Development of genetic transformation protocol for selected elite clone of *Populus deltoides*

A Dissertation

Submitted in partial fulfillment of the requirement
for the award of the degree of

Master of Technology
In
Biotechnology
2014

Under The Guidance of

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CERTIFICATE

This is to certify that the project report entitled, **"Development of genetic transformation protocol for selected elite clone of *Populus deltoides*"** submitted by **Ms Preeti (601204019)** towards the partial fulfilment of the award of the degree of **Master of Technology (Biotechnology)** of the **THAPAR UNIVERSITY, PATIALA**, is a work carried out by her under my supervision. The matter embodied in this thesis has not been submitted in part or full to any other University or Institute for the award of any degree.

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DECLARATION

I hereby declare that the work which is being presented in this thesis "**Development of genetic transformation protocol for selected elite clone of *Populus deltoides***" submitted by me for the award of the degree of Master of Technology in the **Department of Biotechnology**, Thapar University, Patiala, is true and authentic record of my own independent and original research work carried out under the supervision of Dr. Anil Kumar, Associate Professor, Department of Biotechnology, Thapar University, Patiala during June 2013 to June 2014. The matter embodied in this thesis has not been submitted in part or full to any other university or institute for the award of any degree in India or Abroad.

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ACKNOWLEDGEMENT

Words are often less to revel one's deep regards. With an understading that work like this can never be outcome of a single person, I take this opportunity to express my profound sense of gratitude and respect towards all those who have been helpful to me in getting this task of project report to successful end.

First of all I would like to thank the supreme power the Almighty **GOD** who is obviously the one who always guided me to work on the right path of life. Without his grace this project would become difficult to complete. Next to him are my parents to whom I am greatly indebted for having me brought up with love and for their encouragement at every step of my life.

I feel highly obliged in taking the opportunity to sincerely thank **Dr. Anil Kumar** without whose able guidance, regular counseling and wholehearted support this project would not have been ended successfully.

I am thankful to all the faculty members and employees of Department of Biotechnology and TIFAC-CORE at Thapar University, Patiala for providing necessary infrastructural support, assistance and co-operation.

I owe my indebtedness to all the members of Plant Tissue Culture lab for their constant encouragement, suggestions and help. I would like to express my sincere thanks to **Dr. Diwakar Aggarwal and Mr. Khemraj** for their continuous help during the course of this study. I also thank those who could not find a separate name but helped me directly or indirectly.

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ABSTRACT

Various factors influencing shoot regeneration and genetic transformation of a selected elite clone of *Populus deltoides* were standardized. An efficient protocol of *Agrobacterium tumefaciens* mediated T-DNA delivery and subsequent shoot regeneration has been developed. Different factors influenced T-DNA delivery as evident from transient GUS assay. The transient GUS expression was significantly higher in explants that were pre-cultured before bacterial infection on medium supplemented with 100 μM acetosyringone. Method of injury to explant, co-cultivation period of 2 days, bacterial density of 0.6 OD_{600} and 16h photoperiod during co-cultivation showed higher transient GUS expression. Following co-cultivation, shoot organogenesis was achieved from leaf segments on modified MS medium containing 58 mM sucrose. Amongst two strains of *A. Tumefaciens* namely, EHA105 and LBA4404 (harbouring pBI121 plasmid), strain LBA4404 was found to be more efficient. Amongst two clones of *P.deltoides* tested, maximum transient GUS activity was recorded in clone 'L34'. Different concentration of antibiotic was investigated to check the sensitivity of leaf explants. Supplementation of antibiotics (cefotaxime) at 500 mg/l into the medium significantly promoted shoot organogenesis from leaf explants. Stable transformation of regenerated shoots was confirmed on the basis of GUS activity.

ABBREVIATIONS

%	-Percent
°C	- Degree celsius
µg	- Microgram
µl	- Microlitre
µM	- Micromolar
BA	- 6-Benzyladenine
cm	- centimeter
g	- Gram
GUS	- β – glucuronidase
h	- hour
IBA	- Indole-3-butyric acid
IAA	- Indole-3-acetic acid
l	-litre
M	- Molar
Mg	- Milligram
Min	- minute
ml	- Mililitre
mm	- Millimeter
mM	-Millimolar
MS	- Murashige and Skooge (1962) medium
NAA	- α- Naphtalene acetic acid
nos	-Nopaline synthase
Npt II	- neomycin phospho transferase
O.D.	- Optical density
PGR (s)	- Plant growth regulator (s)
UV	- Ultra Violet
X-gluc	-5-bromo-4-chloro-3-indolyl-D-glucuronic acid
w/v	- Weight by volume

TABLE OF CONTENTS

S.No	Chapters	Page No.
1.	Introduction	1
2.	Review of literature	6
3.	Materials and methods	14
4.	Results	24
5.	Discussion	35
6.	References	42

Introduction

Among woody genera, *Populus* has been extensively studied as a model system for biotechnological research. This genus belongs to family Salicaceae and is widely distributed across the Northern Temperate Zone (FAO 2000), composed of five sections: Leuce, Aigerios, Tacamahaca, Turanga, and Leucoides (Dickman and Stuart 1983). All the members of the genus *Populus* are diploid with haploid chromosome number being 19 (2n=38). The genome of *Populus* is interestingly small with the genome size being only ~1.1pg. Leuce is a large group that includes aspen and white poplar, which have great economic importance. Aigeiros, considered the "true *Populus*," include the cottonwoods and black *Populu*s. Tacamahaca, containing the balsam *Populu*s, is the largest *Populus* species group (Dhillon 1987).

It is mainly grown as forestry or agroforestry tree in Haryana, Punjab and Uttar Pradesh because of its superior growth and multipurpose wood properties. It is also known as Aspen, Cottonwood, Carolina *Populus* and Necklace *Populus* in some part of world. *Populus deltoides* is a large tree growing up to 20 to 40 m tall and with a trunk upto 1.8 to 2 m diameter. *Populus* typically lives 70 to 100 years, but they have the potential to live 200 to 400 years if they have a good growing environment. (Eckenwalder 1996)

The genus *Populus* is of economic interest due to their small genome size, fast growth rate and short rotation cycle. *Populus* comprises of over 30 species and have several potential biological advantages such as small genome size, rapid juvenile growth, ease of clonal propagation, simplicity of genetic transformation and regeneration.

Due to its short rotation and fast growth, *Populus* spp., have emerged as important for agroforestry programmes or forestry programmes. World over *Populus* wood is used

Figure 1.1: Elite plants of *Populus deltoides* growing at Thapar Technology Campus, Patiala (Punjab, India)

for the pulp and paper industry (Yang 2008), plywood, match industry and logs of *Populus* provide a growing medium for mushrooms (Beck and Dunlop 2001). *Populus* is also used as energy crop for biofuel (Confalonieri *et al.* 2003). Although, many elite clones of *Populus* has been breed/selected, there is still a scope for traits specific modifications. Therefore, it is important to undertake improvement programme for this selected clones to improve their utility. The only way to achieve this objective is to genetically modify such clones with specific genes to introduce this desired traits.

Opportunities for tree improvement

Compared to traditional methods of tree improvement, plant transformation presents unique opportunities. Perhaps the most significant opportunity is the ability to overcome reproductive barriers for gene transfer. Genes from virtually any organism can be introduced and expressed in any plant (George 1996). Genes from viruses, bacteria, animals, and other plant species have all been expressed in plants (Weising *et al.* 1988). In trees, gene insertion might be useful for overcoming reproductive barriers between closely-related tree species, such as transferring genes for resistance to white pine blister rust (*Cronartium ribicola*) from sugar pine to eastern white pine (Sederoff and Ledig 1985); or to bridge large evolutionary distances, such as inserting the insect toxin gene from *Bacillus thuringiensis* into trees (Strauss *et al.* 1991). Because gene insertion avoids the genetic recombination and segregation that occurs during the sexual cycle, desirable gene combinations can be maintained while new genes are added.

One of the major constraints to traditional tree breeding is the long generation time of trees (Cheliak and Rogers 1990). Because progress from recurrent selection can be slowed by long breeding cycles, much effort has been devoted to enhance early flowering in trees (Ross and Pharis 1985). Using gene insertion, however, genes can be transferred to (or from) trees that are sexually immature. Techniques such as antisense technology (Vander Krol *et al.* 1988) or ribozymes (Bruening *et al.* 1987) could be used to suppress the expression of endogenous genes. This can be helpful in modifying existing metabolic pathway(s).

Although gene insertion presents new options for tree improvement, it also has significant limitations. The primary focus of tree improvement has been on polygenic traits (Zobel and Talbert 1987) few economically important single-gene traits are known in trees (Timmis and Trotter 1989). Using gene insertion, it will be difficult to modify the genes responsible for many of these traits, although it may be possible to engineer improved wood quality and/or carbon partitioning by focusing on key genes (Timmis and Trotter 1989). Individually, most of these "polygenes" probably have small phenotypic effects, and it may be decades before important combinations of genes can be identified, cloned, and inserted into plants. For these reasons, genes inserted into trees will likely come from other species and kingdoms. For many species, especially conifers, constraints are also imposed by the availability of reliable and efficient regeneration systems (Dunstan 1988).

Gene transfer may also be limited by factors such as susceptibility to infection by *Agrobacterium* or other biological transformation vectors. In contrast to most crop improvement programs, tree improvement often relies on the genetic manipulation of plant populations, rather than individual genotypes (Cheliak and Rogers 1990). More efficient and less costly gene insertion techniques are needed for gene insertion to be applied to the improvement of large plant populations. Finally, even if the technical and genetic hurdles are overcome, escape of engineered genes is a concern (Strauss *et al.* 1991).

Moreover, the establishment of a good regeneration system is fundamental for genetic transformation, which can only be achieved through *in-vitro* propagation. The regeneration process generally easy from juvenile parts of the plant, such as cotyledons, hypocotyls and leaf fragments excised from young plantlets from germinating seeds (George 1996).

