Comparison of regeneration efficiency of the Indian potato cultivars during *Agrobacterium*-mediated genetic transformation

A Dissertation
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Masters of Science in Biotechnology

Under the guidance of
Dr. N. DAS
Associate professor
Department of Biotechnology and Environmental Sciences

By:
Sakshi Chawla
Roll No. 301001022

Department of Biotechnology and Environmental Sciences
THAPAR UNIVERSITY
PATIALA-147004
INDIA
July, 2012

DEDICATED TO MY FATHER

CERTIFICATE

This is to certify that the thesis entitled **"Comparison of regeneration efficiency of the Indian potato cultivars during *Agrobacterium*-mediated genetic transformation"** submitted by Sakshi Chawla (Roll no: 301001022) in partial fulfillment of the requirement for the award of Degree of Master of Sciences in biotechnology, to Thapar University (Deemed University), Patiala, is a record of Student's own work carried out by her under our supervision and guidance. The report has not been submitted for the award of any other degree or certificate in this or any other university or institute.

15/07/2012

(Dr. N. Das)
Supervisor
Deptt. of Biotech. and Env. Sciences
Thapar University, Patiala

(Dr. M.S. Reddy)
Head
Deptt. of Biotech. and Env. Sciences
Thapar University, Patiala

(Dr. S. K. Mohapatra)
Dean (Academic Affairs)
Thapar University, Patiala

DECLARATION

I hereby declare that the work which is being presented in this thesis **"Comparison of regeneration efficiency of the Indian potato cultivars during *Agrobacterium*-mediated genetic transformation"** submitted by the undersigned in partial fulfillment of the requirement for the award of Degree of Master of Sciences in biotechnology, Thapar University, Patiala, is true and original record of my own independent and original research work carried out under the supervision of **Dr. N. Das,** associate professor, Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, India. 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.

Date:

Place: **(Sakshi Chawla)**

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Place: Patiala Sakshi Chawla

CONTENTS

ABBREVIATIONS

Name	Abbreviation
bp	Base pair
BSA	Bovine serum albumin
CaMV 35S	Cauliflower mosaic virus 35S
CIM	Callus inducing medium
CPRI	Central Potato Research Institute
DNA	Deoxyribonucleic acid
dNTP	2’-deoxynucleoside-5’-triphosphate
EDTA	Ethylenediamine-tetra acetic acid
GA_3	Gibberillic acid
GBSS	Granule-bound starch synthase
GUS	Glucuronidase
IAA	Indole-3-acetic acid
Kan	Kanamycin
kb	Kilo base
kJ	Kilo joule
L	Litre
LA	Luria agar
LB	Luria broth
M	Molar
mg	Milligram
mg g^{-1}	Milligram per gram
mg L^{-1}	Milligram per liter
mg mL^{-1}	Milligram per milliliter
µg	Microgram
µg mL^{-1}	Microgram per milliliter
min	Minute

mL	Milliliter
mM	Millimolar
MS	Murashige and Skoog
NCBI	National Centre for Biotechnology Information
Nm	Nanometer
O.D.	Optical density
ORF	Open reading frame
PCR	Polymerase chain reaction
PEG	Polyethyleneglycol
RNA	Ribonucleic acid
rpm	Rotations per minute
rRNA	Ribosomal ribonucleic acid
SDS	Sodium dodecyl sulfate
STET	Sucrose Tris EDTA Triton X100
TAE	Tris acetate EDTA
TBE	Tris borate EDTA
TE	Tris EDTA
Tris	Tris-(hydroxymethyl-) aminomethane
V	Volt
w/v	Weight per volume
YEM	Yeast extract mannitol

CHAPTER 1

INTRODUCTION

Potato (*Solanum tuberosum* L.) is now regarded as the world's most important non-grain food crop. After wheat and rice, potato is the most important food crop, with a world-wide production of around 330 million tons in 2009 (http://www.fao.org). The potato is a tuber (somatic storage tissue) grown underground on a specialized plant part known as stolon. Potato is the most important tuber crop in terms of production, accounting for about 45% of the total world production of all tuber crops i.e. potato, cassava, sweet potato, yams, and taro accounting for 90% of total world production. The potato tubers are a globally important dietary source of starch, protein, antioxidants and vitamins. Therefore, it plays a major role with regard to global food security. The cultivated *Solanum tuberosum* subsp. *tuberosum* is considered to be originated from Andean and Chilean landraces developed by pre-Colombian cultivators. The wild species progenitors of these landraces probably derived from a group of ~20 similar wild species referred to as *S. brevicaule* complex, distributed from central Peru to northern Argentina. The Spanish introduced the potato to Europe in the second half of the 16th century. Subsequently, this crop was introduced to many territories and ports throughout the world by European mariners. Potato was introduced to India from Europe in the beginning of early 17th century. The potential of the potato crop was realized in India soon after independence in 1947. The Central Potato Research Institute (CPRI), Shimla was established in 1949 which took a leading role for the improvement of potato crop through conventional breeding techniques. Now India ranks third in terms of area of potato cultivation, and it is the second largest country of around 34.39 million tons production of potato in 2009 (http://www.fao.org). China is now the biggest potato producer in the world.

1.1.Morphology of potato plant

The potato, an herbaceous annual plant can grow up to 20-40 inches (50 to 100 cm) high. The potato is a cool season crop grown in temperate regions. The ends of its underground stems or 'stolons', may enlarge greatly to form a few to more than 20 tubers of variable shapes and sizes. The skin of potato tuber varies in color from brownish white to deep purple. Its flesh normally ranges in color from white to yellow but it also may be purple. The tubers bear lateral buds (eyes) that grow into new plants when the conditions are favorable for growth.

1.2.Taxonomy

The genus *Solanum* consists of approximately 2000 members. Out of this, only about 160 wild and seven cultivated species are able to form tubers. The most common cultivated species of potato i.e., *tuberosum* is a hybrid between the diploid species S. *stentotomum* and the diploid weed *S. sparsipilum* with subsequent chromosome doubling (Ramanna and Hermsen 1979). The potato has a series of ploidy levels, based on a haploid number of 12, ranging from diploid (2n = 2x = 24) to hexaploid (2n = 6x = 72) including triploids, tetraploids, and pentaploids (Spooner et al. 2005). Most potato cultivars are autotetraploid (2n = 4x = 48) and display a high degree of heterozygosity, and suffer acute inbreeding depression.

1.3.Mode of propagation

In contrast to the major food crops (cereals) which are propagated through seeds (products of sexual reproduction), potato plant is propagated through tubers (vegetative or asexual propagation). The tubers meant for propagation are known as "seed tubers" or "seed potatoes". In addition to tubers, a potato plant can also be propagated through botanical seeds, which are known as True Potato Seeds (TPS). TPS is an alternative means of propagation where production of 'seed potatoes' is not feasible. For usual cultivation, TPS is not popularly used; but it is important with respect to potato breeding perspectives. The main advantage of vegetative propagation is that a good potato clone can be maintained with a high degree of genetic purity. It is often colloquial to call a potato plant as a clone, because it is advanced through the generations by clonal propagation, another term of asexual or vegetative propagation. The disadvantage is that many deadly viruses and seed borne pathogens are progressively accumulated in the tubers and carried over repeated multiplications resulting in the gradual degeneration of a clone. For this reason, successful potato cultivation and production depends upon the availability of disease free high quality seed tubers. This is mostly important in tropical and subtropical warm climates as in India where there is an abundance of various vectors e.g. aphids, mites, thrips, white flies, etc. for virus transmission. Potato plants suffer from a variety of viral, bacterial, nematode and fungal diseases which have serious consequences in terms of tuber yield and consumer acceptance. Examples of viral pathogens are potato viruses X and Y. *Streptomyces scabies* and *Erwinia carotovora* represent two bacterial pathogens of tubers that cause serious losses. The most serious losses occur due to fungal pathogens, such as *Phythophthora infestans* that causes potato blight disease.

1.4. Potato crop improvement

The demand for potato crop has been increasing due to overgrowing population and its high nutritional quality. Food and Agriculture Organization of the United Nations has acknowledged potato as the food for the future in order to fight global poverty and hunger. Moreover, shrinking cultivable land, depleted soils, increased abiotic and biotic stresses on account of climate change are some of the perceivable future threats to the potato crop. Hence, there is a need improve the potato crop in terms of productivity, nutritional quality and disease resistance. Crop improvement strategies like Conventional breeding, Molecular breeding followed by genetic transformation could be employed to achieve good agronomic traits.

1.4.1. Conventional Breeding: Conventional breeding develops new plant varieties by the process of selection, and seeks to achieve expression of genetic material which is already present within a species. Conventional breeding employs processes that occur in nature, such as sexual and asexual reproduction. The product of conventional breeding emphasizes certain characteristics that have been present for millennia within the genetic potential of the species. Most new potato varieties are made by crossing together parents with useful characters followed by vegetative propagation of the F' plants to form clones. These clones and their tuber progenies are then screened in gradually increasing plots over several years for favorable combinations of agronomic traits. The cultivated forms originate from a narrow genetic base but 160 wild species are recognized and the global gene pool is relatively untapped (Hawkes, 1978). Commercial cultivars are tetraploid (2n: 4x: 48) and extremely heterozygous, with simplex inheritance (Aaaa) for many characters (Howard, 1978). Some do not flower easily, or have reduced fertility, and others are pollen sterile. This is mainly due to the tetraploid nature of the genome, the high degree of heterozygosity and the absence of homozygous inbred lines or a collection of genetically well-defined marker stocks. Moreover, Conventional breeding is laborious and time consuming process. An alternative approach to overcome the problems associated with Conventional Breeding is Molecular Breeding followed by Genetic transformation.

1.4.2. Genetic transformation: Genetic transformation facilitates introduction of only specifically desirable genes without co-transfer of any undesirable gene from donor species which normally occurs by conventional breeding methods. The introduction of genes into plants by genetic engineering has become an accepted technique in plant breeding. Plant genetic engineering is the process by which useful genes are isolated from donor organisms and incorporated into a plant which normally does not possess it. Genes of agricultural importance, for example viral, fungal or insect resistance genes have been inserted into the genomes of several crop plants (Schaff, 1991). During conventional breeding a susceptible clone can be targeted and crossed with an unrelated resistant parent. However, because potato is tetraploid, its improvement by breeding and selection is very difficult and it is a time consuming process. This makes potato an excellent target for applied molecular genetics which has become a popular tool to obtain disease and pest resistance in potatoes (Mullins et al., 2006). Molecular techniques can be divided into two different sets. Firstly, the molecular breeding techniques that are required for the identification, isolation and modification of specific genes coding for traits of interest and secondly, the cellular techniques such as transformation and the regeneration from transformed cells into plants (Huisman et al., 1992).

