Pak. J. Bot., 46(3): 1079-1086, 2014.

OPTIMIZATION OF *AGROBACTERIUM TUMEFACIENS* MEDIATED TRANSFORMATION IN *POPULUS DELTOIDES*

EDWIN JOHN, ASMA MAQBOOL AND KAUSER ABDULLA MALIK*

Department of Biological Sciences
Forman Christian College, Lahore, 54600, Pakistan.
**Corresponding author's Email: kauser45@gmail.com; Tel.: 92-42-99231581-8*

Abstract

The objective of the study was to develop an efficient protocol for *Populus deltoides* transformation through *Agrobacterium tumefaciens* LBA4404. *Agrobacterium* strain harboring binary plasmid pGA482 with *Gus* (*uidA*) gene under *CamV35S* promoter and *Neomycin phosphotransferase* (*nptII*) gene under *Nos* promoter was used for the transformation. Nodal, internodal and leaf explants from 4-5 months *In vitro* and fieldgrown plants were used for the transformation. Transformation was done under different conditions including, preculture time, optical density, acetosyringone concentration, infection time and co-cultivation time. Confirmation of transformation was done through *GUS* histochemical staining. Highest transformation efficiency was observed in one week precultured leaf explants from field grown source on preculture medium containing 200µM acetosyringone. Precultured explants from *In vitro* source also gave good results for transformation but the callus formation was found to be slow in leaf explant. Calli from the both sources did not show any transformation when infected with O.D A_{600nm} range from 0.3-0.8. Node and internode though showed less transformation rate but the callogenesis was found to be highest in node and internode explants on CIM 1. Leaf explants from field source also gave high callus induction on CIM 5. *A. tumefaciens* O.D A_{600nm} 0.3-0.5 was found to be effective. Infection time of 1-2 hour and co-cultivation time of 1day in dark were found to be optimum for the transformation. 200mg/l of timentin was found the best to control the overgrowth of *Agrobacterium.*100mg/l Kanamycin in growth medium was found to sufficient for selection for transformants. Selected transformants were confirmed through PCR for the presence of transgene. The present protocol for *P. deltoides* was found to be efficient for genetic transformation and can be used to introduce novel traits in the *P. deltoides.*

Introduction

Populus deltoides also known as eastern cottonwood is native to North America and belongs to family *Salicaceae.* Because of its fast growth and wood quality, the genus populus and hybrid cottonwoods, aspens and other poplar species are highly valued and have industrial importance for wood, paper and pulp industry (Stealer *et al.,* 1996).

The production of plants with desired traits is very important from industrial point of view to increase the production of biomass in minimum time. Therefore, production of a poplar species with desired character is highly advantageous for industry and economic growth of a country. That is why there has been a surge in research interest in higher plants having very high value for paper, pulp and biofuel production. The poplar is an important model for woody plant study because its potential for *In vitro* culturing and genetic engineering through *Agrobacterium* mediated transformation (Jehan *et al.,* 1994; Gozukirmizi *et al.,* 1998; Confalonieri *et al.,* 2000; Han *et al.,* 2000; Dai *et al.,* 2003). The *Agrobacterium* mediated efficient transformation system of genus populus has been reported in many studies (Han *et al.,* 2000; Confalonieri *et al.,* 1994), but still there is a need for development of transformation systems with better transformation efficiency. This is due to the fact that transformation parameters and tissue culture varies from species to species.

The introduction of new desirable traits in the poplar species and their hybrids by classical breeding has been delayed because of its large size and long sexual generation cycles. The genetic transformation of cottonwood species has been on the lower side as compared with other genotypes (DeBlock, 1990; Confalonieri *et al.,* 1994, 1995; Huang *et al.,* 1994; Wang *et al*., 1996; Heuchelin *et al.,* 1997).

Despite of progress in the transformation of populus, there is a dire need for better approaches. (Leple *et al*., 1992; Tsai *et al*., 1994; Dinus *et al.,* 1995; Tzfira *et al*., 1996, 1997; Fladung *et al.,* 1997; Han *et al.,* 1997, 2000; Ningxia *et al*., 2012). Although bombardment by biolistic gun and protoplast-based DNA transfer have been employed, *Agrobacterium* mediated transformation system is still preferred and efficient system for genetic transformation (Li *et al.,* 2008, 2009). This work is of importance with relevance to *Populus deltoids*, since majority of the work has been done in other species of populus.

The present study was aimed to study different parameters for transformation of *P. deltoides* i.e., explant source, preculturing, infection time, co-cultivation time, and acetosyringone concentration, to optimize a highly efficient protocol for transformation of *P. deltoids* using *Agrobacterium tumefaciens* strain LBA4404. Future perspectives should focus on genetic improvement of populus with lignin content down regulation and conventional approaches like drought tolerance.