Parsons *et al.* (2002) first reported the genetic transformation of *Populus*. This technology has been applied to various *Populus* species to improve their transformation efficiency. Several factors were systematically analyzed to improve transformation efficiency, including *Populus* genotype, *Agrobacterium tumefaciens* strain for transformation, bacterial concentration, acetosyringone (AS) and different explants including leaf discs and stems.

The pre-requisites for plant genetic transformation are: (a) gene constructs carrying the polynucleotide sequences coding for desired proteins, (b) efficient methods to transform the explants, (c) procedures for selection of plant tissue harboring transgene and d) an efficient plant regeneration protocol form desired explants. In conventional breeding approach, the traits of interest have to reside within the same species. On the other hand, genetic transformation technology enables the scientists to transfer genes for selected traits across genera and kingdoms (Brunner and Li 2007).

The present study is taken up to develop an efficient protocol for the genetic transformation of selected elite clone of *Populus deltoides* using *Agrobacterium tumifaciens*.

Chapter: 2

Review of Literature

Populus species and their hybrids are among the fastest growing temperate trees, and are especially suited to short-rotation intensive culture systems (Isebrands *et al*. 1983). Species of the genus *Populus* are important for a variety of uses, including pulp, veneer for plywood, woody biomass for energy production, and land reclamation (Stettler *et al*. 1988). As *Populus* display marked hybrid vigor and are easy to propagate vegetatively, tree improvement is geared almost exclusively toward the development of improved clones (Gasser and Fraley 1989). Despite their outstanding characteristics, there are number of traits that need improvement; they are highly susceptible to insects and disease, and do poorly under competition from weeds (USDA Forest Service 2000). These traits are amenable to improvement using traditional breeding methods as well as genetic engineering. *Populus* are model species for molecular studies of trees and are ideally suited to tree improvement using genetic transformation. The genome size of *Populus* is considerably smaller than tobacco (Dhillon 1987) and only 3-5 fold larger than that of *Arabidopsis*, which has the smallest plant genome (Dhillon 1987). Due to its importance as forestry and agro-forestry species (Yevtushenko and Misra 2010), a lot of work is being carried out on various aspect with aim of improving productivity, accelerated growth, wood strength, etc. (Ravindranath and hall, 1996). In view of its importance as a source of raw material for pulp and paper industry many superior clones has been selected with higher productivity and more useful characters. Protocol for *in vitro* propagation of *Populus* clones through shoot proliferation and shoot organogenesis has been well worked out (Thakur *et al*. 2005). Clonal propagation of such material is desirable and methods are devised for vegetative propagation using conventional procedures and micropropagation have been developed through shoot multiplication from axillary shoots and shoot regeneration (Padmanabhan and Sahi 2009), however, a lot of variations has been observed from clone to clone (Cseke *et al.* 2007). *Populus* are easily cultured *in vitro*, and were the first trees to be genetically transformed, using both *Agrobacterium* (Fillatti *et al.* 1987), and Biolistics (McCown *et al.* 1991). However, there is a need to develop the clone specific regeneration and transformation protocol for various plants (Aggarwal *et al.* 2010, 2011).

Many fast growing clones have been selected, which help breeder to overcome the limitations of the conventional tree breeding (Conafalonieri *et al.* 2003). This has resulted in increased interest of scientists for the development of efficient *in vitro* propagation (Chung *et al.* 1989) and genetic transformation (Confalonieri *et al.* 2003; Cseke *et al.* 2007) protocols. Many reports appeared on *in vitro* propagation (especially through organogenesis and somatic embryogenesis) in *Populus* spp. (Han *et al.* 2000). Preece *et al.* (1995) has attained the shoot organogenesis using intermodal segments and petiole explants of *Populus nigra*. Somatic embryogenesis has also been achieved using different explants in some of the *Populus* spp. (Park and Son 1988).

Since 1980, *Populus* has been intensively studied for *in vitro* propagation (Chun *et al.* 1988). Such studies reveal that *Populus* tissue exhibits a high degree of developmental plasticity, similar to tobacco a herbaceous species. Many *in vitro* cloning techniques were developed that provide an integral basis for biotechnological applications.

Adventitious shoot formation is amorphous shoots originating from the various explants of leaf, internode, immature embryo, pollen, vascular or bark cambium, root, etc. Cultures for axillary shoot induction are composed of morphous shoots originating from an apical meristem or axillary bud. Apical meristems containing shoot primordia can produce multiple shoots. Adventitious *Populus* shoots can be induced from cultured leaf, internode, petiole, and root explants that normally never reproduce vegetatively (Ernst 1993). Mostly *Populus* and their hybrids produce adventitious shoots on Murashige and Skoog (MS) (Murashige and

Skoog 1962) media or Woody Plant Media (WPM) (Lloyd and McCown 1981) supplemented with different concenteration of 6-benzyladenine (BA) or zeatin as a cytokinin source. Regeneration through shoot organogenesis may generate at much higher rate of shoot production than proliferation of axillary shoots (Park and Son 1988). Morphogenetic responses of explants of cultured *Populus* depend on explant source and the combination of exogenously applied plant growth regulators. Chun *et al.* (1986) demonstrated that abaxial side culture of whole-leaf explants was best to induce adventitious shoot buds from a hybrid aspen (*P. alba x P. grandidentata*) plantlet. Kang *et al.* (1996) successfully developed nodal culture system to maximize shoot production of a cottonwood hybrid (*P. x euramericana*). They used explants: node, internode, node with the axillary bud excised, and reutilized stem. The shoot proliferation capacity of nodal explants and modified explants was compared, and the origin of multiple shoots was investigated. For these explants, most shoots were derived from the axillary meristems. Shoots also originated from the vascular cambium and occasionally from the lenticels. Generally, morphogenetic responses of *in vitro* cultured explants depend on the species, explant source, and combination of exogenously applied plant growth regulators.

Genetic Transformation

The basic approach of genetic modification in plants involves the modification of its genomic DNA by incorporation of new genes using genetic transformation protocols. Various transformation techniques have been developed to assist the transfer of recombinant DNA into recipient plant cells (Hansen and Durham 2000). These transformation techniques involve the insertion of a piece of DNA harbouring one or more genes from one organism into a chromosome/genome of another organism. In plants, it can be mediated either by a biological agents such as *Agrobacterium tumefaciens* (gram negative soil bacterium that have ability to transfer DNA fragment to host) (Tzfira and Citovsky 2006; Gelvin 2003) or by a direct gene delivery system (such as gene gun, electroporation, microinjection, lasers, polyethylene glycol (PEG), silicon carbide fibers) that utilizes physical, electrical or chemical means to deliver gene of interest to a taget cells (Weir *et al.* 1998, Torney *et al.* 2007).

Although genetic transformation of woody plants is a relatively young field, it offers considerable potential for breeding and selecting improved trees for multiple purposes. Conventional breeding programs have produced improved growth rates, adaptability, and pest resistance; however, tree improvement processes are time consuming because of the long generation and rotation cycles (Leple *et al*. 1992). Genetic transformation of trees helps to compensate for conventional breeding disadvantages by incorporating known genes into specific genetic backgrounds. Since the first successful plant transformation was reported in 1983 (Herrera-Estrella *et al*. 1983), several nonsexual gene transfer methods were developed for important agronomic crops and forest tree species.

Report on successful *Populus* transformation appeared more than two decades ago (Parsons *et al.* 1986), wherein a reproducible protocol was reported using wild strain of *A. tumefaciens*. Fillatti *et al*. (1987) reported the successful recovery of transgenic *Populus* plants expressing bacterial *aroA* gene conferring herbicide resistance. Subsequently, many reports described successful transformation using *A. tumefaciens* (Song and Sink 2004). Although there are many reports on the development of transformation protocol but considerable variations have been observed from clone to clone (Cseke *et al.* 2007).

A prerequisite for any genetic transformation work using *Agrobacterium* is the ability of the bacterium to infect the plant. The effect of *A. tumefaciens* strains, A281 and A348, on infection *of P. triclzocarpa x P. deltoides* (Parsons *et al*. 1986) was studied and additional information was gathered on the effect of *Populus* genotypes (Charest *et al.* 1992). Previous studies showed significant differences among the genotypes with in species and the clones within genotype (Confalonieri *et al.* 1994). A differential response of *Populus* section cultivars to infection by *A. tumefaciens* was described by (Nesme *et al.* 1987), and susceptibility of aspen cultivars to *A. tumefaciens* was correlated to cytokinin sensitivity (Beneddra *et al.* 1996). It is critical to select appropriate starting materials (or explants) for *Agrobacterium*-mediated transformation. Potentially, explant material can be derived from seedling, leaf, intenode, petiole, root, callus, or other cells, tissues, and organs. *In vitro* cultured leaves and internodes (stems) have been used most often to transform many *Populus* species. Greenwood stem internode sections *of P. tremuloides* are the most susceptible to tumour formation and leaf disks are the least susceptible (Kubisiak *et al.* 1993). Leple *et al.* (1992) showed that internode explants of *P. tremula x P. alba* produced more transformed calli than leaf explants.

A suspension culture transformation system for inserting genes into *Populus* might offer several advantages including: 1) the ability to screen large numbers of potentially transformed cells; 2) effective inhibition of residual *Agrobacterium* following co-cultivation; and 3) high transformation frequencies due to rapidly dividing suspension cultures that may be amenable to stable integration of foreign DNA (Howe *et al.* 1994). However, it is frequently unknown which cell type within an explant are eaisly transformable or capable of regenerating into a plant. The small amount of data available indicates that the most regenerable cells donot necessarily correspond with the most transformable cells (De Block 1993).

To assure high infectivity levels for effective transformation, the most suitable *Agrobacterium* strain should be determined for each host species, genotype and tissue. Generally, tree species respond better to the nopaline strains than octopine strains of *A. Tumefaciens* (Ahuja 1987).

