17]. Reaction mixture in a total volume of 1 ml contained 1 mM *p*-nitrophenyl *ß*-D-glucuronide and suitable volume of enzyme extract. The reaction mixture was incubated at 37°C temperature for one hour and was stopped using stop buffer. The absorbance was measured at 415 nm wavelength using 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).

Data Analysis

All the experiments were set up in Completely Randomized Design [18] and repeated thrice. The analysis of variance (ANOVA) appropriate for the design was carried out to detect the significance of differences ($P<0.05$) among the treatment means.

Results and Discussion

Plant regeneration from petiole explants

Genetic engineering for incorporation of useful traits is dependent on an efficient and reliable regeneration protocol. In the present investigation, petiole explants showed increase in size and very little callusing was observed at the cut ends in first two weeks of culturing (Figure 2a). The colour of the explant changed from green to greenish-brown and adventitious shoot buds began to originate after 21 days in culture. High frequency shoot regeneration (72%) had been obtained from petiole explants on MS medium containing 0.5 mg l^{-1} BAP, 0.2 mg l^{-1} IAA & 15 mg l^{-1} adenine sulphate, with 3.16 average number of shoots formed per explants (Figure 2b). Adventitious shoot bud induction via direct organogenesis was observed from the petiole explants in all the five media (Table 1). In another study, equal concentrations of Kn and IAA (0.25 mg/l) have also been reported to be optimum for shoot bud induction from leaf, internode and root explants of WIMCO199 and L_{34} clones [10]. Elongated shoots (about 1-2 cm in length, Figure 2c) were excised and cultured on MS medium supplemented with 0.1 mg l^{-1} IAA for rooting and within a period of 8-10 days, profuse rooting (100%) had been observed from all the *in vitro* cultured shoots (Figure 2d). Reproducibility of plant regeneration protocol already developed in our laboratory has been validated well. Chaturvedi et al. [19] had also developed such a regeneration system in *P. deltoides* G_3 clone. Petiole explants had been found as the leading as well as the most efficient explants in *Populus sps* used for plant regeneration studies [9,20].

Kanamycin sensitivity test

Kanamycin resistance is the most widely used selectable marker for plant cell transformation and the sensitivity of a particular species or explant to kanamycin is a key element in the development of any new transformation system where a kanamycin resistance gene will be employed. In the present studies, petiole explants in control medium i.e. shoot regeneration medium without kanamycin, were very healthy and showed appropriate growth on this medium (Figure 3a). 10 and 20 mg l^{-1} concentration of kanamycin could not affect growth of leaf and petiole explants much, however, it was inhibitory. But more than 30 mg l^{-1} of kanamycin had changed the colour of the tissues to pale yellow and slightly brown (Figure 3b). In the control experiment, adventitious shoot bud regeneration was observed after 14-18 days in culture from petiole explants. No shoot regeneration or shoot bud formation was observed from petiole explants even after 5 weeks on the selective shoot regeneration medium containing different concentrations of kanamycin. A gradual decline in fresh weight of the petiole explants was recorded from 10 to 50 mg l^{-1} kanamycin concentration (Figure 4). The maximum decline in fresh weight was observed at 50 mg l^{-1} kanamycin, whereas in case of control (shoot regeneration medium without kanamycin), there was a gradual and maximum increase in fresh weight. The increase in fresh weight observed at higher concentration was perhaps due to expansion of cells. It was observed that the initial stages of shoot regeneration were also affected on kanamycin containing medium. Thakur and Srivastava [21] also found 50 mg l^{-1} kanamycin concentration inhibitory for regeneration from leaf and petiole explants of *P. ciliata*.