Materials and Methods

Explants sources: *Populus deltoides* plants were obtained from Nuclear Institute of Agriculture and Biology (NIAB), Faisalabad. Leaf, node and internode were used for transformation with *A. tumefaciens.*

Micropropagation of *P. deltoides*: Micropropagation of *P. deltoides* was carried out by using young 4-5 months old branches with bud from botanical garden grown *populus* plants. Micro-cuttings of *P. deltoides* were propagated *In vitro* on MS basal medium and MS basal + 5g/L activated charcoal as described by *Byung et al.,* 2009.

Medium preparation: All types of growth media for tissue culture were prepared as described in Table 1. 3% sucrose/maltose, 0.4% Gelzan was added as solidifying agent and final pH was adjusted to 5.8 with 1M NaOH and 1M HCl.

Table 1. Media preparation.

	Medium	Composition
1.	Callus induction medium (CIM1)	MS + 2.5mg/l BAP + 2.0mg/l NAA
2.	CIM2	MS + 2.0mg/l BAP +1.5mg/l NAA
3.	CIM3	MS + 1mg/l BAP + 0.5mg/l NAA
4.	CIM4	MS+ 2 mg/l 2, 4, D
5.	CIM5	MS + 5mg/l IBA
6.	Induction medium	MSO + 200µM Acetosyringone
7.	Co-cultivation Medium	1. CIM2 + 200-400µM Acetosyringone 2. CIM2 without Acetosyringone
8.	Post co-cultivation medium	CIM + 200-400mg/l timentin or 250-500mg/l Cefotaxime
9.	Preculture Medium	CIM + 200µM Acetosyringone
10.	Regeneration Medium 1(RM1)	MS + 1mg/l Zeatin
11.	RM2	MS + 5mg/l BAP + 4mg/l NAA
12.	LB medium	10g/l Tryptone + 5g/l Yeast extract + 10g/l NaCl + 0.8% Gelzan (pH 7.5)

Explants sterilization: Leaf, node, internode and micro-cuttings were surface sterilized by cutting into small pieces of 1-2cm then washed with running tap water for 10-15 minutes. Explants were soaked in 70% ethanol for 1 min, followed by treatment with 50% Clorox for 20 minutes. Afterward explants were washed 2-3 times with autoclaved distilled water, and washed with 70% ethanol for 1-2 minutes. The explants were finally rinsed 5-6 times in sterile distilled water and each washing was carried out for 2-3 minutes. For drying the explants were put on sterile filter paper for 10 min. Leaf, node and internode were put on preculturing medium and micro-cuttings were used for micropropagation on MS medium. All mediums were sterilized by autoclaving. Antibiotics were filter sterilized and added after autoclaving the medium.

Preculturing of explants: Preculturing of explants from *In vitro* and field grown plants was done by inoculating explants on preculture medium (Table 1) for 3-7 days with acetosyringone and preculture medium without acetosyringone.

Bacterial strain used for transformation: *Agrobacterium tumefaciens* strain LBA4404 containing binary plasmid PGA482 with *Gus* (*uidA*) reporter gene driven by CamV35S promoter and *nptII* gene driven by *Nos* promoter *was* obtained from National Institute of Biotechnology and Genetic Engineering (NIBGE), Faisalabad.

Transformation protocol: *A. tumefaciens* LBA 4404 carrying a binary vector was grown overnight at 28°C in liquid Luria Bertani (LB) medium supplemented with 50mg/l of each rifampicin and kanamycin. The *Agrobacterium* was grown until O.D A_{600nm} of 1.0-1.5 was obtained. Cells were collected by centrifugation at 4000RPM for 10 min and resuspended in MS basal medium and O.D A_{600nm} of 0.2-0.8 were made using spectrophotometer in glass cuvettes. 200µM of acetosyringone was added to the cell suspension prior to infection and incubated at 28°C for 2 hours in a shaking incubator using shaking speed of 150RPM. Explants were infected by soaking explants in the bacterial suspension for 1-4 hours at room temperature in dark. The inoculated explants were co-cultivated on CIM with or without acetosyringone at 25°C in the dark for 1-2 days. Explants were put on CIM supplemented with 200mg/l timentin after co-cultivation.

***GUS* Histo-chemical assay:** Infected explants were examined for transient *GUS* expression by histochemical staining method of Jefferson *et al.,* (1987). For infection localization, *GUS* positive explants were washed several times with 70% ethanol to remove chlorophyll for improved visualization of infection sites followed by examination under microscope.

Selection of transformants: Transformants were selected on medium containing 100mg/l kanamycin. The selection was delayed for 10-15 days until the callus induction in the infected explants.

Molecular analysis of transgenic plants: Total genomic DNA was isolated from non-transformed control calli and kanamycin-resistant transgenic calli (100mg) by following Doyle *et al.,* (1990) method. PCR was performed to amplify a fragment of *uidA* gene by using gene specific primers described by Prakash & Gurumurthi (2009). A PCR reaction was setup in total volume of 25 µl containing 200ng of DNA, 2.5 µl of PCR buffer, 1 µl of 2.5 mM dNTP, 2.5 µl of 25mM $MgCl_2$, 10 pM of each primer and 1.5U *Taq polymerase*. Plasmid DNA with *Gus* reporter gene served as a positive control. DNA from a non-transformed control calli served as negative control. The PCR program was consisted of initial denaturing at 94°C for 5 min, followed by 40 cycles at 94°C for 1min, 66°C for 30 sec, 72°C for 30 min, and a final extension at 72°C for 10 min. Amplified PCR product was visualized and photographed using gel documentation system UVP® after electrophoresis on a 1% (w/v) agarose gel containing 0.5µg/ml ethidium bromide as fluorescent dye.