Figure 2.1: Crown gall produced by *Agrobacterium tumefaciens* strain A281 infection of hybrid *Populus (Populus alba x P.grandidentata*) stem after approximately 10 weeks (Jamison 2001).

Most transgenic *Populus* have been produced using nopaline strains of *Agrobacterium* (Han *et al.* 1996). The plasmid rather than the chromosomal background was the most critical determinant for infection (Kubisiak *et al.* 1993). However, influence of plasmid type on infection levels has varied with host species/genotype/tissue type (Kubisiak *et al.* 1993). Two designed vector systems are used in *Agrobacterium-* mediated transformation: 1) co-integrate: T-DNA includes gene(s) of interest with a selectable marker gene instead of oncogenes on the Ti-plasmid; 2) binary: T-DNA is located on a separate vector plasmid instead of the Ti-plasmid. T-DNA also includes the gene(s) of interest and selectable marker gene (Walkerpeach and Velten 1994). No recombination event is necessary for the binary vector system, unlike the co-integrate vector system. Overall, *A. tumefaciens* strains C58, A281, EHA101, and LBA4404 were commonly used with binary vectors for transformation of many *Populus* and seem to generate suitable transformation efficiencies (Klopfenstein *et al.* 1997).

Several factors should be considered to improve transformation efficiency such as the *Agrobacterium* inoculum titer, *vir* inducer, selectable marker system, and *in vitro* tissue culture manipulation techniques. Optimal results were obtained by dipping initial host explants into a bacterial suspension (5 to 6 x 10 cells/ ml) for 20 min to 4 h, then co-cultivating them for 24 to 72 h on a liquid or semisolid regeneration medium that contained plant growth regulators (Wang *et al.*1994). Acetosyringone (AS) and hydroxy-acetosyringone (OH-AS) elicited the expression of *Agrobacterium vir* region genes (Stachel *et al.* 1985). AS and OH-AS occur specifically in exudates of wounded and metabolically active plant cells and perhaps allow *Agrobacterium* to recognize susceptible cells (Stachel *et al.* 1985). Transformation efficiency could be increased during co-cultivation by using a *vir* region inducer such as AS (10 to 200 ~M) (Weigel and Nilsson 1995). A practical selectable marker system is essential to obtain high efficiency transformations while avoiding non transformed plants that escape selection (Leple *et al.* 1992). Selectable marker genes used for *Populus* transformation have encoded traits such as hygromycin resistance (hygromycin phosphotransferase; neomycin resistance (neomycin phosphotransferase II; *nptII*), phosphinotricin (glufosinate) resistance (phosphinotricin acetyltransferase; bar), and chlorsulfuron resistance (mutant acetolactate synthase; crs1-1). Because the *nptII* gene has been frequently employed in several woody plants including *Populus* species to select transformants, kanamycin is one of the most commonly used antibiotics for a transformation selection system (Chun 1994). Even modest kanamycin concentrations (10 mg/1) can inhibit regeneration of untransformed hybrid *Populus (P. alba* x *P. grandidentata*) (Chun *et al.* 1988). Culture on nonselective medium (without selective antibiotics) for 2 days to 2 weeks before transfer to a selective medium (with selective antibiotics) has been used to obtain higher transformation frequencies (Charest *et al.* 1992; Tuominen *et al.* 1995). The transfer of explants to light conditions after decontamination using cefotaxime (250 to 500 mg/1) and/or carbenicillin (250 to 500 mg/1), a preculture (shoot-inducing or callus-inducing medium induding BA, 2, 4-D, NAA, or TDZ) period before *Agrobacterium*-mediated infection, or a prolonged infection period can enhance transformation frequencies dramatically

(Confalonieri *et al.* 1994). Several studies demonstrate that the *Agrobacterium* plasmid, explant type, *in vitro* techniques, and use of a *vir* region inducing compound can substantially influence stable transformation frequency (Confalonieri *et al.* 1994, Kubisiak *et al* .1993). Use of luxF2 as a reporter allows *in vivo* monitoring of gene expression by non destructive imaging (Nilsson *et al*. 1992).

The presence of T-DNA sequences in gall and root tissue confirmed *Populus* as a host *for A. tumefaciens* and *A. rhizogenes* (Pythoud *et al.* 1987). These early pathogenicity studies of *Agrobacterium* provided the basis for its use as a tool to transfer foreign genes into the *Populus* genome.

The process for producing transgenic *Populus* plants includes 5 main components: 1) initiation: starting explants (host species/genotype/tissue type) a reselected for infection and transformation; 2) infection: wounded starting explants are co-cultivated with an *Agrobacterium* strain; 3) selection: after removal of residual *Agrobacterium*, transformed cells are selected for subsequent regeneration into transgenic plants regeneration: transformed cells are regenerated during or after the selection period confirmation: the presence or function of transgenes in the genome of transgenic plants is confirmed using molecular techniques such as polymerase chain reaction, Southern hybridization, northern hybridization, western blotting, enzyme-linked immunosorbent assay (ELISA), or enzyme activity assays (Cseke *et al.* 2007).

A close survey of literature could not reveal any report on development of genetic transformation protocol for the selected elite clone(s) of *P.deltoides.* So, there is a need for the development of genetic transformation protocol for the selected elite clones of *P.deltoides.* The transformation of selected clones will prove to be powerful tool for trait specific modification of existing clones with desirable traits, such as insect and herbicide resistance, and reduction of lignin content (Teulières *et al.* 1994).

Chapter 3

Materials and methods

Plant Material

Elite clone of *Populus deltoides* L34 growing in the plantation area of Thapar University Campus, Patiala was used during this study. These plants were procured from Punjab Agriculture University, Ludhiana and established in the plantation area of Thapar University, Patiala. Aseptic cultures were established on modified MS medium using nodal explants.

Chemicals, glassware and plasticware

All routinely used chemicals (AR Grade) were purchased from HiMedia Laboratories, Mumbai, India. Plant growth regulators (PGRs) like 6-benzyladenine (BA) and indole-3-acetic acid (IAA) and antibiotics were procured from Sigma Chemical Co. (St Louis, MO, USA). Plastic ware such as sterile disposable filter sterilization units were purchased from Merck Millipore (Merck Specialities Pvt. Ltd., Mumbai, India) and petri dishes, microfuge tubes, microtips, measuring cylinders, beakers etc. were procured from Tarsons Products Pvt. Ltd. (Kolkata, India). Glassware such as conical flask, measuring cylinders etc were procured from Borosil Glass Works Ltd. (Mumbai, India). Glass culture bottles of 300 ml capacity were procured from Kasablanka Corporation, (Mumbai, India).

Preparation of culture medium and stock solutions

Modified MS medium (Murashige and Skoog 1962) with 3% sucrose and gelled with 0.8 % (w/v) agar was used for tissue culture experiments. The modified MS medium (Table 3.1) with some alterations was used in this study. Plant growth regulators (PGRs) i.e. BA and IAA were added to the medium in various concentrations and combinations as mentioned in results (Table4.1). The concentrated stock solutions of all the ingredients (macronutrients, micronutrients, vitamins) were prepared individually, which are then used to prepare the medium (Table3.1). Stock solutions of all plant growth regulators (PGRs) in concentration of 2.5 mM were prepared by dissolving them in respective solvents (1N HCl and 1N KOH) and finally volume was made up using Milli Q water. All the stock solutions were kept under refrigeration (~ 4°C). Modified MS medium was prepared from the stocks and used for the tissue culture studies unless otherwise specified. The pH of medium was adjusted to 5.8 with 1N KOH or 1N HCl using pH meter (Cyberscan 510, Eutech Instruments, Singapore) before autoclaving. After preparation of medium it was dispensed (50 ml) into 300 ml glass culture bottles (Kasablanka, Mumbai). After dispensing the medium agar (0.8 %; w/v) was added to the individual culture bottle and medium was sterilized in an autoclave (121 °C; 15 psi; 20 min, Equitron, Mumbai, India). Stock solution of antibiotics such as kanamycin, cefotaxime were prepared in required concentration and filter sterilized using disposable sterile filters of 0.22 µm pore size (Merck Millipore, India) and were stored at -20 °C in deep freezer (Vest frost, India).

Culture/ growth conditions

Unless otherwise mentioned, cultures were incubated at 25±1 °C under cool white fluorescent lights (CFL) (Philips India Ltd, Mumbai) with the light intensity of 42 µmol m^{-2} s^{-1} inside the culture vessel in 16 h light/8 h dark cycle.

Table 3.1 Composition of modified MS medium used in this study.

Sr. No.	**Components**	**Concentration (mg/L)**
1	$(NH_4)_2SO_4$	250.0
2	KNO_3	1900.0
3	NH_4NO_3	1650.0
4	$MgSO_4.7H_20$	450.0
5	$CaCl_2.2H_2O$	440.0
6	KH_2PO_4	170.0
7	H_3BO_4	6.0
8	$MnSO_4.H_2O$	16.9
9	$ZnSO_4.7H_2O$	8.0
10	$Na_2MoO_4.2H_2O$	0.2
11	$CuSO_4.5H_2O$	0.025
12	$CoCl_2.6H_2O$	0.025
13	KI	0.83
14	Nicotinic Acid	0.5
15	Pyridoxine HCl	0.1
16	Thiamine HCl	0.2
17	Adenine Sulphate	15.0
18	Ascorbic Acid	5.0
19	Myo-inositol	100.0
20	FeEDTA. $2H_2O$ (sodium salt)	30.0
21	Sucrose	30000.0

After preparing modified MS medium addition of PGRs i.e. 2.5µM BA and 1.0µM IAA takes place then pH of the final medium was adjusted at 5.8 with 0.1N HCL or 0.1N KOH.