There are many methods that have potential for the production of transgenic plants and can broadly be divided in to two categories namely indirect and direct gene transfer (Schaff, 1991). Currently the most widely used and favored method for the production of transgenic plants is the *Agrobacterium*-mediated gene transfer system. This method is followed by particle bombardment and direct gene transfer of DNA into protoplasts (Vain, 2007). *Agrobacterium* mediated gene transfer is considered to be an indirect method as the gene of interest first has to be transferred to the bacterium and then to the plant. *Agrobacterium tumefaciens* infects the plant cells *in vitro* and transfers the gene of interest to the plant genome (Hansen & Chilton, 1999). In contrast, direct transformation occurs when the foreign DNA is delivered into plant cells by the use of protoplasts, stimulating fusion by electroporation or chemical treatment, or subjecting plant tissue in a particle gun to a shower of high velocity particles coated with the gene of interest (De Block, 1993).

1.4.3. Agrobacterium-mediated transformation: *Agrobacterium tumefaciens* is a gram-negative, soil-borne pathogenic bacterium which has the ability to genetically transform host cells. Most of the plant species tested for the susceptibility to *Agrobacterium* transfer is dicotyledonous plants (Hohn *et al.*, 1989). *Agrobacterium* is the causal organism of widespread crown gall, a disease of dicotyledonous plants (Hooykaas and Schilperoort, 1992). *Agrobacterium* has the ability to transfer a specific DNA segment, the transfer DNA (T-DNA), from the bacterium to the plant. The T-DNA demonstrates three important characteristics for the use in plant transformation. Firstly the development of crown galls is dependent on genetic transfer of a section of the TDNA from *Agrobacterium* to the plant. Secondly, the T-DNA is only transcribed in plant cells and thirdly, any foreign DNA between the borders of the T-DNA can be transferred to the plant cells (De la Riva et al., 1998). *Agrobacterium* was until recently the only natural example of DNA transfer between plants and bacteria (Sheng and Citovsky, 1996).

There are three genetic components of *Agrobacterium* necessary for plant cell transformation (Zupan and Zambryski, 1997). The first requirement is the T-DNA, a segment of DNA present on a large 200 kb extra chromosomal plasmid, called the tumour inducing (Ti) plasmid. The T-DNA is flanked by a left and right border. The borders are thought to be recognition signals for the transfer of the T-DNA into the plant genome. The T-DNA is inserted randomly into the plant chromosome. It has also been found that the copy number of the T-DNA in transformed plant lines is usually low (Sheng and Citovsky, 1996). The next important part is the 30 – 40 kb *virulence* (*vir*) region. It is also located on the Ti plasmid and is composed of six major loci regions: *virA, virB, virC, virD, virE and virG* (De la Riva et al., 1998). The *vir* gene protein products are directly involved in the transfer of the T-DNA. The protein products are regulated in such a way that expression only occurs in the presence of specific compounds (plant phenolics) secreted by wounded plant cells to generate a copy of the T-DNA (Zupan and Zambryski, 1995). The last requirement for transformation to occur is the chromosomal virulence (*chv*) genes. These genes are involved in the attachment of the bacterium to the wounded plant cell (Sheng and Citovsky, 1996).

The plant cells must be wounded in order to allow entrance of the bacterium (Sheng & Citovsky, 1996). The T-DNA must exit the bacterial cell wall and be transferred into the plant cell as a protein-nucleic acid complex. It is known that an *Agrobacterium*-plant cell channel is encoded by the *virB* genes of *Agrobacterium* and is an energy-dependent process. Once inside the plant cell, the T-DNA targets the nucleus and crosses the nuclear membrane after which the T-DNA becomes integrated into the chromosome. The molecular mechanisms by which this is achieved are largely unknown (Zupan & Zambryski, 1995).

The wild-type T-DNA coding region can be replaced by any sequence without any effect on its transfer from *Agrobacterium* to the plant, because the T-DNA element is defined by its borders

(Sheng & Citovsky, 1996). No physical linkage between the T-DNA and the rest of the Ti plasmid is necessary for T-DNA transfer to occur. Therefore it is possible to manipulate the Ti plasmid for the use in plant genetic engineering (Gelvin, 2003).

Genes of agronomical interest, for example disease resistance genes, can be cloned into plasmids between artificial T-DNA borders, which are introduced into an *Agrobacterium tumefaciens* strain already harbouring a Ti plasmid with a *vir* region but lacking the T-DNA. The plant expression vector is transferred from *Escherichia coli* into *Agrobacterium tumefaciens* via a triparental mating process. This process is mediated by helper plasmids which provide mobilisation *(mob)* and transfer *(tra)* functions (Walkerpeace and Velten, 1994).

Many plant expression vectors for the use in *Agrobacterium*-mediated transformations have been created and are available commercially. Each of these vectors contain a bacterial origin of replication and a bacterial selectable marker so that the vector can be manipulated in the bacteria (Gelvin, 2003). The vector also contains a plant selectable marker gene to allow for selection of transformed cells, and a multiple cloning site which contains unique restriction enzyme recognition sequences, often flanked on the 5' end by a plant promoter, for example the cauliflower mosaic virus 35S promoter (Schaff, 1991). Examples of selectable marker genes are *nptII (*kanamycin resistance), *bar* (phosphinothricin resistance), *hpt* (hygromycin resistance) or *uidA* (β-glucuronidase). β- Glucuronidase (GUS) has become a popular plant reporter gene and is widely used in plant transformation studies (Jefferson et al., 1987).

The techniques of plant tissue culture play an important role in plant transformation systems.

1.5. Plant tissue culture

Culture systems provide the experimental system to which techniques of genetic manipulation can be applied. Potato is amenable to a number of tissue culture techniques, ranging from *in vitro* propagation via shoot cultures to regeneration of whole plants from protoplasts .In general terms, these all involve the growth of plants, cells, tissues and organs in sterile conditions, supported by an appropriate culture medium. Media normally contain a mixture of major and minor salts, vitamins, sugar (as a carbon source) and plant growth regulators. The most widely used formulations are based on that of Murashige and Skoog (1962), which is available commercially.

Whole plants can be regenerated from a range of cultured tissues (roots, leaves, tubers, stems) and from single cells. Plant tissues regenerate *in vitro* through two pathways: Organogenesis – is a developmental pathway in which roots or shoots are being induced to differentiate from a cell or cell clusters. Embryogenesis - where usually single cell or a small cluster of cells undergoes differentiation to produce somatic embryos similar to zygotic embryos. Morphogenesis could occur directly from the explants or indirectly *via* the formation of dedifferentiated callus. There are different pathways of regeneration such as:

- Direct organogenesis from explants
- Indirect organogenesis from explants via callus
- Direct embryogenesis from explants
- Indirect embryogenesis from explants via callus

The regeneration of complete plant via tissue culture has made it possible to introduce foreign genes into plant cells and recover transgenic plant. For plant transformation, many different techniques such as *Agrobacterium*-mediated transformation, particle gun bombardment, and microinjection, PEG treatment of protoplast and electroporation of protoplast can be used. However, *Agrobacterium*-mediated and particle gun bombardment are the most extensively used methods. Regeneration via the callus is amenable to *Agrobacterium*–mediated transformation while direct regeneration is amenable for particle gun bombardment (Chandra and Pental, 2003).

1.6. Regeneration by Organogenesis: Tissues from leaves, stems, roots and inflorescence can be cultured to promote direct morphogenesis or indirect morphogenesis to obtain either direct shoot generation (organogenesis) or somatic embryos (somatic embryogenesis) (Balasubramanian et al., 1998). It has been observed from previous studies that plants regenerated via organogenesis are more amenable to transgenic techniques. Organogenesis could be direct or indirect with reference to the intervening callus phase. In direct organogenesis, the roots and shoots are directly induced from cell under specific ratio of plant growth regulators. Direct regeneration is more favourable with respect to transgenesis. The reduced callus phase decreases the chances of occurrence of undesired somaclonal variation in regenerated plantlets. In indirect organogenesis, the intervening callus phase is present. The technique of callus (tissue) culture was first developed in the late 1920s and 1930s and was one of the primary methods of tissue culture for many years. Indirect organogenesis is based on the ability of the highly mature and differentiated cells to regress to a meristematic state. The phenomenon of a mature cell reverting to the meristematic state and forming undifferentiated cell mass called as callus is termed as dedifferentiation. The cells first undergo certain changes to achieve meristematic state these include replacement of non-functional cellular components damaged by lysosomal activity during the processes of cytoquiescence. Under defined ratio of plant growth regulators, the meristematic cells redifferentiates to form plant organ or the whole plant and the phenomenon is known as redifferentiation and and is followed by the process of induction of roots and shoots in callus cultures that is referred as organogenesis. The inherent capacity of a plant cell to give rise to a whole plant, which is retained even after a cell has undergone final differentiation in the plant body, is known as cellular totipotency (Bhojwani and Razdan, 1992).

Regeneration efficiency can be defined in terms of:

- No. of explant regenerated out of total no. of explants
- Time taken by explant to regenerate

Although potato regeneration protocol was establish in many laboratories in the world but still there are very less information regarding the comparative studies among the different varieties of potato. Which genotypes are more efficient for rapid multiplication is need to identify. Need to assess the regeneration potentiality of available release varieties of potato for better utilization in commercial purpose. However, these procedures have major limitations, such as low frequency of transformation and, more importantly, the occurrence of somaclonal variations at very high rates resulting from harsh tissue culture, thus limiting the production of transgenic plants for commercial application (Beaujean and Sangwan, 1998). The success and efficiency of transformation depends on the tissue culture system, and the methods required vary with species. Responses of potatoes to published regeneration regimes have shown cultivar specificity (Wheeler et al., 1985).

Moreover, transformation protocols in potato are genotype-dependent, limiting their usage and making practical applications not easily adaptable to all genotypes (Wenzler et al., 1989, Yee et al., 2001, Banerjee et al., 2006). Previous reports indicated that the competence of plant cells for transformation via *Agrobacterium*- mediated gene transfer could be modified by adjusting physical conditions of the explant or media composition just prior to or during T-DNA delivery (Lin and Zhang, 2005).

1.7. Factors affecting *Agrobacterium* mediated transformation/Regeneration efficiency

Transformation efficiency can be increased by the manipulation of either the plant or bacteria for enhancing competency of plant tissue and *vir* gene expression, respectively (Henzi et al., 2000; Mondal et al., 2001; Lopez et al., 2004; Chakrabarty et al., 2002).

