Factors affecting *Agrobacterium tumefaciens* mediated genetic transformation of Indian potato cultivar 'Kufri Pukhraj'

Dissertation

Submitted in the partial fulfillment of the requirement for

the award of the degree for

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IN

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By

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CANDIDATE'S DECLARATION

I, hereby declare that the work presented in the thesis entitled – **Factors affecting *Agrobacterium tumefaciens* mediated genetic transformation of Indian potato cultivar 'Kufri Pukhraj',** in the partial fulfillment of the requirement for the award of the degree for Master of Technology in Biotechnology, Department of Biotechnology, Thapar University, Patiala, is an authentic record of my work during the period of one year from July 2015 to July 2016, under the guidance of Dr. Anil Kumar, Assistant Professor, Thapar University, Patiala. I have not submitted the matter embodied in this thesis for the award of any other degree or diploma.

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CERTIFICATE

This is to certify that the thesis entitled – **Factors affecting *Agrobacterium tumefaciens* mediated genetic transformation of Indian potato cultivar 'Kufri Pukhraj'** submitted by Anuja Gautam in partial fulfillment of the requirement for the award of Degree for Master of Technology in Biotechnology from Thapar University, Patiala, is a record of student's own work carried out by her. The report has not been submitted for the award of any other degree or certificate in this or any other University or Institute.

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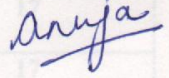

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CONTENTS

ABSTRACT

Factors affecting *Agrobacterium tumefaciens* mediated genetic transformation of Indian potato cultivar '**Kufri Pukhraj'** were studied. *A. tumefaciens* strain EHA105 was found to be more efficient in inducing transient activity in leaf explants whereas strain LBA4404 yielded best results in internodal explants. Transient expression was found to be higher in internodal explants than leaf explants. Explants (both leaves and internodes) precultured for two days, infected for fifteen minutes and co-cultivated for two days yielded maximum transient Gus expression (85%). Prolonged co-cultivation period resulted in bacterial overgrowth and ultimately death of explants. Stable transformation was also observed from both leaf and internodal explants on selection cum regeneration medium. Results indicate that the extended co-cultivation, pre-culture days and reduced infection time results in lower transient expression. Optimized factors for transformation system can be used for genetic insertions in Indian potato cultivars.

Keywords: Potato, transformation, *Agrobacterium tumefaciens, nptII* gene, leaf explants, internode explants, Gus histochemical assay.

LIST OF ABBREVIATIONS

MS	Murashige and Skoog medium
BA	6-Benzylaminopurine
GA_3	Gibberellic acid
PM	Preculture medium
CoM	Co-Cultivation Medium
SRM	Selection cum Regeneration medium
nptII	Phosphotransferase II
X-gluc	5-bromo-4-chloro-3-indolyl glucuronide
NH_4NO_3	Ammonium nitrate
KNO_3	Potassium nitrate
$CaCl_2.2H_2O$	Calcium chloride
$MgSO_4.7H_2O$	Magnesium sulphate
KH_2PO_4	Potassium di - hydrogen phosphate
$MnSO_4.H_2O$	Manganese sulfate
$ZnSO_4.7H_2O$	Zinc sulphate
H_3BO_3	Boric Acid
KI	Potassium iodide
$Na_2MoO_4.2H_2O$	Sodium molybdate
$CuSO_4.5H_2O$	Copper sulfate
$CoCl_2.6H_2O$	Cobalt chloride
Gus	β-glucuronidase

˚C	degree Celcius
mg	milligram
µl	microlitre
ml	millilitre
l	litre
HCL	Hydro Chloric acid
EDTA	Ethylene diamine tetra acetic acid
g	grams
w/v	Weight by volume
PCR	Polymerase chain reaction

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Chapter 1: INTRODUCTION

Potato (*Solanum tubersosum* L.) belongs to the family *Solanaceae* (Nightshade) and the genus Solanum which contains about 2000 species. The genus has been divided into sub genuses, *Pachystemonum* and *Leptostemonum.* According to the latest classification *Pachystemonum* has further been divided into sub-genus *Tuberarium* which includes tuber crops such as potato (Thamburaj and Singh, 2001). Potato is considered to be one of the fourth most important food crop grown worldwide (Dahiya and Sharma, 1994) and India is the second largest producer of potato in the world. The annual production of potato has reached about 300 million tons (Birch *et al.,* 2012). Potato tubers are important dietary source of proteins, vitamins, antioxidants and starch, which helps the plant to grow as a vegetative propagation system as well as a storage organ (Barrrell *et al.,* 2013). The major storage protein in potato is patatin which is one of the most nutritional balanced plant proteins (Liedl *et al.,* 1987). Further, the presence of starch in potato makes the crop industrially important.

Solanum tuberosum is seldomly seen to exist as a wild species (Simon *et al.,* 2010) and is grown in cooler climates during winters, spring season and autumn with elevations typically over 1000 meters (Hijmans, 2001). *S. tuberosum* cannot tolerate frost and dies at temperatures of 3°C or below (Li, 1977). It grows in a variety of soil types, but is unable to tolerate drought conditions and therefore its cultivation requires adequate rainfall or proper irrigation facilities (Haverkort, 1990). Potato is an erect or clambering succulent herb ranging 0.5-1 meter in height. The leaves are usually dark green in colour, very little hairy and pinnately arranged. The flowers are of various colours with fused petals and yellow stamens. Fruits are green and bitter, and its berries bear numerous seeds which are absent in many cultivars (Burbank, 1921). Underground structures called stolons are extended form of stems forming large number of tubers.