Results and Discussion

Micropropagation*:* For micropropagation growth was observed on both types of MS media with or with activated charcoal (AC) after 2-3 weeks of inoculation. In the present study, we did not find any significant difference in growth of plants on MS + AC and plants on MS basal, these results are contrary to Byung *et al.,* (2009) who found activated charcoal to support growth in *Nisqually-1* species of populus (Fig. 1).

For rooting of plantlets, 0.1, 0.5, 1.0 and 2.0 (mg/l) of IBA (*Phyto*Technology Laboratories®) was used in full as well as half MS medium but no rooting was observed in plantlets after 4 weeks of inoculation, this result was also contrary to Cavusoglu *et al.,* 2011 who found MS supplemented with 2.0 mg/l IBA good for rooting of regenerative shoots.

Under influence of 5mg/l IBA plantlets started rooting after 3 weeks of inoculation with thick callus like growth at the bottom of the plantlet. After 3-4 months on MS medium, all the plantlets showed chlorosis and leaf-fall, this might be due the fact that plants were unable to utilize the media, so could not fulfill its energy requirement in the lab conditions. The chlorosis was also same in plants with roots as well, which reveals inefficiency of MS medium for micropropagation of *P. deltoides*. All plants died after 3-4 months of inoculation on MS and MS+AC medium.

Fig. 1. A) Micropropagated plantlet on MS+AC. B) Micropropagated plantlet on MSO.

Relation of explant source with transformation: *In vitro* grown and fieldgrown explants were used for the transformation under different condition of preculture, acetosyringone concentration, infection time and co-cultivation time. No significant difference in transformation efficiency was found between both types of explants sources. *In vitro*-derived materials are generally preferred over field-grown, greenhouse, or growth room grown materials as explants for transformation because they need no surface sterilization, are often more juvenile and thus have superior regeneration capacity, and are less lignified (Divya & Sanjay, 2011). For the transformation, *In vitro* grown plants are more fragile due to their less lignin contents as compared to fieldgrown plants. This makes it more vulnerable to desiccation during transformation. Fieldgrown plants have better viability than *In vitro* plant explants after the infection with *A. tumefaciens*. In the present study we found that the fieldgrown plant explants had better potential to give callus after transformation. An important finding was that the node and internode explants gave efficient calluses while the best transformation was observed in leaf explants.

There was no transformation observed in explants from older stems of *P. deltoides* of fieldgrown plants which may be due to their high phenolics contents. Young leaves gave highest efficiency of transformation rate (Hancock *et al*., 2007). Calli were also infected with *Agrobacterium* at O.D ranging from 0.3-0.8 but no transformation was observed based on *GUS* assay results.

Relation of transformation and pre-culturing: Explants from both *In vitro* and fieldgrown sources were precultured on preculture medium (Table 1). Precultured explants gave significant high transformation efficiency than non precultured explants. Host cell division is required for successful *Agrobacterium* transformation (Diwakar *et al*., 2011). There was no effect of pre-culture

on transient *GUS* expression; it is likely that poplar benefits from pre-culture by increasing the competence of cells for DNA incorporation (Han *et al*., 2000). It was observed that a 7-day pre-culture of fieldgrown plant explants, on preculture medium with 200µM acetosyringone showed significantly increased number of transformants. Generally dicot plants do not need supplementation of phenolics like acetosyringone. However in this case, use of acetosyringone was found to aid the transformation by increasing the transformation efficiency. This is because it increases virulence of *A. tumefaciens* (Godwin *et al.,* 1991).

The preculturing of explants derived from field source, on preculture medium with acetosyringone showed 20-25% (Table 2) higher percentage of transformation. This is in comparison to explants which were precultured and co-cultivated on medium without acetosyringone and explants which were precultured on acetosyringone medium but co-cultivated on medium without acetosyringone.

Therefore, it was found that use of acetosyringone in preculture medium and further medium after transformation e.g., co-cultivation medium, is highly recommended. It increases transformation efficiency by increasing virulence of *Agrobacterium.*

Transformation parameters: Different parameters were considered in order to develop a protocol with highest transformation rate of *P. deltoides*. Bacterial cell culture density is very critical for the transformation; different optical densities of *A. tumefaciens* ranging from 0.2-0.6 were used for transformation. The highest transformation efficiency was found between optical densities ranging from 0.3-0.5. These results are consistent with Han *et al.,* (2000) who found O.D of 0.3-0.4 effective for populus spp. by using strains EHA105, C58 and LBA4404 of *A. tumefaciens*. Transformation efficiency was calculated by *GUS* histochemical staining of the transformed explants. Node, internode and leaves showed a great response for transformation.