Preparation of explants and establishment of aseptic cultures

In vitro cultures were established using young stems and leaves from *P. deltoides* maintained in a growth room. The actively growing shoot cultures were maintained on modified MS medium supplemented with 2.5µM BA and 1µM IAA and further used for micropropagation, regeneration and genetic transformation experiments. Stem (internodes), petiole segments and leaf blades (disks) from microshoots were used for transformation. The temperature of the growth room was maintained at 25°C, with continuous light from cool-white fluorescent lamps

Effect of plant growth regulators on shoot regeneration

Young, expanded leaves (1.5–2.0 cm) from microshoots of selected clone of *P.deltoides*, namely 'L34' maintained on modified MS supplemented with 2.5 µM of BA and 1.0 µM of IAA were taken as explants to induce shoot organogenesis. Leaves were cut transversely along the midrib and 3-4 mm segments were inoculated on modified MS medium supplemented with various concentrations of BA (0.0-12.5 µM) and IAA (0.0-12.5 µM) with their adaxial surface facing the medium.

Shoot proliferation and elongation

After successful establishment of aseptic cultures, shoot clumps of 15-20 shoots (0.3- 0.5 cm) were cultured on modified MS medium variously supplemented with (0.0-12.5 µM) BA, in combination with(0.0- 12.5 µM) IAA to study their effect on shoot multiplication and shoot elongation.

Effect of antibiotics on shoot regeneration

The effect of various antibiotics namely, cefotaxime, carbenicillin and cephalexin (0-500 mg/l) was also tested on shoot organogenesis from leaf segments. Antibiotics (Filtered sterilized, Stock 200 mg/ml) were added to the medium supplemented with BA (2.5 µM) and

IAA (1.0 µM) after autoclaving. Leaf segments were used as explants for this experiment and inoculated with their adaxial surface facing the medium.

Testing of Antibiotic sensitivity of the bacterial strain & leaf cultures

Antibiotic sensitivity test of the bacterial strain and leaf cultures was conducted using different concentrations of antibiotic cefotaxime (0-500 mg/l) for the removal of bacteria and kanamycin (0-100mg/l) to be used for the selection of the transformed tissue.

To test antibiotic sensitivity of bacterial strain, these were streaked on LB medium containing different concentrations of the antibiotics. To test the antibiotic sensitivity of the tissue cefotaxime (Filtered sterilized, Stock 200 mg/ml) and kanamycin (Stock 50 mg/ml) after autoclaving were added on modified MS medium supplemented with IAA, BA.

Clonal variation in shoot regeneration

Shoot organogenesis potential of two different selected clones of *P. deltoides*, namely ‘L34’ and ‘G48’ was also compared. The leaf segment (explant/s) with their adaxial surface facing the medium were inoculated on modified MS medium supplemented with BA (2.5 µM) and IAA (1.0 µM).

Agrobacterium mediated genetic transformation of elite clones of *P. deltoides*

Agrobacterium strain (s) and plasmid vector

The genetic transformation experiments were conducted using *Agrobacterium tumefaciens* strains EHA105 and LBA4404 harboring the binary vector pBI121 (Clontech, San Diego, CA, USA). The *A. tumefaciens* strain EHA105 (Hood *et al.* 1993) and *A. tumefaciens* strain LBA4404 (Hoekema *et al.* 1983) were used for the study. The map of binary vector pBI121 as shown in Figure 3.1 is carrying *uidA* gene (β-glucuronidase) (GUS) as a reporter gene under the control of CaMV 35S promoter and NOS terminator and *nptII* (neomycin phosphotransferase II) gene used as selection marker gene is under the control of NOS promoter and terminator within the t-DNA region. The plasmid was introduced into *A. tumefaciens* disarmed strains EHA105 and LBA4404 by the freeze-thaw method (Holsters *et al.* 1978).

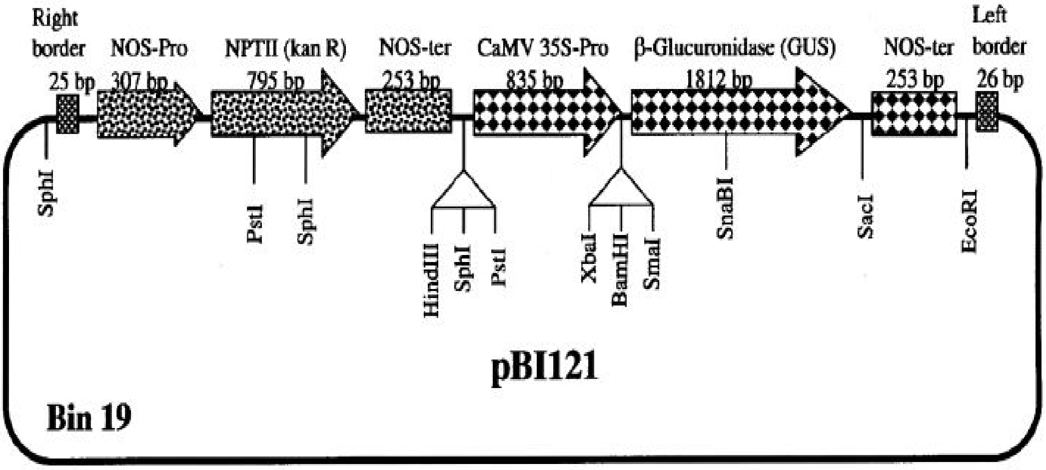

Figure 3.1 Diagrammatic representation of the binary plasmid vector pBI121, T-DNA region indicating the localization of the *nptll* gene, encoding neomycin phosphotransferase (*nptll*) and the *uidA* gene, encoding _-Glucuronidase (GUS), NOS ter (nopaline synthase terminator), NOS pro (nopaline synthase promoter), CaMV35S pro (cauliflower mosaic virus 35S promoter).

Explant Source for genetic transformation

Leaves taken from actively growing microshoot cultures of selected clones of *P. deltoides*, namely ‘L34’ (maintained on modified MS medium) were used as starting material for genetic transformation experiments.

Selection of *A. tumefaciens* strain

For the selection *A. tumefaciens* strain, leaf segments of *P. deltoides* were infected with both strains i.e. EHA105 and LBA4404 harboring binary vector pBI121 and scored for transient GUS expression. Based on transient GUS expression analysis of leaf segments, *A. tumefaciens* LBA4404 was selected for further work on development of *Agrobacterium*-mediated genetic transformation protocol of *P. deltoides.*

Agrobacterium-mediated transformation Protocol

The protocol for *Agrobacterium-mediated* genetic transformation *of P. deltoides* with suspension culture of the disarmed strain of *A. tumefaciens* LBA4404 harboring binary vector pBI121 (having gus gene as reporter gene and *nptII* as a selectable marker gene conferring resistance to kanamycin) was developed. A single bacterial colony of *A. tumefaciens* LBA4404 containing plasmid pBI121 was inoculated in 20 ml Luria broth supplemented with 50 µg/ml kanamycin and 15 µg/ml rifampicin and grown overnight at 28 ℃ on a gyratory shaker (150 rpm). From the overnight grown culture, 0.5 ml was freshly inoculated to 50 ml of Luria broth and grown for 24 h. Bacterial cells were pelleted by centrifugation (4000 x g; 2 min) and suspended in Luria broth supplemented with 100 µM acetosyringone (Sigma Chemical Co., St Louis, MO) to attain the desired OD_{600}. Explants were injured in sterilized petridish by using autoclaved surgical blade and infected with bacterial suspension and then transferred to the modified MS medium for co-cultivation. The explants following co-cultivation were washed 3-4 times with sterile distilled water containing appropriate antibiotic and sub-cultured on the medium containing appropriate antibiotics (both for elimination of bacteria and selection of the transformed tissue) in required dose. The explants were then grown for the several subculture cycles on the antibiotic containing modified MS medium. By using GUS assay actively growing explants on the selection medium was examined for the expression of reporter gene.

Factors affecting *Agrobacterium*-mediated genetic transformation of *P.deltoides*

Effect of pre-culturing

To study the effect of pre culturing of explants prior to infection, leaf explants were cultured on modified MS medium for different interval of times ranging from 0–5 days.

Effect of bacterial concentration and infection time

Leaf segments were infected with suspension of *A. tumefaciens* with different OD_{600} values (0.2, 0.4, 0.6, 0.8 and 1.0) for different time periods (10 ml, 0–30 min) in Petri plates. Following infection, leaves were co cultivated for two days, washed and the transient GUS expression was scored for both the parameters separately.

Effect of co-cultivation period and pH of co-cultivation medium

To find out the effect of co-cultivation period on transient GUS expression, leaf segments were blotted with sterile filter paper to remove the excess of bacterial cells and medium. These were then cultured on modified MS medium supplemented with 100 µM acetosyringone with varying pH (5.2, 5.5, 5.8 and 6) for different time intervals (1–5 days) and scored for transient GUS expression.

Effect of Photoperiod

To find out the effect of photoperiod, cultures were sealed with cling film and incubated under different photoperiod viz continuous light, 16-h light/8-h dark or continues dark. After incubation under different photoperiods leaves were scored for transient GUS expression.

Selection of transformed tissue

Following co-cultivation, leaf explants were washed 4-5 times with sterile distilled water containing 500 mg/l cefotaxime, blotted on sterile filter paper and transferred to modified MS medium, containing 50 mg/l kanamycin and 500 mg/l cefotaxime and incubated at 25±1°C under cool white fluorescent lights with the light intensity of 42 µmol m^{-2} s^{-1} inside the culture vessel in 16- h light/8h dark cycle. The cultures were sub-cultured on same medium at every 20 days interval for shoot regeneration and putatively transformed regenerated shoots were multiplied on modified MS medium supplemented with 2.5 µM BA and 1 µM IAA.