Usually, potatoes are vegetatively propagated from tubers of previous year crops (Ranalli, 1997). This method of propagation has large number of problems ranging from storage area requirements to accumulation of pathogens in tubers (Hoque, 2010). This made the

subsequent generations vulnerable to large no of bacterial, fungal and viral diseases as compared to other crops. Therefore, it became essential to find out alternative methods of production of pathogen and disease free seed tubers. Conventionally, breeding of potato cultivars such as *Solanum bulbocastanum* was used to overcome disease problems and to incorporate desired characteristics in genomes of parental clones. As a result large number of potato cultivars are being released every year. Whereas, each potato cultivar differs in appearance, maturity, storability, resistance to pathogens and physiological disorders. Each variety has its own climatic and nutritional requirements for optimum performance. But, traditional breeding is laborious and time consuming method which can take form 8-12 years for the breeding of new variety depending upon the environmental conditions. It does not assure success and has proved to be difficult to improve the efficiency and selection accuracy for target traits along with increasing the frequency of desired genes. Moreover, tetrasomic inheritance adds complexity to potato breeding in association with heterozygosity (Conner *et al*., 1997) making the buildup of desired cells difficult for breeding as large population of potato seedlings need to be screened. In the long history of conventional breeding the seeds produced are either infertile or have no commercial value. It often results in generating plants with undesired traits which can be potentially hazardous to human health. Disadvantages associated with conventional breeding paved way for the development of modern techniques like micropropagation or plant tissue culture techniques (Sabir *et al*., 2014). These techniques can maximize the yield and minimize the production cost by preventing the use of pesticides and production of disease free plants (Dann and Wilson, 2011). Keeping the importance of potato clones in view, a large number of tissue culture techniques have been applied for better potato production by using means such as micropropagation, disease free propagule development, and preservation of germplasm.

Similarly, genes for important traits can be combined and inserted through genetic transformation technology (Campbell *et al*., 2002). New gene transfer tools make it feasible to preserve the germplasm for future use, so that the desired genes of interest can be isolated and transferred into new commercial varieties. However, there is a requirement of developing an efficient *in-vitro* regeneration protocol for important species. These new gene transfer tools include direct methods like Particle Bombardment [Biolistics], Micro projectile Gun method, Poly Ethylene Glycol [PEG], Protoplast Fusion, Electroporation Silicon Carbide Fibers,

Liposome mediated gene transfer and indirect method of *Agrobacterium tumefaciens* mediated gene transfer.

Nowadays, Particle Bombardment and *Agrobacterium tumefaciens* mediated transformation are preferred because they can cope up with the whole plant tissues such as roots and leaves, which are easier to handle, more stable and require short steps for plant regeneration. *A. tumefaciens* mediated transformation has been the most successful, offering several advantages over other methods (Olhoft *et al.*, 2003). It has the capability of transferring single or multiple copies of DNA fragments which carry the genes of interest with low cost, high efficiency and minimal equipment requirement to transfer large DNA fragments (Liu *et al*., 2002).

AGROBACTERIUM MEDIATED TRANSFORMATION

Creating transgenic plants by using *Agrobacterium tumefaciens* is no longer a vision of the future. Nowadays, large number of agronomically and horticulturally important species are transformed using this bacterium**.** *A. tumefaciens* is a soil born, gram negative, rod shape, motile bacterium and is found in the rhizophere of plants. It has a wide host range, initiates tumors (Crown Gall disease) on most of the dicotyledonous plants along with some monocots (DeCleene & DeLay, 1976). These tumors once initiated in plants do not further require the presence of *A. tumefaciens* for proliferation (White & Braun, 1942) which demonstrates that cells of the plant have been transformed. It is attracted towards the wound site in plants via chemotaxis in response to chemicals such as phenolics and sugar molecules released from the damaged plant cells. The activities of tumor-inducing (Ti) plasmid (~200 kb) residing in the virulent strains of bacteria is the molecular basis behind this transformation (Braun 1982; Binns & Thomashow, 1988; Hooykaas & Beijersbergen, 1994). Specifically, only a small portion (T- DNA) of Ti plasmid is transferred which is flanked by 25 kb homologous sequences (Jouanin *et al*., 1989).

T-DNA transfer is a polar process which begins at the right border and is terminated at the left border. The single stranded T-DNA is converted to double stranded DNA and integrated into the plant genome which occurs in a Mendelian manner (Horsch *et al,*. 1984). Trait can be inherited at a single locus (Carrington and Whitham, 1998). However sometimes it can be

inherited as two independent loci. Once the DNA is transferred into the plant genome it is inherently stable since neither the borders nor the virulence genes are transformed.