The infection time was the second parameter which was found to be critical in case of transformation. Infection time of 1-2 hours without shaking in dark was found to be best for the transformation. The action of shaking had no affect whatsoever on transformation. When the infection time was increased more than 2 hours, explant viability lessened. This is because as it makes the explants more prone to desiccation on the subsequent culturing.

Co-cultivation is another important parameter for the transformation of *P. deltoides*. Co-cultivation of 1 day was found to be most suitable for the transformation. This is because overgrowth of *Agrobacterium* is a major problem during the co-cultivation time. In the present study, we observed that overgrowth of the *Agrobacterium* cannot be controlled even with increasing the concentration of antibiotic (timentin) from 200-400mg/l. Treatment of other antibiotics like cefotaxime at from 250-500 mg/l was not found to control the overgrowth of *Agrobacterium*. Co-cultivation of 1 day was found to be sufficient to aid the transformation and timentin concentration of 200 mg/l was found to be enough to control growth of *Agrobacterium.*

Acetosyringone concentration is also crucial during the transformation. For the induction medium of *Agrobacterium* 200µM of acetosyringone in the cell suspension was found to be optimum for the induction of *Agrobacterium* mediated gene transfer. During the co-cultivation of some transformation experiments the CIM was supplemented with 200 and 400 µM of acetosyringone in growth medium. A concentration of 200 µM of acetosyringone was found enough to support virulence of *Agrobacterium* in co-cultivation media.

***GUS* assay optimization:** After infection of explants with *A. tumefaciens,* explants were examined for transient *GUS* expression; positive transformants gave blue coloration in the *GUS* solution after 1-2 days incubation at 37^{o}C in dark (Table 3). After GUS assay infection localization was done under microscope. In all the explants the most infection sites were observed near leaf veins and midrib, Infection site were also observed near leaf margins and also on margins of node and internode explants (Fig. 2).

Kanamycin selection of explants: 100 mg of Kanamycin was used in the growth medium for the selection of transformants to provide a strict selection system to the transformed calli. Non-transformant showed chlorosis and bleaching due to effects of kanamycin on explants, non-transformant explants died following 6-8weeks of selection.

Callogenesis and embryogenesis: Different growth media for callogenesis were used as described in Table 1. Callogenesis was observed on CIM1. CIM2 and CIM3 were used for callus induction in mature explants from fieldgrown plants. CIM4 and CIM5 were also used for callus induction for explants from both types of sources. All the combinations worked for callogenesis. Different explants gave different response for callogenesis on different callus induction media (Table 4).

Embryogenesis started in callus inoculated on RM2 after 40 days of inoculation (Fig. 3b, A&B). Embryogenesis was observed in calli inoculated on RM1. After 4 months of inoculation embryoid like structures were observed on the calli. (Fig. 3b, C). Some calli also showed morphogenesis on the same medium after 2 months of inoculation (Fig. 3b, D).

Table 2. Effects of acetosyringone on transformation efficiency.

Experiment	Total No. of explants	Explants with positive *GUS*	Percentage efficiency
Exp-1	15	13	86.6%
Exp-2	9	3	33.33%
Exp-3	10	5	50%

Exp-1: 7 days preculture and co-cultivattion on 200µM acetosyringone
Exp-2: 7 days preculture (w/o acetosyringone) and co-cultivation on 200µM acetosyringone

Table 3. Gus assay results.

Agrobacterium O.D A_{600}	No. of explants	*GUS* positive	Percentage efficiency
0.2	10	0	0
0.3	16	10	62.5%
0.4	16	4	25.0%
0.5	20	15	75.0%
0.6	12	6	50.0%