Histochemical GUS assay

GUS assay was carried out using regenerated kanamycin resistant shoots and freshly infected explants after 2 days (transient assay) of incubation for scoring transient as well as stable GUS expression following the method of Jefferson *et al.* (1987). Tissues were incubated overnight at 37 ºC in 100 mM sodium phosphate buffer (pH 7.0), containing 1 mM X-Gluc. Details of the GUS histochemical solution are given in following Table 3.2:

Table 3.2: Composition of GUS histochemical solution

Stock solution Final	**Concentration**	**Reagent Mix (µl/ml)**
1.0 M NaPO4 buffer, pH 7.0	0.1 M	100
0.25 M EDTA, pH 7.0	10 mM	40
0.005 M K-ferricyanide pH 7.0	0.5 mM	100
0.005 M K- ferrocyanide pH 7.0	0.5 mM	100
0.002 M X-glucuronide	1.0 mM	50
10 % triton X-100 (optional)	0.1%	10
Subtotal		400 ml
Distilled water		600 ml
Final volume		1000 ml

After staining, the tissues were fixed by dipping the samples in fixative solution (100 ml- Formaldehyde 10 % v/v - 10 ml, Ethanol - 20 ml, Acetic Acid - 05 ml, Distilled water – 65ml) for 4 h as per the method of Jefferson *et al.* 1987.

[X-Gluc* - For preparation of 1ml of X-Gluc solution, 0.008892gm of X-Gluc was measured and dissolved in N,N-Dimethyl sulfoxide (DMSO) i.e. 5.2mg of X-Gluc in 500µl DMSO and make up the remaining volume with distilled water]

Procedure for GUS histochemical assay:

1. Leaves and/or small plant portions were cut from putative transgenic plants and placed in the 2 ml microfuge tube containing histochemical staining solution.

2. Tube was subjected to vacuum treatment for 1-2 min.

3. After vacuum treatment, tubes were incubated at 37 ºC overnight.

4. Next day, staining solution was removed from the tube and tissue was dipped in fixative solution and incubated at room temp for minimum of 4 h.

5. After that, fixative solution was removed and tissue was again dipped in 50 % solution of ethanol, to remove chlorophyll for better visualization of stained tissue. At this stage 50 % ethanol was changed intermittently.

6. Over the period of time (2 h) the concentration of ethanol solution was slowly raised to 100% by changing the grades of alcohol for complete removal of chlorophyll.

7. After complete removal of chlorophyll, the tissue was observed for the presence of blue color.

Results

Preparation of explants and establishment of aseptic cultures

After surface disinfection, nodal segments were trimmed from cut ends and cultures were established (Figure: 4.1) on modified MS medium supplemented with BA (2.5 µM) and NAA (2.5 µM) following standard tissue culture procedures.

Experiments were carried out to study the effect of different concentrations of BA (0.0-12.5µM) and NAA (0.0-12.5µM) on sprouting of explants. The concentration of cytokinin influenced sprouting from nodal explant, higher concentrations of BA (more than 2.5 µM) did not favour sprouting from nodal explants (Table 4.1). Regeneration of shoot buds from leaf segments was achieved on modified MS medium supplemented with 1.0 µM NAA and 2.5 µM BA. Shoot like structures appeared from the cut ends of the leaf explants, which later grew into complete shoots (Figure: 4.1 E & F). This response of shoot regeneration concentrated to the area of midrib and other veins. Newly formed shoots were excised from the explant and further cultured on modified MS medium supplemented with 2.5 µM BA and 1.0 µM NAA to increase the number of shoots for further work.

Effect of antibiotics on shoot regeneration:

Effect of antibiotics namely cefatoxime, carbenicillin and cephalexin (0-500 mg/l) was evaluated on shoot regeneration potential of leaf explants . Incorporation of cefotaxime in the medium increased the response of explants in terms of both frequency of explants (%) resulting in shoot regeneration and the number of shoots regenerated per explants (Table 4.2). Increase in concentrations of cefatoxime resulted in increased number of explants showing shoot regeneration and an increased number of shoots per explant were also recorded.

Table 4.1: Effect of various concentrations of NAA and BA on regeneration from leaf explant of *P. deltoides* cloneL34

NAA (µM)	BA (µM)	% Nodular callus	% Shoot regeneration
		L34	L34
1.0	1.0	39^{f}	28^{f}
1.0	2.5	69^{a}	53^{a}
1.0	5.0	44^{e}	39^{c}
1.0	12.5	47^{de}	30^{e}
2.5	1.0	48^{d}	35^{d}
2.5	2.5	64^{b}	45^{b}
2.5	5.0	59^{c}	30^{e}
2.5	12.5	2^{h}	0^{i}
5.0	1.0	30^{g}	16^{g}
5.0	2.5	46^{de}	43^{h}
5.0	5.0	40^{e}	25^{f}
5.0	12.5	5^{j}	0^{i}
12.5	1.0	6^{jk}	2^{i}
12.5	2.5	15^{ij}	6^{h}
12.5	5.0	7^{jk}	2^{i}
12.5	12.5	0^{k}	0^{i}

Cultures were sub cultured on the same medium after 4-week interval. Data were scored after 8 weeks of inoculation. Mean values sharing a common letter within the column are not significant at P<0.05 according to Duncan’s multiple range test. All data were analyzed by analysis of variance and the means were compared with Duncan’s multiple range test (Duncan 1955) using GraphPad Prism 4 software.

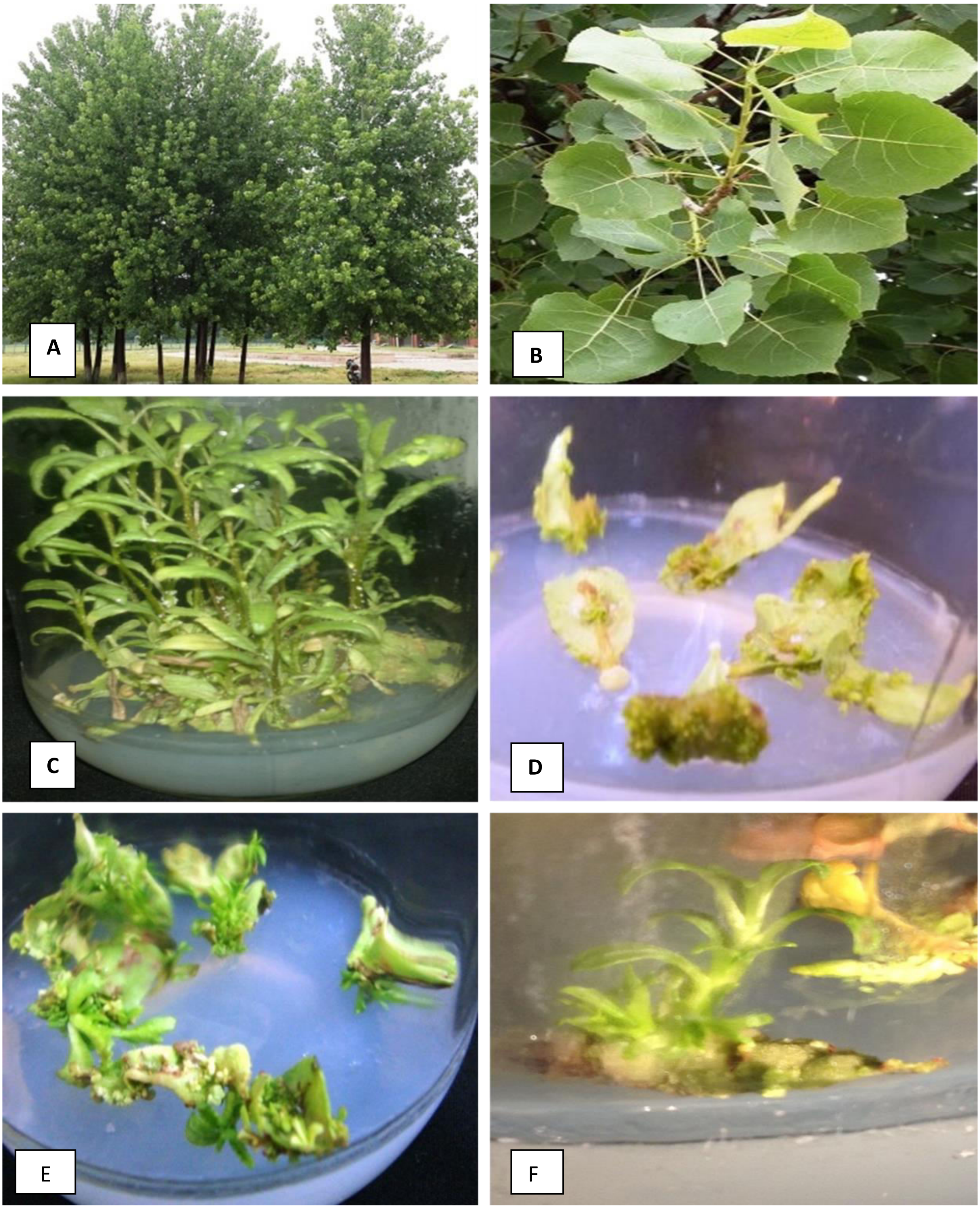

Figure: 4.1 **(A)** Elite plants of *P.deltoides* growing at Thapar University, used in the present study for established of aseptic culture and subsequent experiments (**B**) Actively growing shoots of *P.deltoides* used for collection of nodal explants (**C**) Culture establishment on medium supplemented with 2.5µM NAA and 2.5 µM BA **(D)** Culturing of leaves explants on medium supplemented with 2.5µM NAA and 2.5 µM BA **(E&F)** Regeneration of shoots from leaf explant of *P. deltoides.*

Maximum shoot regeneration occurred in explants cultured on medium containing 500 mg/l cefotaxime (48.6%) in comparison, 29.6% of explants cultured on medium devoid of cefotaxime resulted in shoot regeneration. A complete inhibition of shoot regeneration was observed when 500 mg/l cephalexin was added to the culture medium.