- **Acetosyringone**: Acetosyringone is a phenolic compound, which is secreted at wounded site of dicotyledons. This compound enhances the *Agrobacterium*-mediated gene transformation in dicot. Monocots lack this wound response and it was considered as the limiting factor in *Agrobacterium*-mediated gene transformation in monocots. The addition of acetosyringone in co-cultivation medium induces vir genes, extend host range of some *Agrobacterium* strains (Hiei et al., 1994). Acetosyringone has been commonly utilized in most transformation research for both dicotyledons and monocotyledon plants to enhance transformation efficiency (Frame et al., 2002; Olhoft et al., 2003; Kant et al., 2007). However, the effects of Acetosyringone on *Agrobacterium*-mediated transformation is known to vary according to the plant species. There is even evidence to suggest that Acetosyringone may suppress virulence in some strain/plant species interactions (Godwin et al., 1991).
- **Effect of callus age on transformation:** The age of callus is a major parameter to obtain the high transformation frequency because every age of callus cannot able to withstand infection. Most workers used 3 weeks old calli as explant (Hiei et al., 1994; Rashid et al., 1996) and some reporters have used 8 weeks old callus as explants (Kumar et al., 2005)
- **Bacterial concentration:** It has been considered in several experiments that transformation efficiency might be affected by bacterial growth phase and bacterial cell density. Different concentrations of the *Agrobacterium* have been used by different research groups and for different plant materials. In the standard protocol, cells are grown to the stationary phase (A600nm=2-2.4), pelleted and resuspended in inoculation medium to stationary or log or mid log phase (A600nm=0.1-1.15). High concentrations of bacteria at the stationary phase have normally been used for rice, legume and tobacco transformation (Hood et al., 1987; Kapila et al., 1997; Dillen et al., 1997), and low

concentrations of bacteria at the log or mid-log phase have been used for broccoli (Mets et al., 1995), cabbage (Henzi et al., 2000), wheat (Cheng et al., 1997), cottonwood (Han et al., 2000), tobacco (Krugel et al., 2002) and A600nm – 0.2 for potato (Chakravarty et al., 2010)

- **Bacterial Pre-culture period:** The bacterial pre-culture effect has been tested by Clough and Bent (1998). They preinduced *vir* genes by growing *Agrobacterium* in a standard *vir*-inducing liquid medium for 20 h at 19°C (Liu et al., 1998), and compared bacteria grown on a plate with bacteria grown in rich liquid medium. They reported that transformation efficiency of *Arabidopsis* did not change significantly. They also reported that growth of bacteria on solid *vir*-inducing CIB medium at 19°C led to a four-fold decrease in transformation. Bacteria for pili formation experiments in the microbiology era used to be grown on minimal media for 3 days under 19°C (Fullner et al., 1996). In this study, bacteria were pre-cultured for 3 days before inoculation.

- **Period of co-cultivation:** Co-cultivation for 2 to 7 days has been normally used in *Agrobacterium*-mediated transformation under various co-cultivation temperatures (Han et al., 2000; Mondal et al., 2001; Cervera et al., 1998; SomLeva et al., 2002). 2 to 3 days co-cultivation has been routinely used in most transformation protocols, since longer co-cultivation causes bacterial overgrowth that covers the explant and causes toxicity under room temperature co-cultivation conditions.

- **Age of explant:** The age of explants was observed to have significant effect on shoot tip elongation. Some studies have shown that explant characteristics such as type, source, genotype and history affect the success and commercial viability of tissue culture systems (Bhau and Waklu, 2001; Chan and Chang, 2002; Hoy et al., 2003).

- **Temperature:** The effect of temperature during co-culture on T-DNA delivery was first studied in dicotyledonous species. A temperature of 22°C was found to be optimal for T-DNA delivery in tobacco leaves (Dillen et al., 1997). However, in another report, co culture at 25°C led to the highest number of transformed plants of tobacco, even though 19°C was optimal for T-DNA delivery (Salas et al., 2001). In monocots, co-culture temperature for most of the crops ranged from 24 to 25°C, and in some cases, 28°C was used for co-culture (Rashid et al., 1996; Arencibia et al., 1998; Enriquez-Obregon et al., 1999; Hashizume et al., 1999). The optimum co-culture temperature for *Brassica juncea* was 25°C while higher temperatures resulted in a very low number of transgenic plants (Zhang et al., 2006); in line with previous work suggesting that T-DNA transfer machinery works more efficiently under temperatures below 28ºC (Ditt et al., 2001). Higher transformation frequency was observed in maize immature embryo transformation from 20 to 23°C when using a standard binary vector (Frame et al., 2002). Therefore, it seems optimal temperature for stable transformation should be evaluated with each specific explants and *Agrobacterium* strain involved.

- **Formulation of phytohormones:** Different Concentrations and combinations of hormones (Auxin/Cytokinin) give different results in callusing of potato cultivars. A proper ratio of auxin and cytokinin is needed for early callusing. For early callusing and greater callus weight (callus induction potentiality) slightly more amount of auxins are required than cytokinin. Absence of either auxin leads to delayed callusing or no callusing (Biswas et al., 2010). Auxin alone or in combination with cytokinin is needed for callus profilation but 2, 4 D is most effective for callusing (Shirin et al., 2007) For healthy plantlets with well expanded leaves, moderate amount of cytokinins (1- 1.5 mg mL^{-1}) with very less auxins (0.1 mg mL^{-1}) should be used in shoot regeneration media (Hoque et al., 1996).

CHAPTER 2

REVIEW OF LITERATURE

Potato is a dicotyledonous plant and is an excellent recipient for *Agrobacterium* mediated gene transfer (Vain, 2007). *Agrobacterium*-mediated transformation has been widely used in potato improvement and potato was one of the first plant species that was transformed using *Agrobacterium tumefaciens* (An et al., 1986; Shahin and Simpson, 1986). *Agrobacterium* based DNA transfer system has advantages in plant transformation due to its simplicity, precision, integration of DNA sequence with defined ends, linked transfer of gene of interest along with the transformation marker, high frequency of stable transformation with many single copy insertions, and ability to transfer long stretches of T-DNA (Veluthambi et al., 2003). Over the years, different potato cultivars have been transformed and several transformation procedures have been published and developed that are based either on *Agrobacterium*-mediated transformation or biolistics. *Agrobacterium* has been successfully used for the transformation of leaves, petioles, stems (Trujillo et al*.*, 2001; Andersson et al*.*, 2003), microtubers (Ishida, Snyder and Belknap, 1989; Kumar A, 1995) and a technique of dipping the flower bolts (combination of flowers on a stem) in an *Agrobacterium* solution (Petti et al., 2005). There are only a few reports on potato transformation using the biolistic technique. Romano et al., 2001 reported on potato transformation of leaves, internodal explants and microtubers using particle bombardment. In this report Romano et al., 2001 claimed Agrobacterium would be the preferred technology for potato transformation as long as only a single gene trait is required. Particle bombardment can facilitate the integration and expression of several genes. Biolistic transformation can also be applied when large DNA fragments need to be transferred to potato (Ercolano et al*.*, 2004).
Currently the most widely used and favoured method for the production of transgenic plants is the *Agrobacterium*-mediated gene transfer system.

Genes of agronomical interest, for example disease resistance genes, can be cloned into plasmids between artificial T-DNA borders, which are introduced into an *Agrobacterium tumefaciens* strain already harbouring a Ti plasmid with a *vir* region but lacking the T-DNA. The plant expression vector is transferred from *Escherichia coli* into *Agrobacterium tumefaciens* via a triparental mating process. This process is mediated by helper plasmids which provide mobilisation *(mob)* and transfer *(tra)* functions (Walkerpeace and Velten, 1994).

2.1. *Agrobacterium tumefaciens*-mediated plant transformation

Shongstad et al., 1995 reported that there are three key components required to develop a plant transformation system. The first component consists of the identification of selectable markers, reporter genes and proper selection conditions to isolate transgenic events. The second is the development of a culture system where cells are efficiently converted to plants. The third component involves delivery of foreign DNA to plant cells in a manner that minimizes damage of plant cells.

An efficient system of gene transfer, selection and regeneration of transformed plants can be achieved by the use of tissue culture (Birch, 1997). During *Agrobacterium* transformation it is most important to obtain a large number of regenerable cells that are susceptible to the *Agrobacterium*-mediated gene transfer system, because such potentially regenerable cells may not recover after gene transfer and the capacity for efficient regeneration is short-lived. Prior to transformation, the plant tissue is cut from an *in vitro* plant which allows wounding and plant phenolics to be produced (Stachel et al., 1985). This is referred to as the pre-culture stage in *Agrobacterium*-mediated transformation. The pre-culture period is defined as the time from when the plant tissue is first isolated and cultured before the tissue is inoculated with *Agrobacterium*. During this phase the recipient cells undergo a change to become competent for regeneration (McHughen et al., 1989).

The phenolic compound, 4-acetyl-2, 6-dimethoxyphenol, commonly known as acetosyringone (AS) may be introduced during the pre-culture stage to induce expression of the *vir* genes and transfer from the bacterium to the plant cell (Stachel et al., 1985). After the pre-culture stage, the explants are co-cultivated in the presence of *Agrobacterium*. During co-cultivation gene transfer takes place from the bacterium to the explant. After this period the explants are transferred to callus or shoot inducing medium containing a selection agent to facilitate the selection and identification of modified cells.

Antibiotic resistance marker genes (ARMGs) can be used as selectable markers and the most widely used selection marker is the antibiotic kanamycin *nptII* gene (Bennett et al., 2004). Transformed cells can detoxify kanamycin and survive on medium containing the antibiotic.

Kanamycin kills or inhibits the growth of untransformed cells. One of the following antibiotics, cefotaxime or carbenicillin which eliminates *Agrobacterium* growth, is included in the callus and shoot inducing medium after the co-cultivation phase. The *Agrobacterium* has to be eliminated otherwise it will overgrow the plant culture and the explant culture can die (Borrelli et al., 1992). Callus produced from the explants on the selection medium will mainly consist of transformed cells. Shoots regenerated from these cells are rooted on medium containing the selection agent. The transgenic plants are screened for phenotypic abnormalities and subjected to molecular analysis, greenhouse trials and field trials to confirm stable transgene expression.

2.2. In vitro **regeneration system for potatoes**

The development of a rapid *in vitro* plant regeneration system is necessary for successful plant gene transformation (Birch, 1997). Shoot organogenesis and regeneration from potato plantlets can be divided into three phases. The first phase entails a short callus (undifferentiated cells) proliferation phase. Callus develops from the cut edges of explants stimulated by the addition of a cytokinin plant growth regulator to the growth medium. This phase is followed by shoot initiation from the callus by adding a cytokinin and removal of an auxin from the growth medium. The last regeneration phase is shoot development, which is stimulated by the addition of gibberellins (Hulme etal., 1992). Plant regeneration from potato is indirect and somaclonal variation can take place because of the callus phase and adventitious plantlet origin. Somaclonal variation can be limited by keeping the callus phase as short as possible (Bordallo et al., 2004).

Potato *in vitro* shoot organogenesis protocols published to date can be divided into three methods:

• One-step, where a single culture medium is used for all these phases (Cearley and Bolyard, 1997; Trujillo et al., 2001; Yee et al., 2001)

• two-steps, where callus initiation takes place on one medium and shoot formation and development on another (Wheeler et al., 1985; Wenzler et al., 1989; Yadav and Sticklen, 1995; Hansen et al., 1999)

• Three-steps, where different media are used for each of the three phases (De Block, 1988; Hulme et al., 1992)

Every potato genotype has its own medium requirements with regard to growth regulator composition and concentration and it is necessary to devise a regeneration protocol that works best for each genotype (Visser, 1991).