Foreign genes of interest can be inserted in the T-DNA of Ti plasmid by removing the tumor forming genes for successful transformation of the plant DNA (Sheng and Citovsky, 1996). *Agrobacterium tumefaciens* strain lacking tumor inducing oncogenes is called as the disarmed strain (Klee *et al*., 1987) and can directly be used as a vector or it can be combined with other plasmids, example pBR322 to yield a binary vector such as pGV388. Selection of transgenic cells is done with the help of reporter genes like B-Glucuronidose gene (Gus) for the analysis of gene expression whereas for the selection of transgenic cells is done by antibiotic resistance genes such as neomycin phosphotransferase II (*nptII*) (McElory and Brettell, 1994).

Agrobacterium tumefaciens mediated transformation is the most common means of transformation in plants which has the potential of higher transformation frequency. It is capable of infecting the intact plant cells, tissues and organs thus overcoming much of the tissue culture problems. This is a preferred system as single/multiple copies of genes even with large fragments of DNA can be transformed without undesired gene silencing and fragment action of foreign gene (Kohli *et al.,* 1999). Integration of T-DNA is relatively a precise process and the stability of gene transferred is excellent which makes it simple, cost effective and efficient in most of the cases (Walden and Wingender, 1995).

With the increasing importance of potato as a food crop the important cultivars can be manipulated to incorporate the desired agronomical traits over a short period of time. Moreover, genetic engineering tools have the potential of overcoming various disadvantages associated with the use of conventional breeding techniques for development of pathogen free cultivar(s) of potato. In this present work, efforts have been made to study the factors affecting genetic transformation protocol of Indian potato cultivar **'Kufri Pukhraj'-** a variety released in 1998 by the Central Potato Research Institute (CPRI), Shimla. The effect of pre-culture period, co-cultivation period, mode of injury and time of infection on transformation efficiency in internodes and leaf explants were evaluated. Therefore, the best treatments have been combined for producing efficient and reproducible transformation in Indian potato cultivar 'Kufri Pukhraj' using *Agrobacterium tumefaciens*.

Chapter 2. AIM OF THE PRESENT STUDY

- The present study is undertaken to study the factors affecting genetic transformation of Indian potato cultivar **'Kufri Pukhraj'.**
- The following parameters were optimized for efficient transformation of potato cultivar 'Kufri Pukhraj'.

a) Comparison between two *Agrobacterium* strains (EHA105 and LBA4404)
b) Effect of preculture days
c) Effect of time of infection
d) Effect of co-cultivation days
e) Effect of mode of injury

Chapter 3: REVIEW OF LITERATURE

Potato (*Solanum tuberosum* L.); is an important vegetative rabi crop exceeded in production and area cultivated only by wheat (*Triticum aestivum*), rice (*Oryza sativa*) and maize (*Zea mays*) (Douches *et al.,* 1996). It is an important staple food source consumed on a daily basis in almost 40 countries around the world (Haque *et al*., 2009). Potato was introduced into India barely 40 years after its first introduction into Europe **(**Salaman, 1949). Vegetative propagation through seed tubers has various disadvantages associated with it such as low multiplication rates, high production cost and vulnerable to seed born diseases (Hoque, 2010). Moreover, quick decay of tubers in stores or in the fields have also been reported (Olanya *et al.,* 2009). Besides being propagated vegetatively, it is counted as one of the important food crops across the world which has limited purchasing power but increasing food demand and pressure on land (Struik and Wiersema, 1999). The potato production has begun to decrease in many countries due to three major constraints; seed quality, pest/disease control and limited economic resources (Scott and Suarez, 1992). Tissue culture techniques such as micropropagation have the potential to overcome these limitations successfully. Regeneration protocols for potato have been established in several laboratories all around the world (Hoque, 2010) but still there is an incomplete picture of on efficient and specific transformation protocols among different potato cultivars.

In recent years molecular and cellular approaches including genetic transformation and somatic hybridization have been used for improving the existing varieties of potato (Kumar *et al.,* 1995). Conventional breeding of potato began in 1800's, but due to its complex genetic makeup and tetraploid status, it is time consuming and cumbersome (Bradshaw and Mackay, 1994). It has a narrow genetic base which is linked to be the major problem during breeding (Bradshaw *et al,.* 1995). Potatoes have also been genetically engineered for several agronomic traits such as fungus resistance (Lorito *et al.,* 1998; Song *et al*., 2003), virus resistance (Hoekema *et al.,* 1989; Kawchuk *et al.,* 1991) and insect resistance (Naimov *et al*., 2003). Other factors like herbicide resistance (Jeong *et al.,* 1998) and starch synthesis (Vardy *et al*., 2002) have also been reported. Conventionally, genes from *Solanum bulbocastanum* (a wild relative of potato) were introduced into potato for achieving fungal resistance (Lokossou *et al.,* 2009).