The use of an appropriate antibiotic, which would allow the selective regeneration of transformed cells only, is very important during genetic transformation experiments. Some plant species

Figure 1: Structure of expression vector: T-DNA region of pBI 121, containing (i) transcriptional fusion of CaMV35S promoter with the coding region of *gus* and OCS terminator, ii) transcriptional fusion of NOS promoter with the coding region of *npt-II* and NOS terminator.

and tissues are very sensitive to aminoglycoside antibiotics. One of the possible reasons is that the breakdown products of antibiotics may act as growth regulators, thus modifying defined tissue culture conditions [22]. Another possibility is that kanamycin causes DNA hypermethylation and therefore affects gene expression and plant development [23]. In a similar study, induction of secondary embryogenesis in *Picea omorika* was blocked by the presence of 10 mg l^{-1} kanamycin [24], whereas, Guo-qiang et al. [25] reported the complete inhibition of shoot and root differentiation in stem segments of *Platanus orientalis,* when the media was supplemented with 40 mg l^{-1} and 15 mg l^{-1} concentration of kanamycin, respectively. Xia et al. [26] found 15 mg l^{-1} concentration of kanamycin as the optimal one for transgenic plantlet selection in *Populus euphratica*. However, in our experiments, kanamycin in a concentration of 50 mg l^{-1} was found completely inhibitory for adventitious shoot bud regeneration / callus formation and the petiole explants turned dark brown in this concentration and started dieing.

Genetic transformation in *P. deltoides* clone G_{48}

During pre-culture, petiole explants showed active growth on MS medium containing 0.5 mg l^{-1} BAP, 0.2 mg l^{-1} IAA and 15 mg l^{-1} adenine sulphate. The petiole explants were pre-cultured for 48 hrs before carrying out the co-cultivation experiment and then were co-cultivated with *Agrobacterium tumefaciens* LBA 4404 strain containing the binary vector pBI 121 along with disarmed helper Ti-plasmid for 48 hours and the cultures were incubated in the culture room at 26 ± 2°C (Figure 5a).

Selection and regeneration of putative transformants from petiole explants

After co-cultivation with *Agrobacterium* strain, the explants were transferred to the fresh selective shoot regeneration medium containing cefotaxime (500 mg l^{-1}) and kanamycin (50 mg l^{-1}) to kill the agrobacterial cells and to select the transformed *P. deltoides* cells, respectively (Figure 5b). Callus formation was observed after two weeks at the cut edges of

Figure 2: Plant regeneration in *Populus deltoides* clone G_{48} from petiole explants.
a) Petiole explants cultured initially on shoot regeneration medium.
b) Multiple adventitious shoot bud induction from petiole explants after 14–16 days in culture on shoot regeneration medium.
c) Shoot multiplication and elongation after 28 days in regeneration medium.
d) Root regeneration in *in vitro* developed shoots after 15 days in culture.

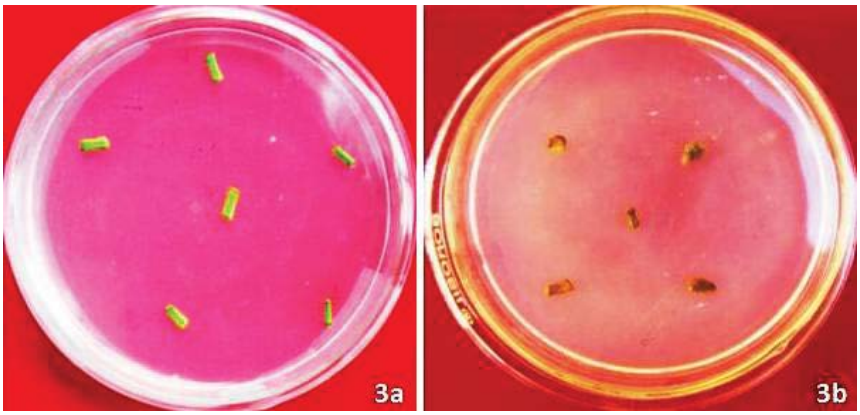

Figure 3: Kanamycin sensitivity in cultured petiole tissues of *Populus deltoides* clone G_{48}.
a) Petiole explants cultured on shoot regeneration medium without kanamycin after 12 days in culture (control experiment).
b) Petiole explants cultured on selective shoot regeneration medium containing 50 mg l^{-1} kanamycin after 28 days in culture.