The frequency of *in vitro* shoot organogenesis of potatoes can be influenced by the type of explant. Foulger and Jones (1986) reported on genotype-dependent regeneration from protoplasts, Venter (1997) reported on the use of cell cultures from leaf explants while Jayasree et al., 2001 and Sharma and Millam (2004) reported on the development of somatic embryogenesis from leaf and internodal segments. The most frequent reports on the regeneration from explants are on the use of leaf tissue (DeBlock, 1988; Hulme et al., 1992, Park et al., 1995; Yadav and Sticklen, 1995; Trujillo et al., 2001).

For the most protocols, potato callus formation is promoted by the presence of a higher cytokinin than auxin concentration in the medium (Hulme et al., 1992). The cytokinin in the medium plays an essential role in cell division and the stimulation of shoot production in combination with auxins. Zeatin is a naturally-occurring cytokinin and it is widely used and highly active in tissue culture medium. The auxin in the medium plays a role in cell elongation, adventitious root formation and the inhibition of shoot formation (Gamborg, 1991). A two step regeneration method can be used for potato. In this method the explants are cultured for two to three weeks on medium containing auxins, cytokinins and gibberellins and then for a similar period on auxin free medium (Hulme et al., 1992). The gibberellins in the medium can influence growth and development in different ways for example increasing stem length (Gamborg, 1991).

The regeneration of *in vitro* potato explants can be inhibited by the accumulation of ethylene (Murray, 1995; Venter, 1997). Biosynthesis of ethylene is increased if the cells are subjected to stress conditions. After wounding of the plant tissue during transformation studies for the infection of plant cells by *Agrobacterium,* ethylene gas is released into the culture vessel (Perl, 1988). The concentration of the gas in the vessel varies according to the type and weight of tissue being grown, the method in which the vessel is sealed and the culture conditions of the explants. Several methods are known to inhibit and overcome ethylene build-up in the culture vessel (Davis et al., 1992). Silver thiosulphate (STS) is very effective in preventing ethylene build-up and is prepared with a molar ratio 1:4 between silver and thiosulphate. All the silver present in the solution is in the form of [Ag (S2O3)2]3. STS has a beneficial effect on the plantlet regeneration of potato explants (Higgins et al., 1992). The use of vented lids with a filter is also very useful. It permits pressure changes within the culture vessel and causes the ethylene to escape from the vessel. Inhibition by ethylene can also be overcome by culturing material in petri dishes sealed with tape that allows gas diffusion (Zobayed et al., 1999).

Beaujean et al., 1998 reported an efficient protocol for *Agrobacterium*-mediated transformation of internodal explants derived from three economically important *Solanum tuberosum* genotypes, namely Bintje, Desiree and Kaptahvandel. Short duration (7–8 weeks), high efficiency, genotype independence and low frequency of somaclonal variation make this system well suited for wider commercial applications of transgenic potato plants.

Trujillo et al., 2001 developed a one-step transformation protocol of two Andean potato cultivars. Leaf explants were transformed using vector pBI121 carrying the β-glucuronidase and kanamycin resistance genes. Calli and shoot formation occurred after 5–8 weeks on a selection medium containing 3 mgL^{-1} zeatin and 1 mgL^{-1} indole acetic acid. The shoot regeneration frequency was 28% and 34% for Diacol Capiro and Parda Pastusa respectively.

Shirin et al., 2007 developed a protocol for rapid callus induction and plant regeneration from the intermodal and leaf explants of four potato (*Solanum tuberosum* L.) cultivars *viz.* Diamant, Multa, Atlus and Lalpakri cultured for callus induction and plant regeneration. The explants were cultured onto MS media supplemented with different concentrations and combinations of 2, 4-D, NAA alone and NAA with BA for callus induction. The highest 80% internodal explants of Atlus induced to develop callus in medium containing 2, 4-D (3.0mgL^{-1}). Among all cultivars, the callusing response of both types of explants was best in 3.0mgL^{-1}, 2, 4-D containing MS medium. MS medium supplemented with different concentrations and combinations of BA, NAA and KIN were employed for shoot regeneration. MS medium containing 4.0 mg L^{-1} KIN + 0.5 mgL^{-1} NAA was the best for maximum shoot regeneration from the internode and leaf derived calli from most the cultivars.

Badr et al., 2008 worked with four potato (*Solanum tuberosum*) cultivars namely, Spunta, Nicola, Hermes and Lady Rosetta as explant sources for tissue culture and genetic transformation with GUS gene. The results revealed that the highest percentage of shoot formation obtained were for Spunta cv. Stem explants resulted in more callus and regeneration after transformation and selection than leaf explants. In all cases, the performance was genotype-dependent. RAPD analysis elucidated the induction of somaclonal variations, which were also found to be genotype-dependent. It occurs more frequently when new plants are formed via leaf explants, while, using stem as explants were genetically stable.

Hoque (2010) worked with the objective to develop a protocol for rapid plant regeneration in potato cultivars namely Diamont, Cardinal, Granulla, Ultra, Dheera and Provinto. Varietal response on *in vitro* regeneration under different hormonal concentrations and combinations were studied. Among the different treatments the combination 2.0 mg L^{-1} of KIN and IAA showed the best response to multiple shoot and root regeneration. The same concentration also took minimum time for regeneration.

Sarker et al., 2009 reported that precise incubation period facilitates significant response to *Agrobacterium* mediated genetic transformation of potato cultivars Atlas and Cardinal. The protocol yielded an average transformation rate of 58.4% for internodal explants compared to 38% for leaf explants in Atlas while 47.6% for intenodal explants compared to 27.6% for leaf explants in potato cultivar Cardinal. Highest survival rates and transient GUS activity was shown by Atlas and Cardinal at 45 min of incubation with *Agrobacterium*. The plants were analyzed histochemically for GUS activity in their leaves and internodes. All these analysis indicated that each independently selected and regenerated plant of cultivar Atlas and Cardinal was GUS positive and transient.

Cingel et al., 2010 woked with Dragaevka and Jelica, serbian potato cultivars. An efficient protocol for *Agrobacterium*-mediated transformation of Serbian potato cultivars Dragaevka and Jelica, enabling the introduction of oryzacystatin genes *OCI* and *OCII*, was established. Starting with leaf explants, a two-stage transformation protocol combining procedures of Webb and Wenzler provided high shoot regeneration efficiency: 84 - 89% for Dragaevka cultivar and 60 - 68% for Jelica cultivar as compared to 76 - 86% for Desiree.

Chakravarty et al., 2010 developed an efficient and rapid *Agrobacterium tumefaciens* mediated transformation protocol to generate activation-tagged mutant lines with the aim of large-scale functional analysis of the potato genome. Various parameters investigated to increase transformation efficiency were the type and age of explant, cultivar, hormone combinations, preculture of explants, period of co-cultivation with bacteria and concentration of bacterial cultures used for transformation. Stem explants from 5 week old plantlets of cv. Bintje which had undergone phytohormone pretreatment for 4 days, inoculation with diluted bacterial concentration of OD_{600} = 0.2 containing acetosyringone followed by 2 days of co-cultivation and selection in media with IAA and *trans*-zeatin all helped in greatly improving the transformation efficiency. The total time required from infection to rooted shoots was 6-7 weeks.

Mutasim et al., 2010 described procedure for plant regeneration from callus culture of potato, *Solanum tuberosum* L. Calli were induced from 1.0 cm^2 tuber segment of potato cultivar Almera on Murashige and Skoog's medium (MS) supplemented with different levels (1.0-5.0 mg L^{-1}) of 2, 4-dichlorophenoxy acetic acid (2, 4-D). The highest degree of callus formation (3.0) and hundred percent (100%) of explants produced nodular calli on MS medium within 7- 12 days when supplemented with 2.0-5.0 mg/l of 2, 4-D. Calli were differentiated into shoot-primordia when subcultured on MS medium supplemented with 1.5 -5.0 mg $L^{-1}$of thidiazuron (TDZ) and 2.0-5.0 mg $L^{-1}$of benzyladenine (BA). The best result for number of shoot per callus (3.3 ± 0.3) and longest shoot (0.8 ± 0.1) were obtained by using TDZ at 5.0 mg L^{-1}. Callus derived shoots were rooted most effectively in full-strength MS medium containing 1.0 mg L^{-1} IBA.

Borna et al., 2010 worked with nodal and internodal segments from three economically important potato (*Solanum tuberosum* L.) varieties namely, Diamant, Cardinal and Granola was conducted using an *Agrobacterium tumefaciens* strain LBA4404 harbouring binary plasmid pBI12 containing the GUS and *nptII* genes. Node and internodal segments were used for direct regeneration as well as regeneration with the intervention of callus. Best responses were obtained for direct regeneration of shoots when the explants were cultured on MS supplemented with 4.0 mg L^{-1} BAP +1.0 mg L^{-1} IAA, 1.5 mg L^{-1} BAP + 0.5 mg L^{-1} IAA and 5.0 mg L^{-1} BAP + 1.0 mg L^{-1} IAA in Diamant, Cardinal and Granola, respectively.

Shin et al., 2011 produced multiple stress-resistant plants containing the glutathione-S-transferase (Gh-5) auxin-regulated gene were produced having stress resistance protein through *Agrobacterium*-mediated transformation method with tested cultivars (Chubaek, Jasim, Jopoong, Namjak and Sumi). There was higher regeneration frequency from intermodal explants than from leaf explants.

Khatun et al., 2012 investigated the genetic transformation ability of two potato varieties namely Cardinal and Heera through *Agrobacterium*-mediated transformation. Leaf and internodes were used as explants. Expression of the transgene (GUS) was confirmed by histochemical analysis.

The variety Cardinal was found more suitable for expressing best GUS response (80% GUS positive) over Heera (Khatun et al., 2012).

Ahmad et al., 2012 studied the different factors affecting *Agrobacterium*-mediated transformation by using nodes as explant of potato cultivars Desiree and Sh-5. *Agrobacterium tumefacien*s strain LBA4404 harboring a pB1333-EN4-RCG3 plasmid having chitinase gene and selectable marker hygromycin gene under the control of the CaMV 35S promoter was used. The parameters optimized for the potato transformation includes co-cultivation time, cefotaxime concentration, and days to pre-selection. The results show that the infection time (2 min) gave best mean value of transformation efficiency, that is, 2.9 and 2.1 in Desiree and Sh-5, respectively. The over growth of *Agrobacterium* were controlled with a concentration of 200mgL^{-1} of cefotaxime and obtained maximum mean value of transformation efficiency of 3.38 and 3.10 in Desiree and Sh-5, respectively. The pre-selection period seven days prior to selection were considered effective for regeneration of explants and high transformation efficiency. The high mean value of regeneration (3.08 and 2.82) and transformation efficiency (3.00 and 2.60) was observed in Desiree and Sh-5, respectively.

ORIGIN OF THE PROBLEM

Although potato regeneration protocol was established in many laboratories in the world but still there are very less information regarding the comparative studies among the different varieties of potato as they vary with regard to over all genetic makeup, vegetative growth and maturation time. Transgenic approach is a powerful tool for making designer crops with useful trait(s). Therefore, assessing regeneration potential of the individual potato cultivars is an important prerequisite. In other words, the successful transformation depends on regeneration efficiency of the suitable explants. For this we need to adopt simple and efficient methods such as various media formulations during *Agrobacterium*-mediated co-cultivation and subsequent regeneration in the selective media. Till to date there are no comprehensive reports available in the literature with regard to regeneration efficiency of different popular Indian potato cultivars. Keeping in view with this, the objectives are framed in the next section.