Potato highly responds to a broad range of tissue culture techniques and thus can be improved through modern genetic techniques (Bajaj, 1981). Basically two major methods have been commonly used for transferring foreign genes into various plant species. First, is the use of biolistic devices or Microprojectile Bombardment method (Klien *et al.,* 1987; Shirgurkar *et al*., 2006) and second, *Agrobacterium tumefaciens* mediated transformation (Andersson *et al.,* 2003). The era of plant genetic transformation started in 1980's with escapade of first potato plants carrying foreign gene using *A. tumefaciens* mediated gene delivery (Ooms *et al.,* 1983). One of the first crop for which transgenic plants were regenerated is potato (An *et al.,* 1986) and efficient gene transfer protocols for important cultivars using *Agrobacterium tumefaciens* mediated gene transfer were quickly adopted (De Block, 1988). Transformation using *Agrobacterium tumefaciens* has remarkable advantages in comparison to other transformation methods such as T-DNA can be integerated preferentially into transcriptionally active parts of chromosomes (Olhoft *et al.,* 2003). Transformation protocols using *A. tumefaciens* differ from one species to another and between different cultivars of the same species. It requires various factors to be studied in detail. Firstly, the bacterium and plant interaction needs to be optimized on competent cells of different tissues capable of regenerating. Secondly a proper method needs to be developed for regenerating the transformed cells (Gustavo *et al.,* 1998). Use of binary vectors have also been reported by many scientists (Sheerman and Bevan, 1988).

In recent years transformation of potato has been attained through direct DNA uptake (Feher *et al.,* 1991; Valkov *et al.,* 2011) and recently *Vir* gene mediated transformation from *Rhizobium* species (Wendt *et al*., 2012) and *Ensifer adhaerens* (Wend *et al.,* 2012) have been reported. Development of efficient transformation protocol depends upon important factors like healthy, vigorous explant source and method of wounding (Banerjee *et al*., 2006). Several reports on transformation of potato via *A. tumefaciens* have been cited (Kumar *et al.,* 1995; Trujillo *et al.,* 2001; Anderson *et al.,* 2003). Productive plant regeneration for several potato genotypes have been reported from a range of explant tissues, including leaf, stem and tuber for several potato genotypes (De Block, 1988; Wenzler *et al*., 1989). Leaves of *in-vitro* grown plantlets are commonly used due to their easy availability and aseptic nature (De Block, 1988; Moravcikova *et al.,* 2004). However, mini tuber segments (Sheerman and Bevan, 1988; Ishida

et al., 1989; Stiekema *et al.,* 1988) or internodal segments (Chang *et al.,* 2002; Heeres *et al.,* 2002) have been equally successful.

Successful gene transformation using leaf explants of different cultivars of potato for analyzing the effect of co-cultivation days have been reported in many studies (An *et al.,* 1986; De Block 1988). Results revealing that internodal segments show better transformation ability than the leaf explants have also been reported (Ducreux *et al.,* 2005). Whereas, leaf explants were more sensitive to cuts than the internodes (Ducreux *et al.,* 2005). Transient Gus expression efficiency of *Agrobacterium tumefaciens* strain LBA4404 is reported to be superior than EHA105 in many studies (Wenzler *et al*., 1989; Trujillo *et al.,* 2001).

Identification of transformed plant cells is done with the help of a selectable marker gene which enables the transformed cells to survive while the non-transformed cells to die in media (Draper and Scott, 1991). Selection of transformed cells is done by their resistance to phytotoxic concentrations of specific chemicals (Grant *et al*., 1991). Genetic engineering in potato is well established and routinely relies on kanamycin resistance which acts as a selectable marker (Vayda and Belknap, 1992; Conner *et al.,* 1997). The most common marker gene *nptII* conferring kanamycin resistance is exclusively used for potato transformation and is highly effective (Flavell *et al.,* 1992; Nap *et al.,* 1992). The marker gene *nptII* has originated from bacterial transposon Tn5 (Conner and Meredith, 1989). Confirmation of transformed gene as well as stable integration in plants have been reported through Polymerase Chain Reaction (PCR) and Southern Analysis (Yevtushenko and Misra, 2010).

Chapter 4: MATERIALS AND METHODS

4.1) Glassware and Plant Material

The cultures of Indian cultivar 'Kufri Pukhraj' were already procured from CPRI and maintained in Plant Tissue Culture Laboratory of Thapar University, Patiala. Cultures were sub cultured at regular interval of 21 days on Murashige and Skoog (1962) medium containing 3% sucrose and 0.7% (w/v) agar as solidifying agent. Plant Tissue Culture Grade Chemicals were purchased from Hi Media Laboratories Ltd. (Mumbai). Experiments were carried out in 300 ml culture vessels (Kasablanka Pvt. Ltd, Mumbai) containing 30 ml medium in each bottle. The pH of medium was adjusted to 5.8 before autoclaving. Bacterial cultures were maintained in 250 ml conical flasks containing 25 ml of Luria Broth medium. Microshoots of 4 weeks old cultures were used as a source of explant for experimentation. Cultures were incubated at 25 ± 1°C and photoperiod of (16 h light/8 h dark) under light intensity of 42 µ mole m^{-2} s^{-2} provided by cool flouroscent lamps (Philips Ltd.) Bacterial cultures were maintained in 250 ml flasks containing 25 ml of Luria Broth medium (Hi Media Laboratories Ltd, Mumbai).