Exp-3: 7 days preculture and co-cultivation without acetosyringone

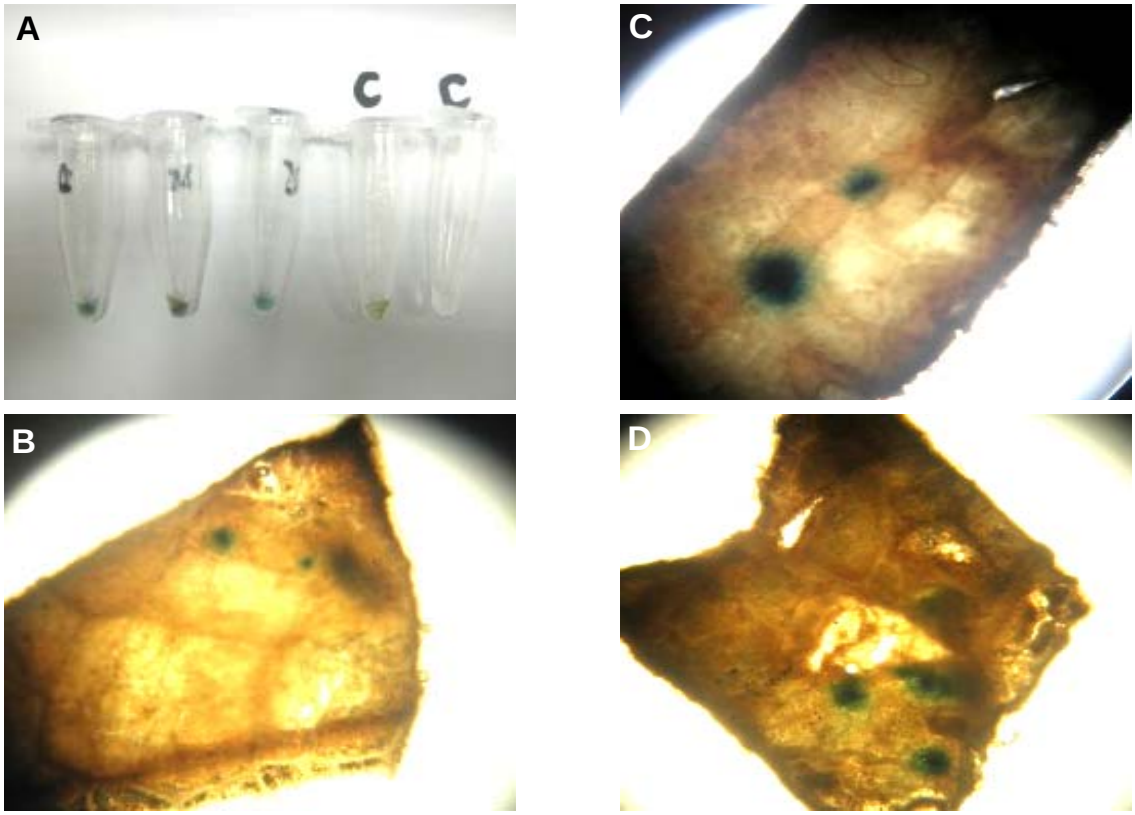

Fig. 2. A) Blue color production after GUS assay with two negative controls at left and three positive explants at right hand side. B,C& D) Blue spots on explants showing infection localization on leaf explants.

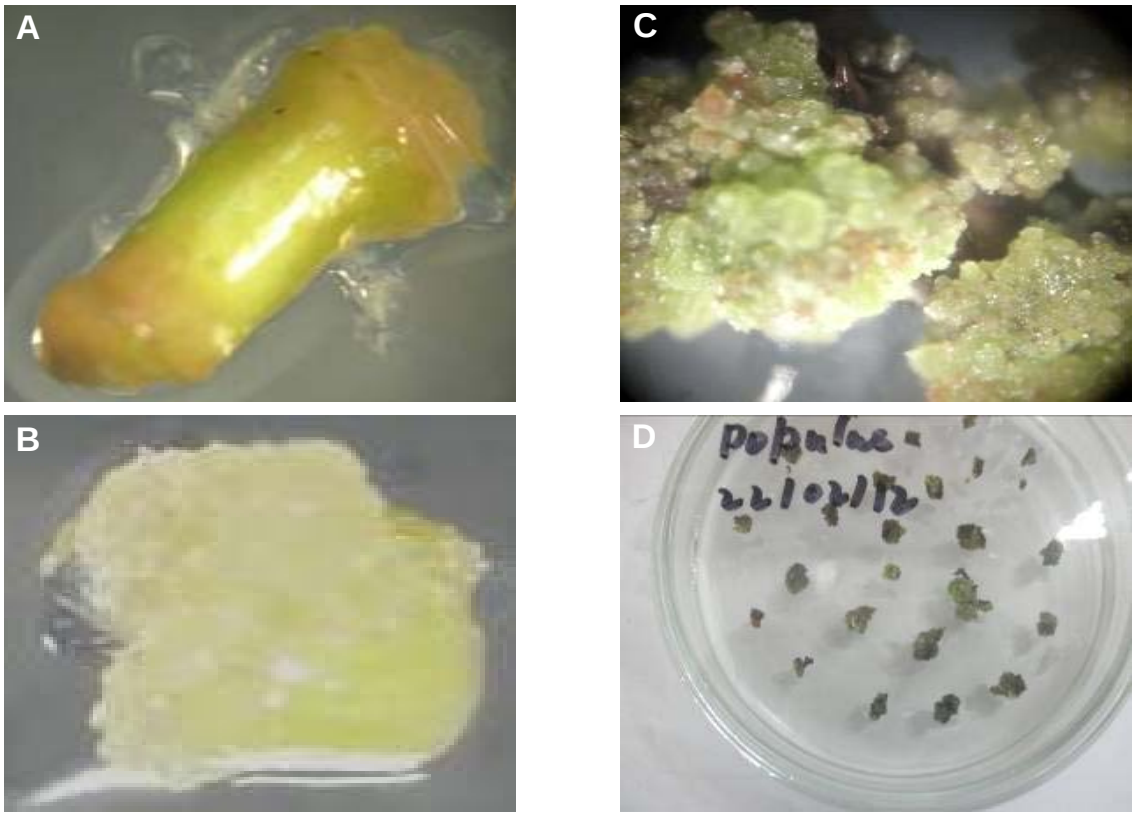

Fig. 3. A) Start of Callogenesis after 21 days. B) Callus formation after 40 days. C & D Callus formation after 4 months in light.