Table 4.2: Effect of various concentrations of different antibiotics on shoot regeneration from leaf segments taken from microshoots of *P. deltoides* on modified MS medium supplemented with 2.5µM BA and 1µM NAA**.**

Antibiotic	Concentration (mg/l)	Percent explants showing shoot regeneration	Average no. of shoots/explants
Cefotaxime	0	29.6^{de}	12.9^{ef}
	100	29.9^{de}	13.8^{cd}
	300	39.0^{b}	14.2^{b}
	500	48.6^{a}	16.0^{a}
Carbenicillin	100	32.3^{c}	13.3^{cd}
	300	26.2^{fg}	13.0^{ef}
	500	25.7^{fg}	11.0^{g}
Cephalexin	100	20.5^{h}	9.7^{h}
	300	11.5^{i}	6.8^{i}
	500	0^{j}	0^{j}

Data were scored after 8 weeks of inoculation and sub-culturing was carried out at 4 weeks interval on same medium. Mean values sharing a common letter within the column are not significant at $P< 0.05$.

Effect of clonal variations:

The regeneration potential varied amongst different clones of *P. deltoides*. A higher frequency of explants resulted in shoot regeneration when the ‘L34’ clone was used as compared to the ‘G48’ clones. The shoot regeneration frequency of ‘L34’ and ‘G48’ clones of *P. deltoides* were 40% and 28% respectively, and the callusing frequency of ‘L34’ and ‘G48’ clones were 55% and 45% respectively. So, in terms of shoot regeneration and callus formation, clone ‘L34’ performed better than clone ‘G48’.

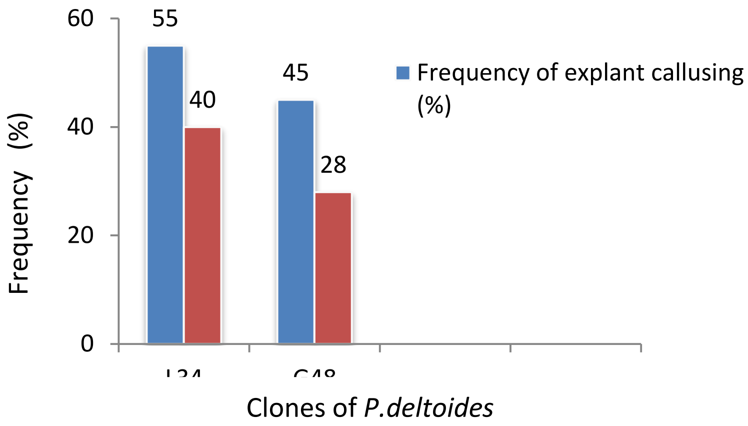

Figure 4.2 Shoot regeneration and callus formation potential of different clones of *P. deltoides* from leaf segments, taken from microshoots, on modified MS medium supplemented with 2.5 µM BA and 1.0 µM NAA. Mean value data were scored after 8 weeks of culture and sub-culturing was carried out at 4-week intervals on the same medium

Genetic transformation studies

Determination of antibiotic sensitivity:

The sensitivity of the leaves of *P. deltoides* L34 for kanamycin for the selection of the transformed tissue was determined by culturing the leaf segments on modified MS medium containing 2.5 µM BA and 1.0µM NAA supplemented with different concentrations of kanamycin (0, 10, 20, 30, 40, 50, 70, 100 mg/L).

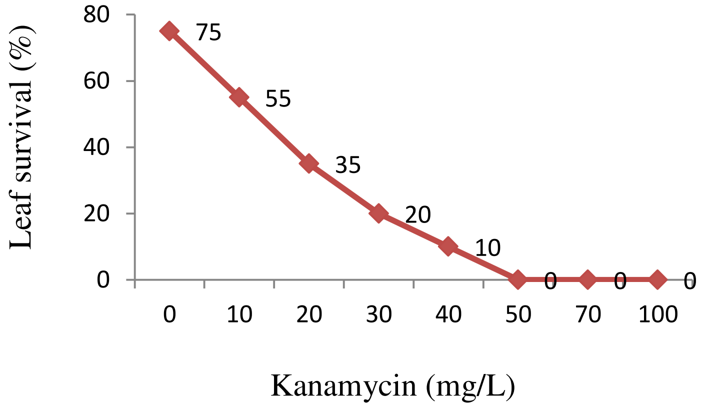

Figure 4.3: The effect of kanamycin on the survival of leaf explants taken from microshoots of *P. deltoides* L34. Data were recorded after 7 weeks of culture. Values are the means of two experiments consisting of four replicates each (ten explants in each replicate).

Experimental results investigating the sensitivity of the leaf explants showed that the presence of kanamycin in the medium caused considerable toxicity to explant and resulted in drastic decline in shoot regeneration potential compared to those cultured on kanamycin-free medium. On medium lacking kanamycin, about 75% of explants survived, whereas incorporation of kanamycin inhibited shoot organogenesis and all the explants died on media containing 50 µg /ml kanamycin. Therefore, the concentration of kanamycin was kept at 50 µg /ml in all experiments.

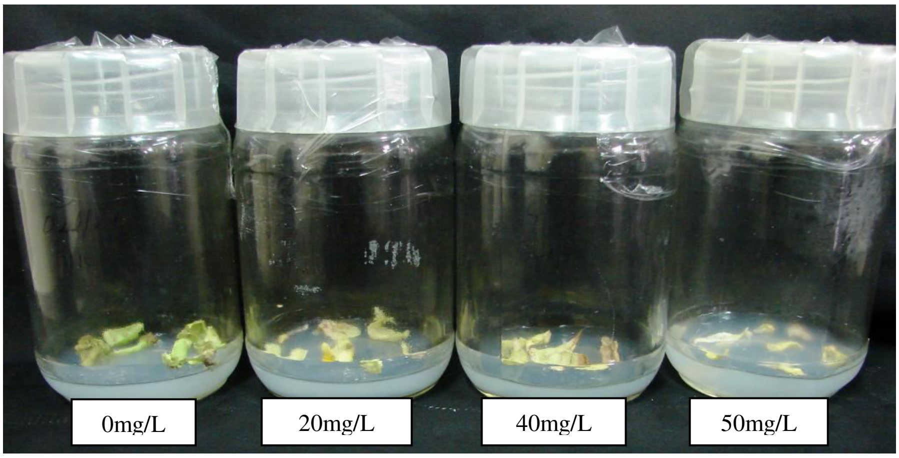

Figure 4.4: Effect of different concentrations of kanamycin on the survival of leaf explants taken from *P. deltoides*.

Selection of *A. tumefaciens* strain for genetic transformation

The efficiency of two strains of *A. tumefaciens* namely, EHA105 and LBA4404 were tested for genetic transformation. Strain LBA4404 induced significantly higher transient GUS activity (58.3 % explants) as compared to strain EHA105 (47.5 % explants). Therefore, strain LBA4404 was used in all subsequent experiments.

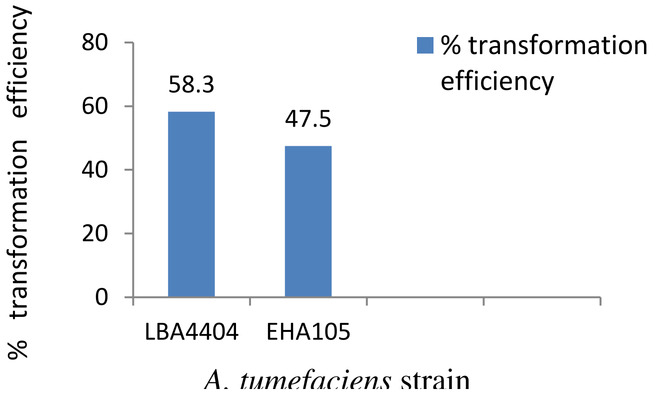

Figure 4.5 Effect of different *Agrobacterium* strains (transformed with binary vector pBI121) on transient gene expression on *P. deltoides* leaves. Data based on 20 explants per treatment and each experiment repeated thrice. Mean values sharing a common letter are not significant at P< 0.05.

Standardization of various factors affecting *Agrobacterium*-mediated genetic transformation

In the present investigation, factors influencing genetic transformation and subsequent shoot regeneration of selected elite clone of *P. deltoides* were studied. Various factors namely pre-culture, bacterial density, incubation conditions, photoperiod, infection time etc., influenced transformation efficiency of leaf explants.

Leaves from different clones of *P. deltoides* L34 were pre-cultured on modified MS medium containing PGRs and 50 µM acetosyringone for 2 days and incubated under 16 h light cycle showed maximum transient GUS activity 59 % and 57% respectively.

The pH of medium during co-cultivation also influenced the efficiency of T-DNA delivery. Higher frequency explants showed transient GUS activity when cultured on medium with pH of 5.8 as compared to pH of 5.2, however, these differences were statistically non significant (Table 4.3).

The density of bacterial suspension used for infection of the explant also influenced transient GUS activity (Table 4.3). Maximum transient GUS activity was obtained in explants that were infected with the bacterial suspension having an OD_{600} of 0.6. At higher bacterial density, the decrease in transient GUS activity was observed (Table 4.3).

An approppriate co-cultivation period following *Agrobacterium* infection has significant role in delivery of T-DNA and influenced the expression of transient GUS activity (Table 4.3). A maximum explants showed transient GUS activity when these were cocultivated for 2 days. Co-cultivation period of less than two days and more than two days resulted in decrease in transient GUS activity. In this study, co–cultivation period of more than three days caused excessive bacterial growth leading to necrosis of explants.

Photoperiod during co-cultivation also had an impact on the transient GUS activity (Table 4.3). With different clones of *P. deltoides* L34, a photoperiod of 16-h was found to induce maximum transient GUS activity 58.5% and 52.5% respectively.