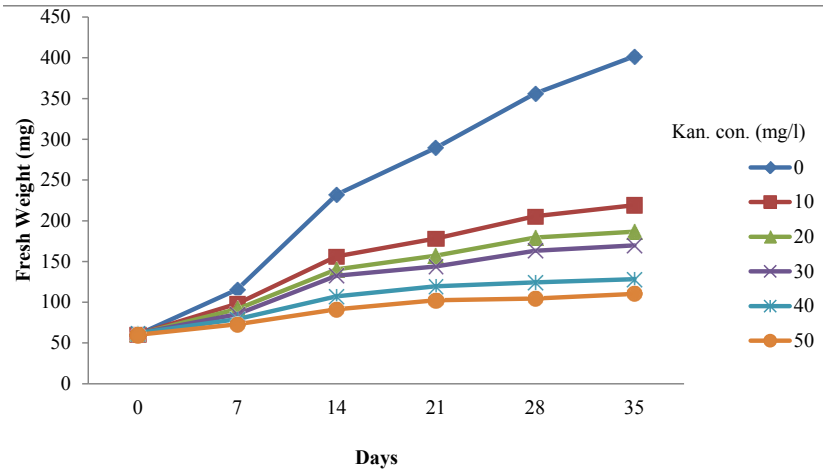

Figure 4: Effect of different concentrations of kanamycin on the relative growth (fresh weight) of petiole explants of *Populus deltoides* clone G_{48} at different intervals of time.

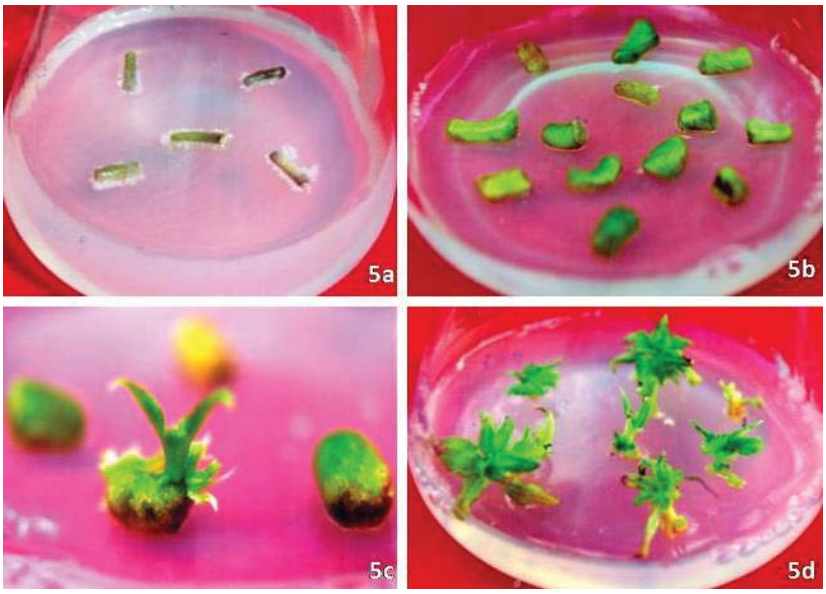

Figure 5: *Agrobacterium tumefaciens*-mediated genetic transformation in *Populus deltoides* clone G48.
a) Co-cultivation of petiole explants on regeneration medium for 48 hours.
b) Petiole explants subcultured on the selective regeneration medium after co-cultivation.
c) Shoot bud induction from petiole explants after three weeks of culturing on selective regeneration medium.
d) Multiplication and elongation of putative transgenic shoots on selective regeneration medium