OBJECTIVES OF THE PRESENT STUDY

- To establish virus-free potato germplasms through apical meristem culture
- Transformation of *Agrobacterium tumefaciens* with Ti-plasmid based vectors
- To optimize the various factors that affect the regeneration efficiency of the selected Indian potato cultivars during *Agrobacterium*-mediated genetic transformation

CHAPTER 3

MATERIALS AND METHODS

3.1. Materials:

3.1.1. Procurement of potato germplasm and other materials:

The germplasm of various potato cultivars such as Kufri Chipsona-1 (CS-1), Kufri Chipsona-2 (CS-2), KufriJyoti (KJ), were procured from Central Potato Research Institute (CPRI), Shimla, India and are routinely maintained in our laboratory on MS basal medium.

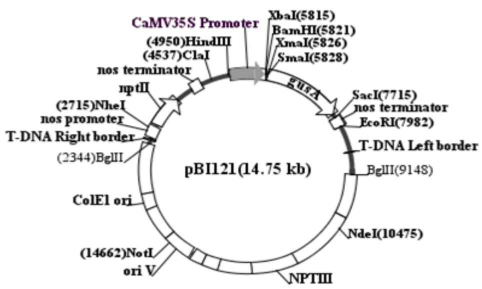
Cultivar	**Year of release**	**Salient features**	**Areas of adaptation**
KufriJyoti (KJ)	1968	Medium maturing; tuber white, large, oval with fleet eyes; resistant to wart; moderately resistant to early and late blight.	North Indian plains and hills, South Indian hills
KufriChipsona-1 (CS1)	1998	Medium maturing; tuber white, medium to large ,oval with fleet eyes; resistant to late blight, suitable for processing	North Indian plains
Kufri Chipsona-2 (CS2)	1998	Medium maturing; tuber white, medium round-oval with fleet eyes; resistant to late blight, tolerant to frost, suitable for processing	North Indian plains
Desiree(De)	1962	Late maturing; tuber medium oval-round red skinned tuber with fleet red eyes, low to medium resistance to late blight	Origin at Netherlands

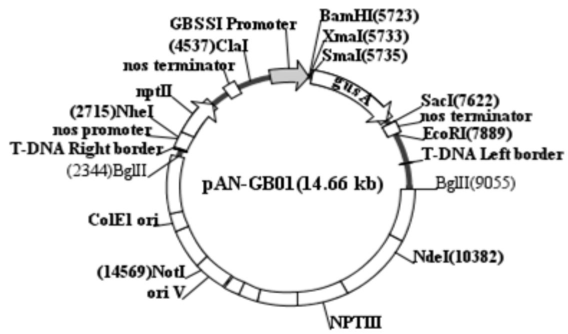
The required chemicals were purchased from Sisco Research Laboratory Pvt. Ltd. Mumbai, Qualigens Fine Chemicals, Merck, CDH Pvt. Ltd., New Delhi, and HiMedia Laboratories Mumbai. Various enzymes used were purchased from Bangalore Genei Pvt. Ltd., Bangalore and Amersham Biosciences Ltd., Hongkong. All salts and additives were purchased from

HiMediaLabs Limited, India and growth hormones from sigma chemicals, USA. Glasswares and Plasticwares were purchased from Borosil and Tarsons Products Pvt. Ltd.

3.1.2. Bacterial strains and plasmids

***E. coli* DH5α:** supE44 ΔlacU169 (Φ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 *rel*A1 *E.coli* DH5α strain was maintained on Luria agar medium, whereas, those transformed with pBI121(CaMV35S-GUS construct) and pAN-GB01(GBSS-GUS construct) plasmid were maintained on Luria agar medium containing 50 μg mL^{-1} of kanamycin.





***Agrobacterium tumefaciens* LBA4404 strain: LBA4404 (Ach5 pTiAch5) Sm/Sp(R) in the virulence plasmid (from Tn904); all T-DNA of pTiAch5 eliminated in pAL4404 (Hoekema et al., 1983).** LBA4404 strain was maintained on YEM medium containing rifampicin (15 μg mL^{-1}) and streptomycin (50 μg mL^{-1}). During triparental mating, *E. coli* pRK2013 and *Agrobacterium tumefaciens* LBA4404 strains were used as helper and recipient, respectively.

3.1.3. Various Primers used in the present study

CaMV 35S promoter-specific primers: Forward primer AF35-01, 5'-ATTCAAATAG AGGACCTAAC-3' corresponding to the bases 5291-5310, and reverse primer BR3502, 5’CCGTGTTCTCTCCAAATGA-3' complementary to the bases 5791-5809. The primers were designed based on the available pBI121 binary vector sequence in the database (GenBank Accession No. AF485783).

GBSSI gene-specific primers: The different primers were designed based on the available granule-bound starch synthase (*GBSSI*) gene sequence in the database (GenBank Accession No. X58453) corresponding to the wild type monoploid potato clone, AM79.7322. Forward primers:

GB1-F01, 5'-AATGCAACAGTATCTTGTAC-3' corresponding to the bases 54-73 and GB1-F02, 5'-AGACATAGGAATGTCAAGTG-3′ corresponds to the bases 514-533; reverse primers: GB1-R01, 5'-AAGAACATCACCTAGTCCAC-3' complementary to the bases 1363-1382 and corresponds to the N-terminus of mature peptide; and GB1-R02, 5'-CTTGTT GAGC TGTGT GAGTG-3' complementary to the bases 785-804 and consists of the predicted transcription start site (TSS) region.

3.1.4. Composition and Stock Preparations for Murashige and Skoog (MS) Basal Medium:

MS Major Salts:

S. No.	MS Major Salts	MS Basal conc. (mg L^{-1})	Amount required for 100X stock (g L^{-1})	Use of stock for 1 L medium (mL)
1.	KNO_3	1900.0	190.0	10.0
2.	NH_4NO_3	1650.0	165.0	10.0
3.	$MgSO_4.7H_20$	370.0	37.0	10.0
4.	$CaCl_2.2H_2O$	440.0	44.0	10.0
5.	KH_2PO_4	170.0	17.0	10.0

Note: All the MS major salts stock solutions to be prepared separately.

MS Minor Salts:

S. No.	MS Minor Salts	MS Basal conc. (mg L^{-1})	Amount required for 1000X stock (g L^{-1})	Use of stock for 1 L medium (mL)
1.	H_3BO_4	6.20	6.20	1.0
2.	$MnSO_4.4H_2O$	22.30	22.30	1.0
3.	$ZnSO_4.7H_2O$	8.60	8.60	1.0
4.	$Na_2MoO_4.2H_2O$	0.25	0.25	1.0
5.	$CuSO_4.5H_2O$	0.025	0.025	1.0
6.	$CoCl_2.6H_2O$	0.025	0.025	1.0
7.	KI	0.83	0.83	1.0

Note: The $Na_2MoO_4.2H_2O$ to be added first followed by the H_3BO_4 during preparation of Minor salts stock solutions.

MS Vitamins:

S. No.	Name of Vitamins	MS Basal Conc. (mg L^{-1})	Amount required for 1000X stock (mg mL^{-1})	Use of stock for 1 L medium (mL)
1.	Nicotinic Acid	0.5	0.5	1.0
2.	Pyridoxine HCl	0.5	0.5	1.0
3.	Thiamine HCl	0.1	0.1	1.0
4.	Glycine	2.0	2.0	1.0
5.	Myo-inositol	100.0	100.0	1.0

Note: All the MS vitamins stock solutions to be prepared separately.

S. No.	Name of Chemical	MS Basal Conc. (mg L^{-1})	Amount required for 1000X stock (mg mL^{-1})	Use of stock for 1 L medium (mL)
1.	$Fe_2EDTA.2H_2O$ (sodium salt)	30.0	30.0	1.0

Note: Preparation of MS basal medium included major salts, minor salts, vitamins, $Fe_2EDTA.2H_2O$, 3.0% sucrose, 0.7-0.8%. agaragar. The pH of medium was set to 5.8 using 0.01N HCl or 0.01N NaOH.

3.1.5. Various phytohormones:

Sr. No.	Phytohormons	Stock conc (mg mL^{-1})	Details of preparation
1.	Zeatin	2.5	Dissolve zeatin, in 0.1 N HCl, heat gently and made to the volume by adding sterile water. Adjust the pH to about 5.0.
2.	IAA	2.0	Dissolved auxin such as IAA (Indole acetic acid) 0.1 N KOH, stirred gently and made up the volume by adding distilled water.
3.	2,4-D	2.0	Dissolved 2,4-D (2,4-dichlorophenoxy- acetic acid) 0.1 N KOH, stirred gently and made up the volume by adding distilled water.
4.	Gibberellins (GA_3)	3.0	Dissolved GA_3 in 95% ethanol, stirred gently and made up to the volume by adding sterile water.

3.1.6. Various Antibiotics:

Sr. No.	Antibiotics	Stock conc. (mg mL^{-1})	Solvent used
1.	Ampicillin	50	Sterile water
2.	Kanamycin	70	Sterile water
3.	Rifampicin	15	Methanol
4.	Streptomycin	50	Sterile water
5.	Cefotaxime	250	Sterile water

3.1.7. Various media formulations:

Sr. No.	Media	Composition
1.	Luria Bertani (LB) Medium	0.5% (w/v) Yeast extract, 1.0% (w/v) Tryptone, 1.0% (w/v) NaCl, 1.5% (w/v) Agar-Agar Volume was made up by single distilled water and autoclaved.
2.	YEM Medium	0.4 g L^{-1} Yeast extract, 10.0 g L^{-1}Mannitol, 0.2 g L^{-1} $MgSO_4.7H_2O$, 0.5 g $L^{-1}$$K_2HPO_4$, 0.1 g L^{-1}NaCl, 1.5% Agar-Agar
3.	Callus inducing medium	MS medium, Zeatin (0.8 mg L^{-1}), 2, 4 D (2.0 mg L^{-1})
4.	Selective Callus inducing medium	MS medium, Zeatin (0.8 mg L^{-1}), 2, 4 D (2.0 mg L^{-1}),kanamycin (70 mg L^{-1}) and cefotaxime (200 mg L^{-1})
5.	ZIG medium	MS medium, Zeatin (2.0 mg L^{-1}), GA_3 (3.0 mg L^{-1}), IAA (0.1 mg L^{-1}), kanamycin (70 mg L^{-1}) and cefotaxime (200 mg L^{-1})
6.	Shoot inducing medium	MS medium, Zeatin (1.0 mg L^{-1}), GA_3 (2.0 mg L^{-1}), kanamycin (70 mg L^{-1}) and cefotaxime (200 mg L^{-1})
7.	Root inducing medium	MS medium, IAA (0.1 mg L^{-1}), kanamycin (70 mg L^{-1}) and cefotaxime (200 mg L^{-1})

Various Buffers:

Sr. No.	Buffers	Composition and preparation
1.	0.5 M Tris-HCl (pH 8.0)	For 100 mL stock, 6.05 g of Tris base was dissolved in 50 mL water and pH was adjusted with 6.0 N HCl and made up volume with water and autoclaved
2.	0.5 M EDTA (pH 8.0)	For 100 mL stock, 18.6 g of sodium salt of EDTA was dissolved in 50 mL water and pH was adjusted with concentrated NaOH and made up volume with water and autoclaved
3.	3.0 M Sodium acetate (pH 5.5)	For 50 mL stock, 12.3 g of Sodium acetate was dissolved in water and adjust the pH with glacial acetic acid and final volume 50 mL
4.	0.5 M Sodium acetate (pH 4.7)	For 50 mL stock, 2.05 g of Sodium acetate was dissolved in water and adjust the pH with glacial acetic acid and final volume 50 mL.
6.	5 M Potassium acetate	49 g of potassium acetate was dissolved in water and made final volume 100 mL and autoclave
7.	3 M Potassium acetate (pH4.8)	29.4 g of potassium acetate was dissolved in water and 11.5 mL of glacial acetate was added and made final volume 100 mL and autoclave
8.	0.5 M Sucrose	17.1 g of sucrosewas dissolved in sterile water and final volume 100 mL
9.	The buffer for plasmid isolation by boiling method (STET)	8.0 % (w/v) Sucrose, 0.5 % (w/v) Triton X 100, 50 mM EDTA (pH 8.0), 10 mM Tris-HCl (pH 8.0) Volume was made up by double distilled water and autoclaved.
10.	STE Buffer	0.3 M NaCl, 50 mM Tris-HCl (pH 8.0), 5 mM EDTA (pH 8.0)
11.	Saline EDTA	0.15 M Sodium chloride, 0.1 M EDTA (pH 8.0)

12.	TE Buffer (1X)	10.0 mMTris-HCl (pH 8.0), 1.0 mM EDTA (pH 8.0) Volume was made up whit distilled water and autoclaved
13.	TBE Buffer (5X)	54 g L^{-1}Tris base, 28 g L^{-1} Boric acid, 3.8 g L^{-1} EDTA The pH of the buffer was set at 8.0 Volume was made up with distilled water and autoclaved
14.	DNA Gel Loading Buffer (5X)	35 % (w/v) Sucrose or 40% glycerol, 20.0 mM EDTA (pH 8.0), 0.1 % (w/v) Bromophenol blue Volume was made up with sterile water.
15.	DNA extraction buffer	50 mM Tris-HCl pH 8.0, 50 mM EDTA (pH 8.0), 250 mM NaCl, 15% sucrose

3.1.8. Description of different Enzymes:

Sr. No.	Various enzymes	Stock conc.	Working conc.	Other relevant details
1.	Lysozyme	10 mg mL^{-1}	300-400 µg mL^{-1}	Sterile water was used for stock preparation
2.	Ribonuclease A	10 mg mL^{-1}	10-20 µg mL^{-1}	Dissolved in 10 mM Tris-HCl (pH 8.0) and 15 mM NaCl. buffer and kept in boiling water bath for 10 min followed by slow cooling to room temperature and stored at -20 ºC for subsequent use
5.	*Taq*DNA Polymerase	3U μL^{-1}	3 U μL^{-1}	

3.2. METHODOLOGY

3.2.1. Establishment of potato germplasm

The high-yielding Indian potato cultivars namely Kufri Chipsona-1, Kufri Chipsona-2 and Kufri Jyoti as used in this study were procured from Central Potato Research Institute (CPRI), Shimla, India. These cultivars vary with regard to their genetic makeup, maturation time and growth in different agro-climatic zones of the Indian subcontinent. All the cultivars early are medium maturing. All these cultivars along with Desiree (a late maturing exotic cultivar) were routinely micropropagated in our laboratory under controlled conditions (16 h light/8 h dark, 25-27ºC, 70% relative humidity) for four to five weeks on MS basal medium. The aseptically grown micropropagated potato plantlets of the above cultivars were used *Agrobacterium*-mediated genetic transformation.

3.2.2. Genetic transformation of *Agrobacterium* strain

Transformation by triparental mating: In triparental mating, the donor strain (*E. coli* harboring Ti plasmid with gene of interest) was mated with conjugal helper strain (*E. coli* harboring broad host range plasmid pRK2013) and a recipient *Agrobacterium* strain (harboring *vir* plasmid). The Ti plasmid in *E. coli* was mobilized to recipient *Agrobacterium* strain due to mobilization function of pRK2013 (broad host range plasmid). After mating, *A. tumefaciens* strains harboring the engineered plant transformation vector (Ti plasmid with gene of interest) were selected by growth in the presence of antibiotics for which resistance is provided by genetic markers unique to those recipient *Agrobacterium* and Ti plasmid vector (Ti plasmid with gene of interest). The steps involved were: the recipient *Agrobacterium tumefaciens* strain LBA4404 was grown on Luria agar medium containing rifampicin (15 µg mL^{-1}) at 28ºC. The donor *E. coli* strain harboring engineered Ti plasmid and conjugal helper *E. coli* strain (pRK2013) were grown on Luria agar medium containing kanamycin (50 µg mL^{-1}) at 37ºC. A single colony of each freshly grown strain was patched separately close to each other on Luria agar plates. The three patches were mixed with sterile loop and the plates were incubated at 28ºC for 24 hrs. The small portion of triparental patch was picked with the help of loop and serially diluted in 0.9% saline and 100 µL of it was spread on Luria agar containing antibiotics rifampicin (15 µg mL^{-1}) and kanamycin (50 µg mL^{-1}) and incubated at 28ºC. Single colonies of transformed *Agrobacterium* were streaked on YEM medium containing rifampicin (15 μg mL^{-1}) and kanamycin (50 μg mL^{-1}) for further use.

3.2.3. Isolation of plasmid DNA by boiling method

Plasmid isolation in mini scale was carried out by boiling prep method as described by Holmes and Quigley (1981). In this process bacterial transformant colonies were inoculated aseptically in 4.5 mL LB containing ampicillin in test tubes. The culture was incubated at 37°C/120 rpm for overnight. Cells were harvested from 1.5 mL overnight grown culture in microfuge tubes. The pellet was loosened by vortexing, followed by resuspension in 800 μL of STET buffer. 30 μL of lysozyme was added to the bacterial suspension and mixed well. Each microfuge tube containing cell suspension was kept in boiling water bath for 1.5 min. After cooling down to room temperature high speed centrifugation (12,000 rpm) was carried out for 15 min. After removing the pellet, 2.0 μL of RNase solution was added to the supernatant to remove the contaminating RNA. After incubation at 37°C for 45 min equal volume of phenol: chloroform was added, mixed for 5-7 min and centrifugation was performed at 10,000 rpm for 10 min. To the upper aqueous layer, $1/10^{th}$ volume of 3M sodium acetate (CH_3COONa) and equal volume of isopropanol was added and incubated at 4°C for 45 min for the precipitation of plasmid DNA. Then the tubes were centrifuged at 12,000 rpm for 15 min. The DNA pellet was washed with chilled 70 % ethanol to ensure the removal of excess salts and other impurities. Finally, DNA pellet was air dried at room temperature and dissolved in 20-30 μL of TE buffer.

3.2.4. Analysis of isolated plasmids through agarose gel electrophoresis

The integrity of isolated plasmids pBI121 (CaMV35S-GUS construct) and pAN-GB01 (GBSS-GUS construct) was checked with the help of Agaroge gel electrophoresis.

Preparation of 100 mL of 0.8% agarose gel: 0.8 g of agarose in 100 mL of 0.5 X TBE was boiled and cooled up to 55ºC. 10 μL of ethidium bromide (0.5 μg mL^{-1}) was added in the gel and was casted in gel casting apparatus and was allowed to set for one hour at room temperature. 0.8% agarose gel is suitable for resolving DNA in the 0.5-8.0 kb size range. The gel was submerged in the 0.5 X TBE electrophoresis buffer. The mixture of plasmid samples (2μL), 5X DNA gel loading buffer (2μL) and sterile double distilled water (6μL) was loaded in the gel and electrophoresis was carried out at 4-5 V cm^{-1} until the bromophenol blue was migrated to the end.

3.2.5. Further analysis of cloned insert through PCR

PCR is iterative process, consisting of three cycling parameters, heat denaturation of DNA template, annealing of oligonucleotide primers to single stranded DNA templates, and extension of the annealed primers by a thermostable DNA polymerase. The composition for a typical 50 μL PCR reaction was set as follows: 5 μL of 10X PCR buffer, 0.5-1.0 μg Template DNA, 10 pmoles forward primer, 10 pmoles reverse primer, 2.5 μL of 2.5 mM dNTP mix, 1.0 μL (1U μL^{-1}) Taq DNA polymerase and finally the volume was made up to 50 μL with sterile water. After initial denaturation at 94ºC for 1 min 30 s, the thermal cycling parameters were: denaturation at 94ºC for 1 min, annealing at 55ºC or 50ºC for 2 min; polymerization at 72ºC for 2 min or 1 min for 30 cycles followed by final extension at 72ºC for 5 min.

3.2.6. Agrobacterium-mediated transformation of potato plants

The well characterized transformed *Agrobacterium* strain (corresponding to individual genetic constructs) was used for co cultivation. The single colony of transformant strain was grown in YEM broth up to required O.D.Inter-nodal stem segments of potato plantlets (Kufri Chipsona-1, Kufri Chipsona-2, KufriJyoti and Desiree) of required age, grown in MS medium, were co-cultivated with the culture for particular time period, blot the internodal stem segments on sterile filter paper, placed horizontally on MS basal medium and incubated in dark for 48 hrs in growth room. Internodal stem segments were washed in cefotaxime (200 mg L^{-1}) and shifted to the required selective medium for regeneration and primary selection of the transgenic potato lines. The initial shoots were transferred to the rooting media to obtain complete potato plantlets.

3.2.7. Factors taken in to consideration in the present study

- Type of cultivar: Kufri Jyoti, Kufri Chipsona-1, Kufri Chipsona-2, Desiree (exotic variety)
- Age of explant: 30 and 45 days
- Bacterial concentration: OD_{660}= 0.2,0.4 and 0.6
- Period of co-cultivation with bacteria: 20 min,30 min,45 min
- Binary vectors used :

  pBI121 (CaMV35S-GUS construct)

  pAN-GB01 (GBSS-GUS construct)
- Type of regeneration media:

  Callus inducing media

  Zeatin based media (ZIG medium)

CHAPTER 4

RESULTS AND DISCUSSION

The present study aimed in the studies on regeneration through direct and indirect regeneration from internodal stem segments of the micro plants of some selected Indian potato cultivars. The various work components of this study are presented in the following sections:

4.1. Establishment of potato germplasms

The potato germplasms Kufri Chipsona 1, Kufri Chipsona 2, Kufri Jyoti and Desiree were routinely subcultured on MS-Basal medium by using nodal segments under controlled conditions (16 h light/8 h dark, 25–27^{0}C, 70% relative humidity) for 4–5 weeks on MS-Basal medium. The subcultured potato plantlets of the cultivars are shown in Figure 1.