Table 1: Different combinations of media used for genetic transformation of Indian potato cultivar 'Kufri Pukhraj'

MEDIUM	COMPOSITION
Pre-culture Medium(PM)	MS+2. 5 µM 6-Benzylaminopurine (BA) and 5 µM Gibberellic acid (GA_3)
Co-cultivation Medium (CoM)	MS+2. 5 µM 6-Benzylaminopurine (BA) and 5 µM Gibberellic acid (GA_3) + 50 mg/l acetosyringone
Selection cum Regeneration medium (SRM)	MS+2. 5 µM 6-Benzylaminopurine (BA) and 5 µM Gibberellic acid (GA_3) + 200 mg/l cefotaxime and 100 mg/l kanamycin

4.2) Bacterial Cultures for Transformation

A single colony of *Agrobacterium tumefaciens* strain (EHA105 and LBA4404) was inoculated in 25 ml of liquid LB medium (Appendix 1) containing selection antibiotics - kanamycin (50 mg/l) and rifampicin (15 mg/l). Filter sterilized antibiotics were added to autoclaved medium after cooling it down to 40°C. Antibiotics were purchased from Sigma Laboratories Ltd. (Mumbai) The culture was allowed to grow overnight at 25°C/150 rpm. Next day 100 ml of the overnight grown culture was taken to freshly inoculate LB medium (Appendix 1) and was used for transformation until O.D. reaches 0.5 at 600 nm. *Agrobacterium tumefaciens* strain EHA105 and LBA4404 (harbouring pBI121 plasmid) were used in this study. The binary vector pBI121 contains *uidA* gene (*β*-glucuronidase) and neomycin phosphotransferase (*nptII*) gene as a selection marker gene for genetic transformation. Both the genes are present on T-DNA and are controlled by *CaMV* 35S and *nos* promoters, respectively.

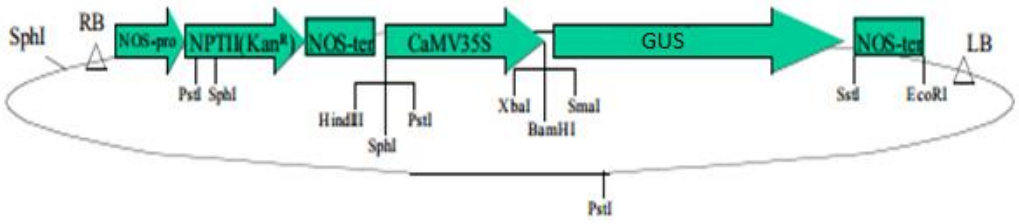

Figure: Structure of pBI121 plasmid (Source: google images)

4.3) Antibiotic Sensitivity for Selection of Transformants

Sensitivity or tolerance limit of potato cultivar 'Kufri Pukhraj' was resolved by culturing the leaf explants on previously optimized selection cum regeneration medium supplemented with 2.5 µM 6-Benzylaminopurine (BA) and 5 µM Gibberellic acid (GA_3). Kanamycin sensitivity was checked at 0, 10, 30, 50, 70 and 100 mg/l kanamycin by culturing the leaf explants on MS medium. Explants were observed for necrosis and results for noted after 4 weeks of initial culture. Filter sterilized Kanamycin was added to the autoclaved MS (Appendix 1) media after cooling it down to 40°C.

4.4) Generalized Procedure for Transformation

Internodes and leaf explants (100 each) were cut from both the ends with the help of a sharp scalpel and inoculated on MS basal medium (mentioned in appendix 1). After 48 hours co-cultivation of pre-cultured explants was done with suspension cultures of both the strains of *A. tumefaciens* (EHA105 and LBA4404) at OD 0.5 (600 nm). Bacterial pellets were resuspended in basal MS (Appendix 1) medium after allowing the cultures to centrifuge at 4000 rpm at 4 °C for 15 min. Suspension cultures were supplemented with acetosyringone (50 mg/l) 10 minutes prior to infection, then poured in sterilized petriplates containing leaf and internodal explants by slightly excising them. Removal of excess suspension cultures was done by soaking the explants on sterilized blotting papers and were finally placed on the MS medium containing acetosyringone at 25°C culture conditions. After two days, *Agrobacterium tumefaciens* infected explants were washed 6 times with sterile distilled water and once with sterile distilled water containing cefotaxime. The washed explants were transferred onto selection medium containing cefotaxime (200 mg/l) and kanamycin (100 mg/l) supplemented with 2.5 µM 6-Benzylaminopurine (BA) and 5µM Gibberellic acid (GA_3) for required number of days till the regeneration of shoots was achieved. Histochemical assay was done for Gus activity using X-gluc as substrate.

4.5) Factors affecting transformation efficiency.

An efficient transformation protocol for potato cultivar 'Kufri Pukhraj' through *Agrobacterium tumefaciens* mediated transformation has been standardized by performing one on one with following experiments.

4.5.1) *Effect of bacterial strains on transient Gus expression*

Two different bacterial strains EHA105 and LBA4404 were used for carrying out the transient Gus expression using leaf and internodal explants. Rest of the procedure for preculture, co-cultivation and infection was kept exactly the same for analyzing the superior strain of *Agrobacterium tumefaciens.*

4.5.2) *Effect of preculture days*

Leaves and internodes (100 each) were used as explants for carrying out transformation. Leaves were used as explants by cutting them into smaller strips or squares. In this protocol apical and basal part of leaves were separated after detaching the leaves from the petiole. Wounds made during explant preparation are sufficient but additional cuts were made during inoculation. Wounding of the explants was done using a sharp scalpel. Laterally inverted internodes and adaxial sides of the leaves was kept in contact with preculture medium (Table 1). In this protocol, experimental treatment ranged from freshly isolated explants to those precultured upto 8 days using both the strains of *Agrobacterium tumefaciens* (EHA105 and LBA4404). The effect of preculture days was studied after transferring the precultured explants onto selection cum regeneration (Table 1).