petiole explants and also at the wound site where the tissues were damaged during inoculation. The tissues which were not transformed turned brown or black on selective medium after 20-25 days in culture. The transformed petiole explants which were growing on the selective regeneration medium, gave rise to independent putative transgenic shoots after 25 days in culture (Figure 5c). The putative transformed shoots were green colored reflecting the presence of *npt-II* gene and indicated that regeneration has occurred only from transformed cells of petiole explants (Figure 5d). A selective advantage was given to the transformed cells through the introduction of *npt-II* gene conferring resistance against kanamycin, which allows only the transformed cells to grow under the conditions where non-transformed cells were unable to survive. Along with kanamycin, another antibiotic cefotaxime is used extensively to eliminate agrobacterial cells from plant cultures after co-cultivation. In our experiments, we have used a concentration of 500 mg l^{-1} cefotaxime to inhibit the growth of agrobacterial cells. In another study, it had been reported that cefotaxime exerts a positive effect and promotes shoot regeneration in *Eucalyptus tereticornis* [27]. LBA 4404 strain was found quite infective to *P. deltoides* cells. It has been used successfully for genetic transformation in a number of tree species including eucalyptus [28], *Populus ciliata* [29] etc.

In the present studies, a pre-conditioning and co-cultivation period of 48 hours had been found suitable for effective genetic transformation from petiole explants. It has been proposed that during pre-culturing, the explants undergo a physiological and developmental shift to enter the morphogenetic competency. When

Table 2: β-glucuronidase activity (n mole p-nitrophenol liberated/ hr/gm fresh weight) in control and transformed leaf tissue of *Populus deltoides* Marsh clone G_{48}.

Sr. No.	Sample	Enzyme activity (nmol *p*-nitrophenol liberated $h^{-1}g^{-1}$)
1.	Control tissue (non-transformed)	0.00
2.	Transformed leaf tissue	2147.00 ± 50.76*

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

the T-DNA is inserted after this period, the recipient cells have already entered the regeneration pathway. In similar studies, medium composition and pre-culturing incubation conditions of the explants, before *Agrobacterium* infection were found to enhance T-DNA transfer and transformation frequency in some plant species [30,31]. Shoot elongation occurred at a slow rate on medium containing kanamycin.

Transient gene expression in transgenic shoots of *P. deltoides* clone G_{48}

Although the kanamycin resistant shoots growing on selective regeneration medium containing 50 mg l^{-1} kanamycin were indicative of the expression of *npt-II* gene, yet *GUS* activity was also examined to further confirm the expression of these newly incorporated genes. In the present studies, leaf tissues obtained from ten putative transgenic shoots of *P. deltoides* clone G_{48} were assayed by biochemical or spectrophotometric analysis to confirm the gene integration and expression into the genome of *P. deltoides* cells and all of them showed β-glucuronidase activity. The enzyme activity was not observed in the non-transformed shoots (Table 2). β-glucuronidase is easily and sensitively assayed fluorometrically. This enzyme catalyses the hydrolysis of a wide variety of β-glucuronides. In similar studies, spectrophotometric examination of *GUS* activity had also been carried out successfully to confirm the gene expression in transgenic plants of *Eucalyptus tereticornis* [27], *Populus ciliata* [7,8] and *Phoenix dactylifera* [32].

Conclusion

In conclusion, an efficient *Agrobacterium tumefaciens*-mediated genetic transformation protocol and a kanamycin sensitivity regime has been developed for *P. deltoides* clone G_{48} using *gus* and *npt-II* genes through direct organogenesis, followed by subsequent regeneration of transgenic shoots. In future, this protocol may be used to genetically improve *P. deltoides* clone G_{48} by incorporating various silviculturally important genes like; a) Insect resistance genes: *'Bt* gene (*cry*) and proteinase inhibitor-II gene; b) Lignin reducing/modifying genes; c) Herbicide tolerance genes and genes for tolerance to water and salt stress etc [33].

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