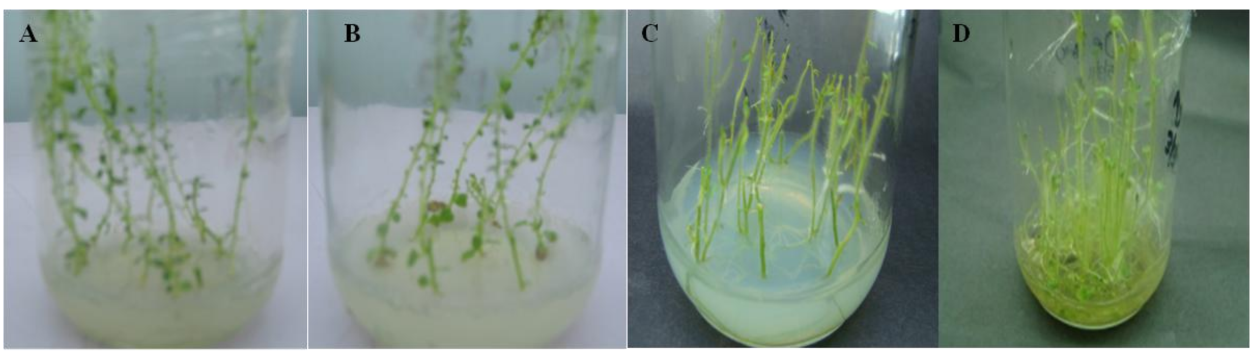


Fig. 1. Potato cultivars subcultured on solid MS media. A) Kufri Chipsona 1; B) Kufri Chipsona 2; C) Kufri Jyoti; D) Desiree

4.2. PCR Characterization of *Agrobacterium* based *Ti* plasmids

The *Agrobacterium* based *Ti*-plasmids (pBI121 and pAN-02) were mobilized from *E. coli* DH5α to *Agrobacterium tumefaciens* LBA4404 with the help of helper strain *E. coli* DH5α (pRK2013) through Triparental mating. The *Agrobacterium* transformants harbouring *Ti*-plasmids were isolated and characterized through PCR by using their respective promoter specific primer pairs as shown in Figure 2A and 2B.

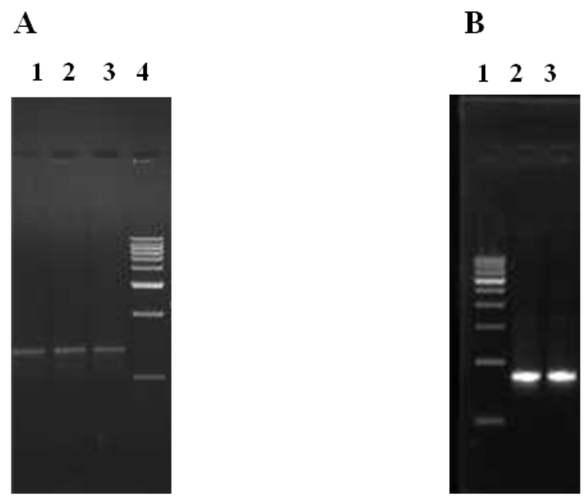


Fig. 2. PCR Characterization of *Ti*-plasmid based vectors A) Lanes, 1, 2 and 3, amplified promoter region of pBI121 (CaMV 35S-GUS construct); Lanes 4, 0.5 kb DNA ladder; B) Lane 1, 0.5 kb DNA ladder; Lanes 2 and 3, amplified promoter region of pAN-GB01 (GBSS*I*-GUS construct)

4.3. *Agrobacterium*-mediated genetic transformation of potato plants

The *Agrobacterium* harbouring *Ti*-plasmids (pBI121 and pAN-02) were co-cultivated with intermodal explants of different potato cultivars to study the regeneration efficiency of the cultivars by varying different parameters like age of the explants, bacterial concentration, co-cultivation period and hormonal combinations of the media components. The results corresponding to each parameter are discussed in the subsequent sections.

4.3.1. Effect of bacterial concentration

Inorder to study the effect of bacterial concentration on regeneration of the potato cultivars, different concentrations of bacterial cells i.e. OD_{600}= 0.2, 0.4 and 0.6 were taken for the co-cultivation of internodal explants. At OD_{600}= 0.2, the callusing percentage is more and the time taken for callusing is less as compared to OD_{600}= 0.4 and 0.6. The callusing percentage at OD_{600}= 0.2 is more in the case of CS2 followed by CS1, De and KJ respectively as shown in Table 1. On shoot regeneration media, the time taken for regeneration is less for all the cultivars at OD_{600}= 0.2 when compared to OD_{600}= 0.4 and 0.6. The number of shoots regenerated per explant are more in the case of CS2 followed by De, KJ and CS1 at OD_{600}= 0.2. However, at

OD_{600}= 0.4 and 0.6, there was no much difference with regard to callusing percentage on CIM and shoot regeneration on ZIG media. From the results, it is quite evident that the cell-density influences the transformation efficiency. Although, prolonging the incubation period usually yields more efficient T-DNA delivery, but higher cell damage causes necrosis resulting in the death of the tissue. Thus, diluting the bacterial suspension to OD600 = 0.2 significantly increased the transformation rate which confirms the previous findings (Li et al., 2003; Wang-Pruski et al., 2002; Zaragoza et al., 2004).

Diluting the bacterial concentration also reduced the number of explants with overgrowth of bacteria thus increasing the viability period of the explants on regeneration media.

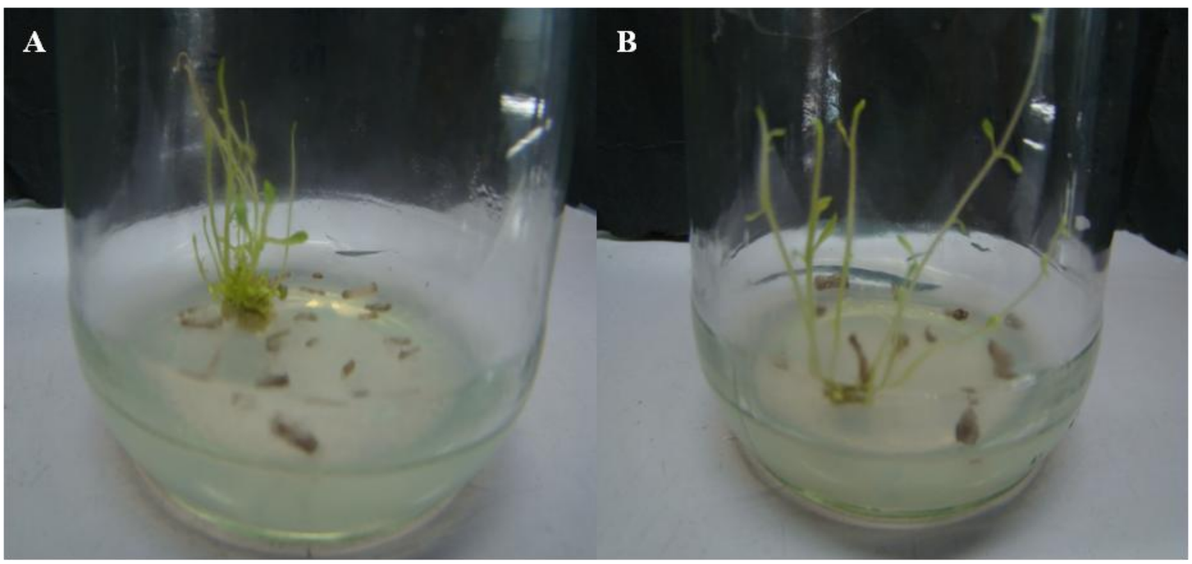


Fig. 2. Effect of bacterial concentration (OD_{600nm}=0.2) on multiple shoot regeneration per explants on ZIG media A) cv. CS-2; B) cv. De

Cultivar	Plasmid Vector	Hormonal combination	Bacterial Cell Concentration		
			A_{260} = 0.2	A_{260} = 0.4	A_{260} = 0.6
CS-1	pBI-121	Callusing % (CIM)	++	+	+
		Direct shoot regeneration(ZIG)	+	++	++
	pAN-GB01	Callusing % (CIM)	+++	-	+
		Direct shoot regeneration(ZIG)	+	+	++

CS-2	pBI-121	Callusing % (CIM)	+++	++	+
		Direct shoot regeneration(ZIG)	+	-	+
	pAN-GB01	Callusing % (CIM)	++	++	+
		Direct shoot regeneration(ZIG)	+++	+	-
KJ	pBI-121	Callusing % (CIM)	+	+	++
		Direct shoot regeneration(ZIG)	+	-	+
	pAN-GB01	Callusing % (CIM)	++	+	+
		Direct shoot regeneration(ZIG)	++	+	-
De	pBI-121	Callusing % (CIM)	+	+	+
		Direct shoot regeneration(ZIG)	++	+	-`
	pAN-GB01	Callusing % (CIM)	+	++	++
		Direct shoot regeneration(ZIG)	+	-	-

Table 1. Effect of bacterial cell density on regenereation of the potato cultivars
+ = less response; ++ = medium response; +++ = higher response; - = poor response

4.3.2. Effect of age of explants

The effect of age of explants (30 and 45 days) on the regeneration efficiency of Indian potato cultivars are presented in Table 2. Observations suggest that the percentage callusing is more in case of 45 days old explants in all the four cultivars irrespective of the type of vector used whereas age of explants has no direct effect on direct shoot regeneration in case of KJ and De. But, in the case of CS1 at OD_{600}=0.2, both 30 and 45 days old culture responded equally on shoot regeneration media, where as at OD_{600}=0.4 and 0.6, 30 days culture responded better , for CS2, OD_{600}=0.2 and 0.4, 45 days culture responded better. From the data we can conclude that direct shoot regeneration is dependent on OD but not on the age of explants.