4.5.3) *Effect of co-cultivation days*.

Explants were precultured (three days) onto PM (Table No.1) with the help of a sharp scalpel and transferred on co-cultivation medium (Table 1). In this experiment co-cultivation period was ranged from day 1 to day 4 with each *A. tumefaciens* strain (EHA105 and LBA4404). Washing was done after completion of required number of co-cultivation days and the explants were then transferred onto selection cum regeneration medium (SRM) (Table 1). The explants transferred onto SRM were kept at 25 ± 1°C and photoperiod of (16 h light/8 h dark)

4.5.4) *Effect infection time*

The bacterial suspension of each *A. tumefaciens* strain was used for infecting the explants precultured for three days. The explants were injured using a sharp scalpel and then transferred into sterile petri plates for different time intervals of 5, 10, 15, 20 and 30 minutes. These explants were then transferred for two days onto CoM (Table 1) and later to RSM (Table 1) in order to analyze the time of infection which results in maximum transformation.

4.5.5) *Mode of injury*

In present study three different modes of injury were used for excision of explants- sharp scalpel, glass beads and sand paper. The entire procedure for transformation remained the same in each case except for the mode of injury so that the best mode of injury leading to efficient transformation could be analyzed. Following co-cultivation for two days, the explants were then cultured on SRM (Table 1)

4.6) Gus (β-glucuronidase) histochemical assay

Transformation ability of survived co-cultured tissues was examined through transient Gus histochemical assay (Jefferson *et al.,* 1987) with little modifications. Explants were immersed in 100 mM Sodium Phosphate Buffer (pH=7) containing X-gluc (5-bromo-4-chloro-3-indolyl glucuronide) as the substrate and incubated overnight at 37℃. The stained explants were washed with 70% methyl alcohol to remove excess of chlorophyll. Expression of *uidA* gene (*β*-glucuronidase) in plant tissues was confirmed by characteristic blue coloured spots. Transformation efficiency was evaluated by the no. of explants expressing blue spots in comparison with the total no. of explants. Composition of Gus solution is given below.

Composition of Gus (*β* -Glucuronidase) solution

COMPONENTS	**CONCENTRATION**
Sodium Phosphate Buffer	100 mM
Sodium EDTA	0.25M
Ferro Cyanide	0.5 mM
Ferri Cyanide	0.5 mM
X-gluc	10 mg/l
Triton-x 100	(0.1%) w/v
Water	

4.7) Statistical Analysis

Results of transient Gus expression were analyzed after seven days of explants (leaves and internodes) cultured on selection cum regeneration medium. Experiment was repeated two times with 50 explants in each experiment. Entire data has been reported as mean ± standard error.

Chapter 5: RESULTS

Before proceeding with the transformation experiment, regeneration protocol for potato optimized previously was re-tested for Kufri Pukhraj. Leaves and internodal explants cultured on 2.5 µM 6-Benzylaminopurine (BA) and 5 µM Gibberellic acid (GA_3) showed regeneration within 4 weeks.