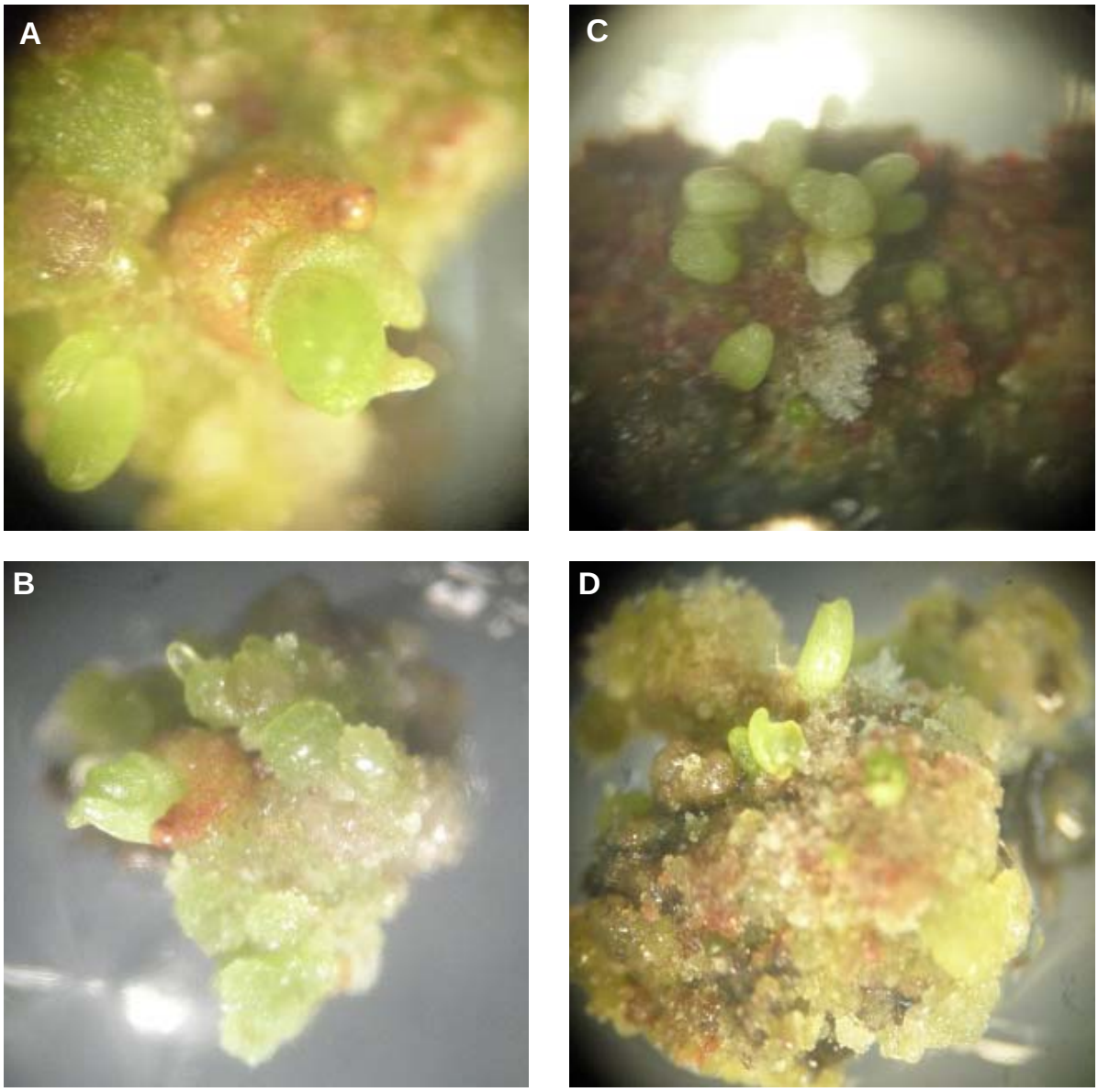

Fig. 3b. A&B) Embryogenesis on regeneration medium 2. C) embroids like structure emerging on regeneration medium1. D) Morphogenesis on regeneration medium 2.

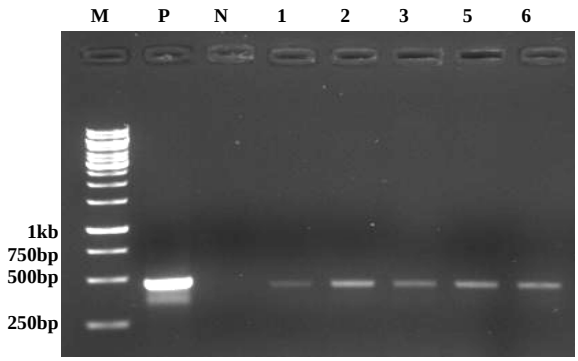

Fig. 4. PCR results of transformants.
M = 1kb DNA ladder, P = Positive control, N = Negative control (non-transformant), Lane1-5: Samples from transformants.