The duration of the exposure interval to *Agrobacterium* cells also influences the transformation frequency of explants. Leaf explants incubated for 5 mins with *Agrobacterium* cells showed a significantly increased frequency of transformation, while exposure to *Agrobacterium* for more than 20 mins resulted in decline in transformation frequency as shown in (Table 4.3). However the 30 mins treatment was associated with problems such as elimination of the *Agrobacterium* subsequent to co-cultivation and loss of viability of the explants resulting from the overgrowth of bacteria.

After optimization of various parameters of *Agrobacterium* mediated genetic transformation of elite clone of *P. deltoides*, the best transformation procedure initially involves preculturing of leaf explants of *P. deltoides* on modified MS medium for two days, then in sterilised petridish infect the leaf explants for 5mins with disarmed strain of *A .tumefaciens* LBA4404 suspension culture grown overnight having density 0.6 at O.D.$_{600,}$ Explants were then injured either with autoclaved surgical blade or needle, After infection, tissues were blotted with sterile filter paper to remove the excess of bacterial cells and medium. These were then cultured for two days on modified MS medium having pH 5.8 supplemented with 100 µM acetosyringone (Sigma Chemical Co., St Louis, MO). Cultures were sealed with cling film and incubated under photoperiod of 16 h.The leaf explants after two days of co-cultivation period were then washed 3-4 times with sterile distilled water containing 500 µg/ml cefotaxime, blotted on sterile filter paper and transferred to culture bottles containing modified MS medium supplemented with 2.5 µM BA, 1 µM IAA, 50 µg/ml kanamycin and 500 µg/ml cefotaxime

Table 4.3: The effect of different transformation parameters on transient *gus* expression in leaf explants of *P. deltoides* clone L34 co-cultivated with *Agrobacterium tumefaciens* LBA4404.

Factors	**Variable**	**% *GUS* expression**
		L34
Infection time	5 min	53.4^{a}
	10 min	51.4^{b}
	15 min	46.0^{c}
	20 min	40.5^{de}
	30 min	39.6^{de}
Bacterial density (O.D)	0.2	32.5^{e}
	0.4	41.4^{d}
	0.6	61.0^{a}
	0.8	56.3^{b}
	1.0	46.2^{c}
Pre culture	0 day	35.8^{f}
	1 days	45.4^{c}
	2 days	57.0^{a}
	3 days	51.2^{b}
	4 days	41.5^{de}
	5 days	40.8^{de}
pH of co-cultivation medium	5.2	42.8^{c}
	5.5	49.0^{b}
	5.8	53.4^{a}
Co-cultivation days	1 day	42.5^{cd}
	2 days	52.4^{a}
	3 days	47.8^{b}
	4 days	42.3^{cd}
	5 days	35.0^{e}
Photoperiod	24 h light	41.3^{c}
	24 h dark	46.7^{b}
	16 h light/8 h dark	52.5^{a}

Data based on 30 explants per treatment and each experiment was repeated thrice**.** Mean values sharing a common letter within the column are not significant at $P<0.05$ according to Duncan's multiple range test.

Histochemical GUS assay was carried out using regenerated kanamycin resistant shoots and freshly infected explants after two days of incubation on modified MS medium supplemented with 2.5 µM BA, 1 µM IAA, 50 µg/ml kanamycin and 500 µg/ml cefotaxim, for scoring transient expression following the method of Jefferson *et al.* 1987. The tissue showing blue colour was scored.

The cultures were sub-cultured on same medium at every 20 days interval. Following genetic transformation of tissue, shoot regeneration was observed as shown in Figure: 4.6.

Although, most of the parameters were found to influence T-DNA transfer, yet a lower transformation efficiency was observed in this study (Table 4.4). This could be due to the use of explants taken from the mature plants of selected elite clones, which is essential in retaining the superior genetic makeup of the existing genotype. It has been reported earlier that tissue taken from selected clones results in lower transformation and organogenesis as compared to juvenile tissues taken from seedlings (Tournier *et al.* 2003; Aggarwal *et al.* 2010)

Table 4.4: Transformation efficiency of regenerated shoots of *P.deltoides* on modified MS medium

Experiment	**Explant types**	**Preculture (day)**	**No. of leaf explants incubated**	**No. of Kanamycin resistant shoot**	**Transformation efficiency (%)**
1	Leaf	1	300	3	1^{b}
2	Leaf	2	300	5	1.6^{a}
3	Stem	2	300	1	0.3^{c}

Transformation efficiency based on surviving shoots on modified MS medium supplemented with antibiotic. Mean value in each experiment is average of ten sets of experiment with 30 leaf explants in each set

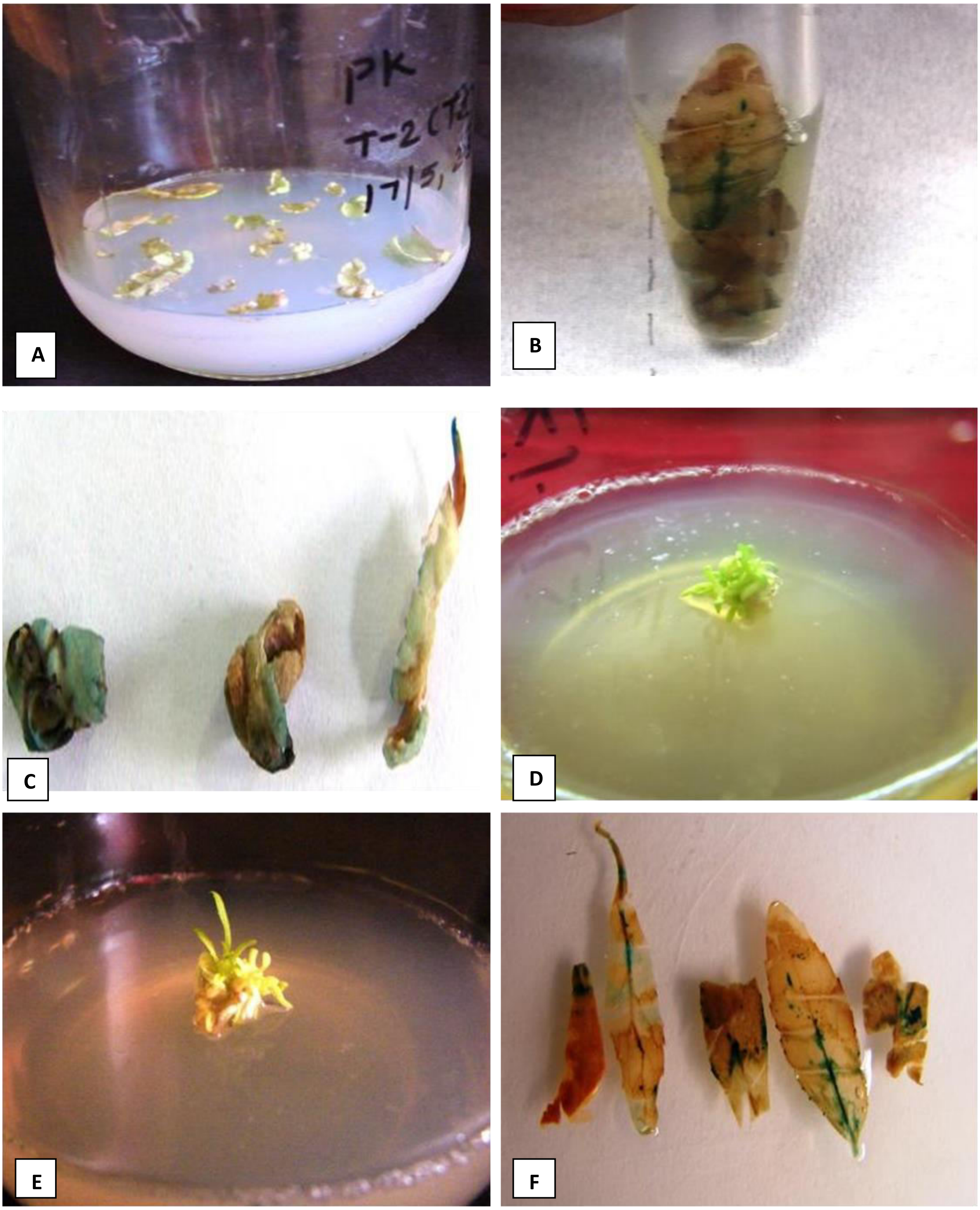

Figure:4.6 Regeneration of transgenic *P.deltoides*(**A**) *A. tumefaciens* mediated transformation experiment of *P.deltoides* leaf explants (**B**).*P.deltoides* leaf explants showing transient GUS activity **(C)** Transformed *P.deltoides* callus showing GUS activity. **(D&E)** Regeneration of transformed *P.deltoides* shoots on selection medium containing kanamycin. **(F)** Transformed *P.deltoides* shoots showing Stable GUS activity.

Discussion

Micropropagation, at present is one of most reliable method for the mass multiplication of plants (Altman and Loberant, 1998). The number of species cultured *in vitro* has been progressively increasing over the last two decades (Hartman *et al.* 1990). However, studies on tree tissue culture have received little attention, Consequently the success of *in vitro* work on forest trees has been rather slow and limited (Bisht *et al.* 1998). Nevertheless, micropropagation protocols for several trees species have been developed (Aggarwal *et al.* 2012; Vengadesan and Pijut 2009) but reproducibility has been achieved only in a few cases. Therefore, in the present work we was focused to optimize and standardize each step in micropropagation protocol of selected elite clone of *P. deltoides*, so that large number of plantlets can be obtained in shorter time frames. The factors affecting micropropagation and transformation of elite clone of *P. deltoides* have been also investigated. Furthermore, highly efficient and reproducible *in vitro* regeneration systems via somatic embryogenesis or organogenesis are a prerequisite for clonal propagation.