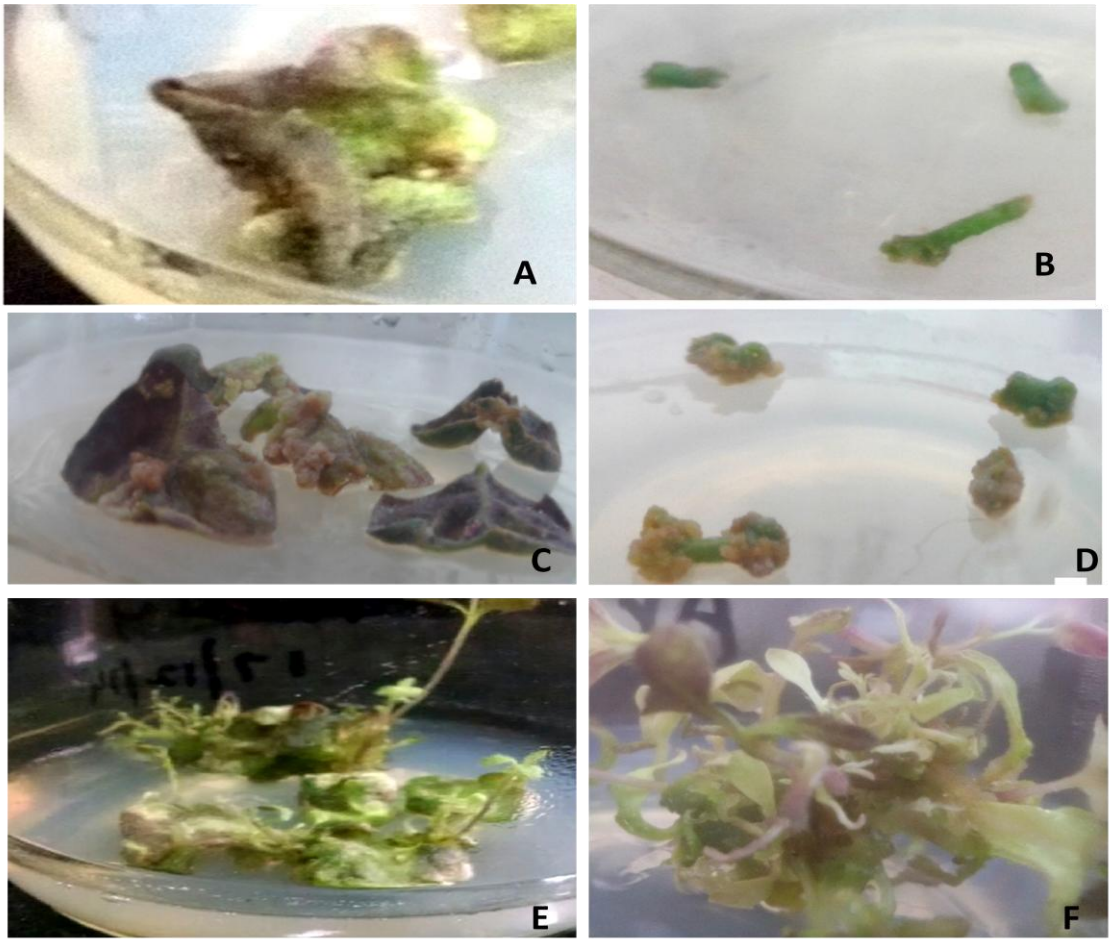

Figure 1:- (A-B) Leaf and internodal explants of potato cultivar 'Kufri Pukhraj' showing (C-D) callogenesis and (E-F) shoot organogenesis within 4 weeks of culture on MS medium supplemented with 2.5 µM 6-Benzylaminopurine (BA) and 5 µM Gibberellic acid (GA_3) showed regeneration within 4 weeks

5) Optimization of transformation using *Agrobacterium tumefaciens*

Transformation in *Agrobacterium tumefaciens* is affected by different parameters like *Agrobacterium tumefaciens* strain, preculture days, duration for co-cultivation, time of infection and mode of injury. These various factors have been optimized to increase the transformation efficiency using leaf and internode explants.

5.1) Effect of kanamycin on survival of explant

The proper concentration of kanamycin was selected by placing the leaf explants on MS medium (Appendix 1) containing kanamycin at concentration of 0, 10, 30, 50, 70 and 100 mg/l of kanamycin. Percentage of explants showing survival and explants showing necrosis were recorded. It was determined that with increase in kanamycin concentration the percent explants showing growth and survival decreased. Media containing no kanamycin (control) showed 100% survival of explants. Whereas, the media containing 100 mg/l kanamycin concentration showed complete necrosis of explants. A distinct decline in percent explants showing survival was seen as the kanamycin concentration was increased from 70 mg/l to 100 mg/l. (Fig:- 1)

It was analyzed that at kanamycin concentration of 50 mg/l and above, the explants started to become whitish yellow and failed to grow completely as compared to media containing lower or no concentration (Fig:- 2.A). It was also observed that more than 80% of explants were killed at a concentration of 70 mg/l within 28 days. Moreover, a negative effect on growth and survival of explants was observed at higher levels of concentration. Therefore, 100 mg/l of kanamycin concentration was used for selection of transformed plants as this resulted in complete necrosis of explants.

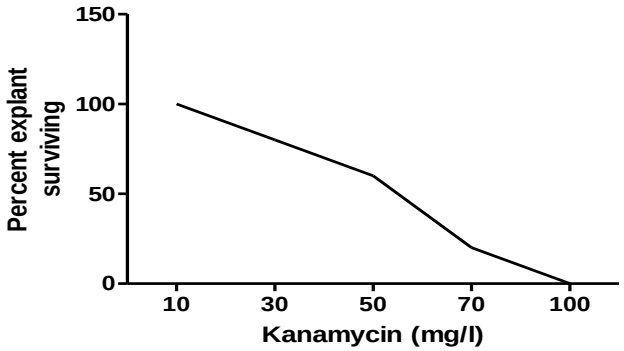

Figure 2:- The effect of kanamycin concentration in MS medium on survival of leaf explants.

5.2) Effect of bacterial strains on transient Gus expression

Agrobacterium tumefaciens strains LBA4404 and EHA105 harbouring plasmid pBI121 were compared for their efficiency in producing transformants using leaf and internodal explants. *A. tumefaciencs* strain EHA105 was preferred over LBA4404. Maximum transient expression was shown by leaf explants on infection with *A. tumefaciencs* strain EHA105. Whereas different results were observed in internodal explants as infection with strain LBA4404 was found to be more successful than strain EHA105.