Table 4. Results of callogenesis on different callus induction media

Medium	No. of explants	Callus formed	Average callus formation in weeks	Percentage efficiency
CIM I	19	7	6	36.84%
CIM II	13	1	6	7.69%
CIM III	7	1	8	14.28%
CIM IV	32	17	3	53.12%
CIM V	35	33	3	94.28%

PCR confirmation of transgene: A 461bp PCR product of *Gus* (*uidA*) gene was amplified by using gene specific primers. The amplification was observed in all kanamycin resistant transformant calli DNA. This amplification confirms the stable integration of tDNA into plant cell

genome (Fig. 4). The overall transformation efficiency was 10.95%, calculated on the basis of PCR results.

Conclusion

This study provides an efficient transformation protocol with 10.95% overall transformation efficiency. Best results were observed in field plants, hence the practicality. CIM5 was found to be best for the callus induction in the fieldgrown explants with minimum time required. The development of present system for transformation can be helpful for introduction of desirable traits in *P. deltoides* through genetic transformation using *A. tumefaciens.*

Acknowledgement

We are very thankful to Dr. Aftab Bashir from National Institute of Biotechnology and Genetic Engineering (NIBGE), Faisalabad, for providing the *Agrobacterium tumefaciens* strain LBA4404 with binary plasmid pGA 482 and Higher Education Commission, Pakistan (HEC) for financial support.

References

Byung-guk, K., O. Lori, K. Dean, A.T. Gerald and M.C. Zong. 2009. Micropropagation of *Populus trichocarpa* 'Nisqually-1': the genotype deriving the Populus reference genome. *Plant Cell Tissue Org. Cul.,* 99: 251-257.

Civinova, B. and Z. Sladky. 1990. Stimulation of the regeneration capacity of tree shoot segment explants *In vitro*. *Biol. Plant*, 32: 407413.

Confalonieri, M., A. Balestrazzi and S. Bisoffi. 1994. Genetic transformation of *Populus nigra* by *Agrobacterium tumefaciens*. *Plant Cell Rep.,*13: 256-261.

Confalonieri, M., A. Balestrazzi, S. Biosffi and R. Celia. 1995. Factors affecting *Agrobacterium tumefaciens*-mediated transformation in several black poplar clones. *Plant Cell Tissue Organ Cult*., 43: 215-222.

Cavusoglu, A., Z. Ipekci-Altas, K. Bajrovic, N. Gozukirmizi and A. Zehir. 2011. Direct and indirect plant regeneration from various explants of eastern cottonwood clones (*Populus deltoides* Bartram ex Marsh.) with tissue culture. *African Journal of Biotechnology*. 10(16): 3216-3221.

Dai, W., Z.M. Cheng and W. Sargent. 2003. Plant regeneration and *Agrobacterium*-mediated transformation of two elite aspen hybrid clones from *In vitro* leaf tissues. *In vitro Cell. Dev. Biol. Plant*, 39: 6-11.

DeBlock, M. 1990. Factors influencing the tissue culture and the *Agrobacterium tumefaciens*-mediated transformation of hybrid aspen and poplar clones. *Plant Physiol*., 93: 1110-1116.

Dinus, J.R., J.C. Stephens and S. Chang. 1995. *Agrobacterium tumefaciens* mediated transformation of *Populus deltoides* leaf sections. Southern Forest Tree Improvement Conference.

Divya, N. and Sanjay S. 2011. Micropropagation of Bambusa balcooa Roxb. Through axillary shoot proliferation. In: *Vit. Cell & Developmental Bio-Plant*., 47: 604-610.

Diwakar, A., K. Anil and M.S. Reddy. 2011. *Agrobacterium tumefaciens* mediated genetic transformation of selected elite clones of *Eucalyptus tereticornis. Acta Physio Plantarum*., 33(5): 1603-1611.

Doyle, J. J. and J. L. Doyle. 1990. A rapid total DNA preparation procedure for fresh plant tissue. *Focus*., 12:13-15.

Fladung, M., S. Kumar and M.R. Ahuja. 1997. Genetic transformation of Populus genotypes with different chimaeric gene constructs: transformation efficiency and molecular analysis. *Transgene Res.,* 6: 111-12.

Godwin, L., G. Todd, B. Ford-Lloyed and H.J. Newbury. 1991. The effects of acetosyringone and pH on *Agrobacterium* mediated transformation vary according to plant species. *Plant cell Rep*., 12: 671-675.

Gozukirmizi, N., K. Bajrovic, Z. Ipekci, M. Boydak, T. Akalp, K. Tunctaner, H. Balkan, H. Tanrıyar, M. Calikoglu, T. Ogras, O. Ozden, M. Tulukcu and T. Tank. 1998. Genotype differences in direct plant regeneration from stem explants of *Populus tremula* in *Turkey. J. For. Res.,* 3: 123-126.