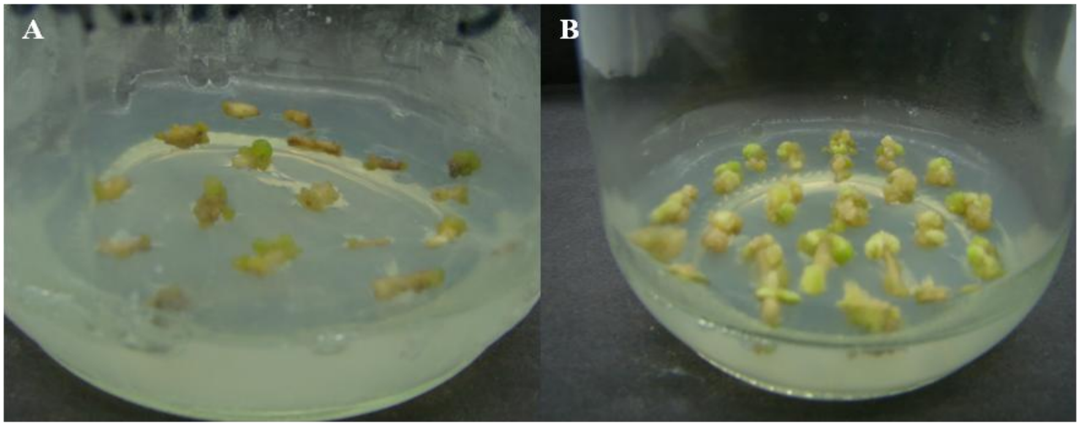


Fig. 3. Effect of age of explants on callusing percentage in the cv. Kufri Jyoti A) 30 days old explants; B) 45 days old explants

Cultivar	Plasmid Vector	Hormonal combination	Age Of Explant	
			30 days	45 days
Cs – I	pBI-121	Callusing % (CIM)	+	+++
		Direct shoot regeneration(ZIG)	+	+
	pAN-GB01	Callusing % (CIM)	+	+++
		Direct shoot regeneration(ZIG)	++	+
Cs – II	pBI-121	Callusing % (CIM)	+	++
		Direct shoot regeneration(ZIG)	+	+
	pAN-GB01	Callusing % (CIM)	+	+++
		Direct shoot regeneration(ZIG)	-	++
KJ	pBI-121	Callusing % (CIM)	+	++
		Direct shoot regeneration(ZIG)	+	+
	pAN-GB01	Callusing % (CIM)	+	+++
		Direct shoot regeneration(ZIG)	+	+

De	pBI-121	Callusing % (CIM)	+	+
		Direct shoot regeneration(ZIG)	+	++
	pAN-GB01	Callusing % (CIM)	+	+++
		Direct shoot regeneration(ZIG)	+	+

Table 2. Effect of age of explant on regenereation of the potato cultivars

+ = less response; ++ = medium response; +++ = higher response; - = poor response

4.3.3. Effect of co-cultivation period

The effect of co-cultivation time period on regeneration was studied at different time periods i.e. 20 min, 30 min and 45 min respectively. At OD_{600nm}=0.2, during 20 min of co-cultivation, the cvs. CS-2 and De responded better in terms of shot regeneration where as the cvs. CS-1 and KJ showed efficient regeneration at 30 min. This clearly suggests that the time period required for co-cultivation differs from cultivar to cultivar for efficient regeneration.

Cultivar	Plasmid Vector	Hormonal combination	Period of Co- cultivation with bacteria		
			20 min	30min	45min
CS-1	pBI-121	Callusing % (CIM)	+	+	+
		Direct shoot regeneration(ZIG)	+	++	++
	pAN-GB01	Callusing % (CIM)	+	++	++
		Direct shoot regeneration(ZIG)	-	+	+
CS-2	pBI-121	Callusing % (CIM)	++	++	+
		Direct shoot regeneration(ZIG)	++	-	-
	pAN-GB01	Callusing % (CIM)	+	++	++
		Direct shoot regeneration(ZIG)	-	+	+

KJ	pBI-121	Callusing % (CIM)	+	++	+++
		Direct shoot regeneration(ZIG)	+	+	-
	pAN-GB01	Callusing % (CIM)	-	++	++
		Direct shoot regeneration(ZIG)	-	+	+
De	pBI-121	Callusing % (CIM)	++	++	++
		Direct shoot regeneration(ZIG)	++	+	-
	pAN-GB01	Callusing % (CIM)	+	++	+++
		Direct shoot regeneration(ZIG)	+	-	-

Table 3. Effect of co-cultivation time period on regenereation of the potato cultivars

+ = less response; ++ = medium response; +++ = higher response; - = poor response

4.3.4. Effect of phytohormonal formulations

In order to study the effect of hormonal combination, different concentrations of phytohormones (auxins/cytokinins) were added in the regeneration media CIM and ZIG. For early callusing and greater callus weight (callus induction potentiality), slightly more amount of auxins are required than cytokinins (Biswas et al., 2010). The percentage callusing of the explants on CIM media is in the order CS-2 > CS-1 > De > KJ. But, the shoot regeneration from the callus is more in the case of CS-2 followed by KJ. In other words, shoot regeneration from the callus was found to be poor in the case of the cvs. CS-1 and De. The explants of potato cultivars CS-2 and KJ showed efficient regeneration on CIM media, where as the potato cvs. CS-1 and De showed better response on ZIG media. This clearly implicates the difference in the hormonal requirement of the potato cultivars required for regeneration and reflects the genotypic response.

Cultivar	Plasmid Vector	Hormonal Combination	
		CIM	ZIG
CS-1	pBI-121	+	+++
	pAN-GB01	+	+++

CS-2	pBI-121	+++	+
	pAN-GB01	+++	+
KJ	pBI-121	+++	-
	pAN-GB01	+++	-
De	pBI-121	+	+++
	pAN-GB01	+	+++

Table 4. Effect of phytohormonal combinations on regenereation of the potato cultivars

+ = less response; ++ = medium response; +++ = higher response; - = poor response

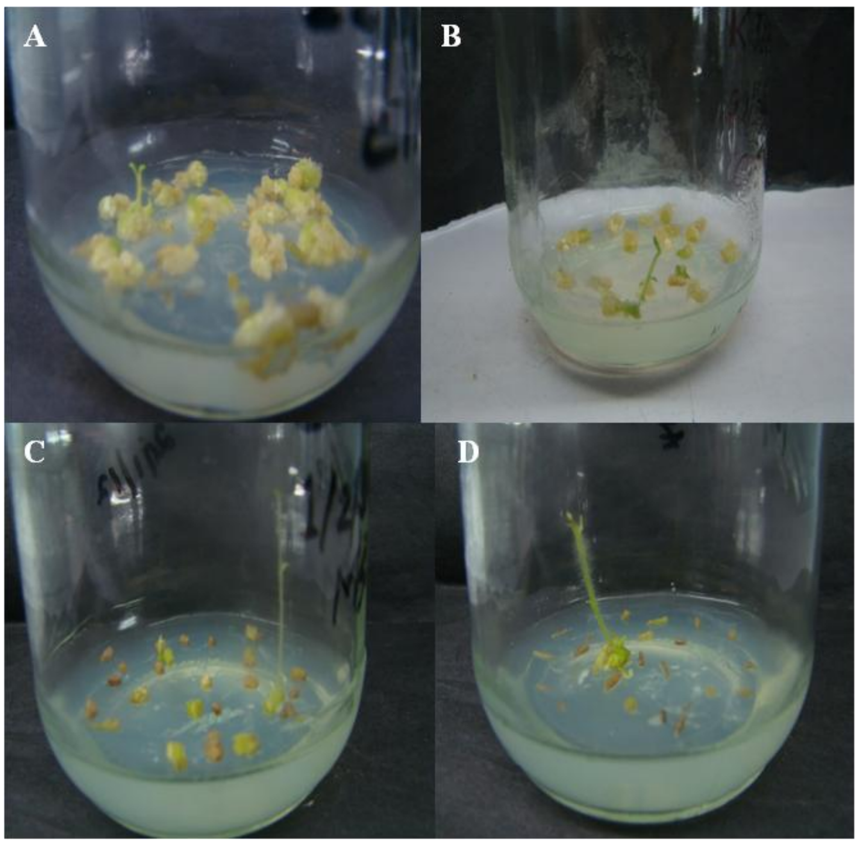


Fig. 5. Effect of hormonal combinations on direct and indirect shoot regeneration (A) & (B) indirect shoot regeneration from explants of cv. CS-2 and KJ on CIM media; (C) & (D) direct shoot regeneration from explants of cv. CS-1 and De on ZIG media

CONCLUSIONS

The regeneration efficiency in potato is believed to be genotype dependent. Therefore, each and every potato cultivar needs separate attention to optimize the various parameters during *Agrobacterium*-mediated co-cultivation of the explants and subsequent regeneration. Some of the important parameters include bacterial cell density, age of the explants, time period of co-cultivation, combination of phytohormone in the regeneration media. In this study, efforts were made to study the effect of various parameters on regeneration of some popular Indian potato cultivars during *Agrobacterium*-mediated genetic transformation. At bacterial cell density of OD_{600}= 0.2, the regeneration efficiency was found to be effective for all the potato cultivars under study as compared to higher cell densities as used during co-cultivation. The explants corresponding to the cultivars Kufri Chipsona I and Desiree showed efficient regeneration on zeatin-based media, whereas the cultivars Kufri Chipsona 2 and Kufri Jyoti performed better after passage through callus inducing medium (CIM) for a period of around two weeks (however, to avoid somaclonal variations a minimum intervening callus phase was allowed prior to shoot regeneration). In CIM, it was noted that the explants of 45 days old culture showed more efficient regeneration as compared to the response of explants from 30 days old plantlets. 20 min duration of co-cultivation of the explants with *Agrobacterium* was found to be optimum in case of CIM formulation, whereas for zeatin-based media the duration of co-cultivation was around 30 min. for optimium regeneration efficiency. All these observations together will provide clues to ensure efficient regeneration of the individual potato cultivars during transgenic approaches.

Chapter 5

Summary

The present study was focused to study the effect of various parameters on potato regeneration in some selected Indian potato cultivars namely Kufri Chipsona-1, Kufri Chipsona-2, Kufri Jyoti along with an exotic cultivar Desiree. These cultivars vary with regard to the maturation period, genetic makeup and adoptability to different agro-climatic zones of the Indian subcontinent.

- The germplasms of the cultivars under study were routinely subcultured on MS Basal medium and maintained in our laboratory under controlled conditions.
- The *Agrobacterium*-based *Ti*-plasmid vectors (pBI121 and pAN-GB01) were mobilized to *Agrobacterium tumefaciens* LBA4404 through triparental mating. The plasmid DNA was isolated from the *Agrobacterium* transformants and the orientation of the different components of T-DNA of the vectors were further checked by PCR approach.
- The *Agrobacterium* strain harbouring Ti-plasmid based vectors was co-cultivated with the explants of the individual potato cultivars under varying parameters such as, bacterial cell density, age of the explants, time period of co-cultivation, phytohormone formulations to see their effects on overall regeneration efficiency.
- The regeneration response of the individual potato cultivars was noted carefully. The salient observations of this study include: At bacterial cell density of OD_{600}= 0.2, the regeneration efficiency was found to be effective for all the potato cultivars under study as compared to higher cell densities as used during co-cultivation. The explants corresponding to the cultivars Kufri Chipsona I and Desiree showed efficient regeneration on zeatin-based media, whereas the cultivars Kufri Chipsona 2 and Kufri Jyoti performed better after passage through callus inducing medium (CIM) for a period of around two weeks (however, to avoid somaclonal variations a minimum intervening callus phase was allowed prior to shoot regeneration). In CIM, it was noted that the explants of 45 days old culture showed more efficient regeneration as compared to the response of explants from 30 days old plantlets. 20 min duration of co-cultivation of the explants with *Agrobacterium* was found to be optimum in case of CIM formulation, whereas for zeatin-based media the duration of co-cultivation was around 30 min. for optimium regeneration efficiency. All these observations together will provide clues to ensure efficient regeneration of the individual potato cultivars during transgenic approaches.

CHAPTER 6

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