In general, different concentrations of BA in combination with the auxins like NAA and/or IAA were beneficial for the induction of shoot buds *in vitro* (Kaur *et al.* 1999). Higher concentrations (2.5 µM) of BA promoted shoot multiplication (Table 4.1). Earlier, beneficial effect of BA over other cytokinins for shoot multiplication/organogenesis was reported (Vengadesan and Pijut 2009). Cytokinins were the first compounds recognized for their ability to induce cell division in certain plant tissues, are now known to evoke a diversity of responses in plants (Letham 1978). Roots are considered as site of cytokinins biosynthesis and known to move to xylem to the shoot where these are known to control of both development and senescence (Letham and Palni 1983). Moreover, higher concentrations cytokinins are known to suppress apical dominance and thus stimulate shoot multiplication

(George 1996). The requirement of NAA for shoot multiplication/elongation could be due to its reported role in elimination of phenolic substances by competing for the active sites of auxin oxidase enzyme involved in oxidization of phenols (Perez-Tornero *et al.* 2000), thus helping BA in production of multiple shoots (Sugimura *et al.* 2005). Earlier, presence of NAA along with BA in the medium has been reported to improve shoot induction and multiplication (Saritha and Naidu 2008). Among the different combinations of PGRs tested, shoot organogenesis was observed on media containing combinations with higher concentrations of BA and lower concentrations of auxins (NAA) (Table 4.1). The requirement of BA for the induction of shoot organogenesis has been reported (Ganeshan *et al.* 2006).

Many other compounds, in addition to PGRs, are known to influence growth and morphogenesis under both natural and tissue culture conditions (Yu and Wei 2008). The most important of these are antibiotics, which are used in plant tissue culture for different purposes (Tiwari *et al.* 2006). In our study, the incorporation of cefotaxime into the medium significantly improved shoot organogenesis from 29.6% to 48.6% explants (Table 4.2). The beneficial effect of cefotaxime on somatic embryogenesis in wheat has also been reported (Yu and Wei 2008). The growth regulatory activity of antibiotics has been attributed to their interference with the metabolism of PGRs, cefotaxime has been shown to interfere with ethylene biosynthesis (Pius *et al.* 1993).

Despite the clear potential of genetic engineering for improving woody plants due to their vast commercial interest, progress has been slow on these plants especially *Populus* because of its recalcitrant nature. In particular, regeneration after transformation is often very poor, probably due to the high concentration of phenolic compounds in the cells or due to low endogenous cytokinin content. Moreover, organogenesis or embryogenesis capacity is even lower on a selective antibiotic containing medium making it often impossible to recover transgenic shoots even when stable transformation has been achieved. Nevertheless, transgenic *Populus* has been recovered in a few species (Tournier *et al.* 2003), mainly from juvenile material that showed better transformation and regeneration capacity than adult clones. However, an efficient improvement programme for the selected clones can only be taken up using mature tissue. Therefore, it is important to develop transformation protocol from the mature selected plants of *P. deltoides.* In this present work, factors influencing efficiency of T-DNA delivery into the selected elite clones of *P. deltoides* using *A. tumefaciens* was studied and developed a *Agrobacterium* mediated genetic transformation protocol for *P. deltoides*.

In order to select the transformed tissue on the selectable marker, the sensitivity of the tissue to this antibiotic is required to be tested. Kanamycin is an aminoglycoside derivative of penicillin and is widely used to select the transformed cells with *nptII* gene. Kanamycin sensitivity appears to be species dependent. A wide range of concentrations has been reported to inhibit organogenesis in various plant species such as, apple (Yao *et al.* 1995), grape (Scorza *et al.* 1996) and citrus fruits (Yao *et al.* 1996). Leaves taken from microshoots of *P. deltoides* were tested for their sensitivity towards kanamycin. It was found that kanamycin restricted the growth to a greater extent and completely inhibited growth at concentration above 50 mg/l (Figure 4.3). This sensitivity test of the tissue to kanamycin will be useful in further screening of transformants during genetic transformation.

Transformation efficiency often depends on the strain of *A. tumefaciens* used (Kumar and Rajam 2007; Hood *et al.* 1993). In this work, we were also used two strains of *A. tumefaciens* namely LBA4404 and EHA105 (both transformed with binary vector pBI121) and the transient GUS frequencies resulting from the infection of these starins were compared (Figure 4.6). Earlier, several studies have reported that EHA105 is more effective than other strains for transformation in sugarcane (Manickavasagam *et al.* 2004) and blueberry (Song and Sink

2004). The different *Agrobacterium* strains are defined by their chromosomal and plasmid genomes. The different strains were reported to vary in virulence because of the presence or absence of the *virF* locus on Ti plasmids. The *A. tumefaciens* strain EHA105 is more infectious than LBA4404 because EHA105 is a derivative of the super virulent strain A281 (Hood *et al.* 1993), while LBA4404 is derived from the less virulent strain Ach5 (Hoekema *et al.* 1983). However, the *A. tumefaciens* strain LBA4404 has also been used for many plant transformations because, the elimination of LBA4404 from plant tissues is relatively easy at low concentration of antibiotics (Maheswaran *et al.* 1992). In contrast, it is difficult to eliminate EHA105 from plant tissues. Therefore, in this investigation concentration of cefotaxime was kept 500 µg/ ml in subsequent subculture cycles to completely eliminate the bacteria. Further, it was observed that cefotaxime enhanced shoot organogenic potential of leaf explants of *P. deltoides* (Table 4.2)

The various factors namely pre-culture, bacterial density, incubation conditions, photoperiod, pH of co-cultivation medium etc., influenced transformation efficiency of leaf explants (Table 4.3). Culture medium and incubation conditions of the explants, prior to *Agrobacterium* infection have been reported to enhance T-DNA delivery in some plant species (Yevtushenko and Misra 2010).

Preculture of the explants has also been reported as a significant factor for transformation of several plants (Padmanabhan and Sahi 2009). In present study, leaves pre-cultured on modified MS medium (Table 3.1) containing 50 µM acetosyringone for two days showed maximum transient GUS activity (59 % with L-34 and 57% with G-48; Table 4.3). The higher GUS activity is probably due to the presence of cytokinin resulting in increased cell division and/or presence of acetosyringone that is known to induce *vir* genes and enhance T-DNA transfer (Stachel *et al.* 1985). Earlier, pre-culturing of explants on a particular medium prior to infection with *Agrobacterium* has been reported to enhance transformation efficiency in many plant species (Padmanabhan and Sahi 2009).

Wounding of tissue before infection could allow bacterial penetration deep into the tissue facilitating the accessibility of plant cells to *Agrobacterium* on one hand and may stimulate the induction of *vir* genes as a result of phenolics secretion on other, which could be the main reasons for enhanced bacterial efficiency for T-DNA delivery (Binns and Thomashow 1988). Method of injury to plant tissue before cocultivation has also been shown to influence transformation frequency in the plants such as *Vitis vinifera* (Dutt *et al.* 2007).

Although *Agrobacterium tumefaciens* was reported to show the best growth at neutral pH (Li *et al.* 2002), previous reports on the *Agrobacterium*-mediated transformation showed that an acidic pH favoured the optimal expression of the *vir* genes (Godwin *et al.* 1991). The transient GUS expression was the strongest when the pH of the co-cultivation medium was adjusted at 5.8 indicating the importance of maintaining the required pH of medium during co-cultivation. Low pH during co-cultivation was reported to be beneficial for *Agrobacterium* mediated transformation across the species by Godwin *et al.* (1991). Thus, we can conclude that low pH (5.8) was responsible for the maximum frequency of observed transient GUS expression (Table 4.3).

A critical factor in *Agrobacterium* mediated transformation systems is the density of the *Agrobacterium* inoculum in the inoculation medium. We inoculated leaf explants (as described in materials and methods) with an *Agrobacterium* suspension which was applied at range of cell densities. Maximum transient GUS activity was obtained in explants that were infected with the bacterial suspension having an OD_{600} of 0.8. At higher bacterial density, the decrease in transient GUS activity was observed (Table 4.3). A reduction in the mean transformation frequency was observed following inoculation with higher densities of

Agrobacterium, possibly due to increased damage, necrosis of plant tissue and increased production of harmful compounds .

The effect of varying length of co-cultivation period was also investigated. Co-cultivation is one of the most important steps for *Agrobacterium*-mediated transformation of plants (James *et al.* 1993). A maximum number of explants showed transient GUS activity when these were infected with *A. tumefaciens* for 10 minutes and further co-cultivated for two days (Table 4.3). It is known that co-cultivation of explants with *A. tumefaciens* for an appropriate duration improves the transformation efficiency, but prolonged co-cultivation period were reported to result in death of explants, due to overgrowth of bacteria (James *et al.* 1993). In this study, co–cultivation period of more than three days caused excessive bacterial growth leading to necrosis of explants. Although, an increased transformation frequency has been positively correlated with longer cocultivation periods (2–5 days), yet it has been emphasized that for higher transformation efficiency, an optimum co-cultivation period is required.

Different light conditions during co-cultivation also have a strong affect on the efficiency of transient expression (Zuker *et al.* 1999). A photoperiod of 16-h was found to induce transient GUS activity in maximum explants (58.5%, Table 4.3). Beneficial effect of light on *Agrobacterium* mediated T-DNA transfer to *Phaseolus acutifolius* and *Arabidopsis thaliana* has been reported (Zambre *et al.* 2003). Thus various factors influencing the T-DNA delivery to the *Populus* has been worked out and a standard protocol is available for genetic transformation of *P. deltoides* L34.

Conclusion

In conclusion, an efficient, reproducible micropropagation and shoot regeneration has been obtained from the selected elite clones of *P. deltoides* L34. Also, various factors effecting the genetic transformation protocol for the selected elite clone of *P. deltoides* was obtained and on the basis of that the protocol for delivery of T-DNA using *A. tumefaciens* has been developed and subsequent regeneration of transformed shoots has been achieved in selected clones of *P. deltoides*. This seems to be first report of successful transformation of elite clones of *P. deltoides* using *A. tumefaciens*. This protocol has the potential to facilitate work on genetic modification of these clones incorporating genes of important traits.

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