Han, K.H., M.P. Gordon and S.H. Strauss. 1996. Cellular and molecular biology of *Agrobacterium*-mediated transformation of plants and its application to genetic transformation of Populus. In: *Biology of Populus and its implication for management and conservation*. (Eds.): Bradshaw Jr., H.D., R.F. Stettler, P.E. Heilman and T.M. Hinckley. National Research Council, Ottawa, Ont., Canada, pp. 201-222.

Han, K.H., M.P. Gordon and S.H. Strauss. 1997. High frequency trans-formation of cottonwoods (genus Populus) by *Agrobacterium rhizogenes. Can. J. For. Res.*, 27: 464-470.

Han, K.H., R. Meilan, C. Ma and S.H. Strauss. 2000. An *Agrobacterium tumefaciens* transformation protocols effective on a variety of cottonwood hybrids (genus *Populus*). *Plant Cell Rep.,* 19: 315-320.

Hancock, J.E, W. M. Loya1, C. P. Giardina, L. Li, L. Vincent and K.S. Pregitzer. 2007. Plant growth, biomass partitioning and soil carbon formation in response to altered lignin biosynthesis in *Populus tremuloides*. *New Phytologist*, 173(4): 732-742.

Heuchelin, S.A., H.S. McNabb Jr and N.B. Klopfenstein. 1997. *Agrobacterium*-mediated transformation of *Populus euramericana* using the chimeric CaMV 35S-pint gene fusion. *Can. J. for Res.,* 27: 1091-1048.

Huang, F.H. 1994. Putatively *Agrobacterium tumefaciens* mediated transformation of glyphosate tolerant hybrid poplar strain NC 5331. *Q. J. Chin. For*., 27: 43-50.

Jefferson, R.A., T.A. Kavanagh and M.W. Bevan. 1987. *GUS* fusion: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J*., 6: 3901-390.

Jehan, H., S. Brown, D. Marie, M. Noin, M. Proutea and D. Chriqui. 1994. Ontogenesis and ploidy level of plantlets regenerated from *Populus trichocarpa* x *Populus deltoides* cv. Hunnegam root, leaf and stem explant. *J. Plant Phsiol.,* 144: 576-585.

Leple, J.C., A.C.M. Brasileiro, M.F. Michel, F. Delmotte and L. Jouanin. 1992. Transgenic poplars: expression of chimeric genes using four different constructs. *Plant Cell Rep.,* 11: 137-141.

Li, J.Y., A.M. Brunner, R. Meilan and S.H. Strauss. 2008. Matrix attachment region elements have small and variable effects on transgene expression and stability in field-grown Populus. *Plant Biotechnol. J.,* 6(9): 887-896.

Li, J.Y., A.M. Brunner, R. Meilan and S.H. Strauss. 2009. Stability of transgenes in trees: Expression of two reporter genes in poplar over three field seasons. *Tree Physiol.,* 29: 299-312.

Ningxia, D., L. Xin, L. Yun, C. Shouyi, Z. Jinsong, H. Da, D. Wenguang, S. Chunkui, Z. Yingzhi and M.P. Paula. 2012. Genetic transformation of *Populus tomentosa* to improve salt tolerance. *Plant Cell Tiss Organ Cult.*, 108: 181-189.

Prakash, M.G. and K. Gurumurthi. 2009. Genetic transformation and regeneration of transgenic plants from precultured cotyledon and hypocotyl explants of *Eucalyptus tereticornis* Sm. using *Agrobacterium tumefaciens*. *In vitro Cell Dev. Biol. Plant.*, 45: 429-434.

Stealer, R.F., H.D. Bradshaw Jr., P.E. Heilman and T.M. Hinckley. (Eds.) 1996. Biology of Populus and its implication for management and conservation. National Research Council Canada, Ottawa, Ont.

Tsai, C.J., G.K. Podila and V.L. Chiang. 1994 *Agrobacterium*-mediated transformation of quaking aspen (*Populus tremuloides*) and regeneration of transgenic plants. Plant *Cell Rep.,* 14: 94-97.

Tzfira, T., H. Ben-Meir, A. Vainstein and A. Altman. 1996. Highly efficient transformation and regeneration of aspen plants through shoot-bud formation in root culture. *Plant Cell Rep.,* 15: 566-571.

Tzfira, T., C.S. Jensen, W. Wang, A. Zuke, B. Vincour, A. Altman and A. Vainstein. 1997. Transgenic *Populus tremula*: A step by step protocol for its *Agrobacterium*-mediated transformation. *Plant Mol. Biol. Rep.,* 15: 219-235.

Wang, G., S. Castiglione, Y. Chen, L. Li, Y. Han, Y. Tian, D.W. Gabriel, Y. Han, K. Mang and F. Sala. 1996. Poplar (*Populus nigra* L.) plants transformed with *a Bacillus thuringiensis* toxin gene: insecticidal activity and genomic analysis. *Transgene Res.,* 5: 289-301.

(Received for publication 12 September 2012)