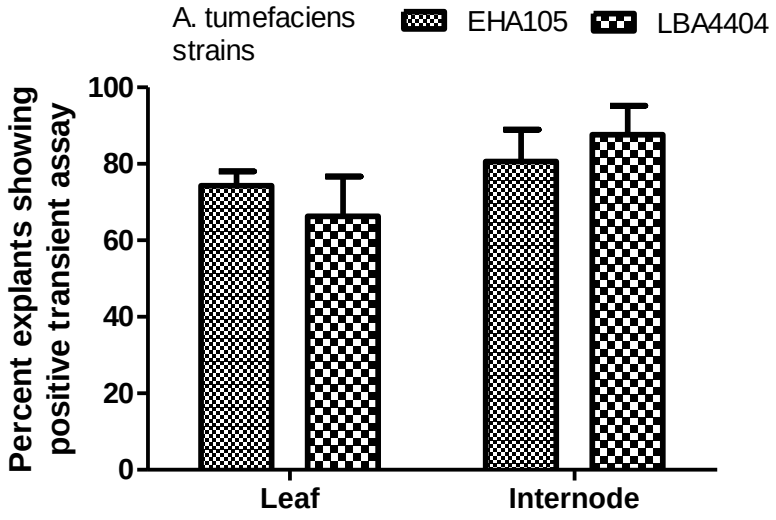

Figure 3:- Comparative analysis of transient *Gus* expression in leaf and internodal explants of potato cultivar 'Kufri Pukhraj' with two different strains of *A. tumefaciens* (EHA105 and LBA4404).

5.3) Effect of preculture days on transient Gus expression

Preculture days were found to have an important role in transformation. Maximum transient expression was observed when leaf and internodal explants were cultured on PM only for one to two days. Transient expression decreases drastically with increase in number of preculture days in leaf and internodal explants infected with *A. tumefaciens* strains EHA105 and LBA4404. It was observed that more transient expression was shown by internodes in comparison to leaf explants (82.04 to 96%)

Table 2:-Effect of preculture days on transient Gus expression in leaf and internodal explants of potato cultivar 'Kufri Pukhraj' using *A. tumefaciens* strains.

PRECULTURE DAYS	STRAIN of *A. tumefaciens*	% EXPLANTS SHOWING TRANSIENT EXPRESSION (Mean+S.E)	
		LEAVES	INTERNODES
1	EHA 105	82.04±1.72	96.0±4.00
	LBA 4404	66.12±7.69	55.89±1.75
2	EHA 105	76.12±2.82	98.43±1.56
	LBA 4404	63.19±2.79	73.33±6.67
3	EHA 105	70.26±3.07	90.92±6.22
	LBA 4404	86.19±2.86	90.47±2.03
4	EHA 105	81.78±3.12	69.86±0.86
	LBA 4404	59.16±1.50	71.93±1.93
5	EHA 105	55.36±6.62	35.32±0.68
	LBA 4404	59.59±4.59	63.34±4.16
6	EHA 105	86.94±4.72	77.78±10.55
	LBA 4404	58.75±10.42	52.53±4.54
7	EHA 105	71.47±0.48	62.19±5.62
	LBA 4404	42.55±1.22	67.23±0.81
8	EHA 105	32.42±11.57	78.33±2.33
	LBA 4404	64.71±15.99	57.00±10.00

Gus expression was analysed after 7 days of co-cultivated explants with two strains of *Agrobacterium tumefaciens* (EHA105 and LBA4404) harbouring plasmid vector pBI121. Experiment was repeated two times with fifty explants in each experiment.

5.4) Effect of co-cultivation period on transient Gus expression

Explants co-cultivated with *A. tumefaciens* strains EHA105 and LBA4404 showed almost similar variation with change in co-cultivation days. Co-cultivation days were found to have an inverse relation with transient Gus expression. With increase in number of co-cultivation days from two, Gus expression was reduced significantly. On one day completion of co-cultivation with both the strains, more transient Gus expression was observed in leaf explants than the internodal explants. Whereas opposite results were recorded with increase in co-cultivation days above 1 day. It was also interesting to note that the explants infected with strain LBA4404 *A. tumefaciens* sowed maximum transient expression.

Table 3:- Effect of co-cultivation days on transient Gus expression in leaf and internodal explants of potato cultivar 'Kufri Pukhraj'using *A. tumefaciens* strains.

CO-CULTIVATION DAYS	STRAIN of *A. tumefaciens*	% EXPLANTS SHOWING TRANSIENT EXPRESSION (Mean+ S.E)	
		LEAVES	INTERNODES
1	EHA105	67.29±3.35	56.32±1.35
	LBA 4404	74.29±4.14	57.56±0.34
2	EHA 105	75.98±2.46	87.07±2.85
	LBA 4404	78.33±5.0	83.81±3.15
3	EHA 105	63.68±10.61	69.53±11.11
	LBA 4404	77.66±1.33	87.09±0.52
4	EHA 105	60.74±6.24	79.98±2.00
	LBA 4404	63.82±6.72	73.79±15.53

Gus expression was analysed after 7 days of co-cultivated explants with two strains of *Agrobacterium tumefaciens* (EHA105 and LBA4404) harbouring plasmid vector pBI121. Experiment was repeated two times with fifty explants in each experiment.

5.5) Effect of infection time on transient Gus expression

Infection time was found to play a key role in transformational efficiency. Fifteen minutes of incubation of leaf and internodal explants with both the strains of *Agrobacterium tumefaciens* was found to be optimum for effective transformation. Decreasing or increasing infection time from fifteen minutes resulted in decreased transformational efficiency. It was also observed that EHA105 strain of *A. tumefaciens* was more active even if incubation time was reduced upto 5 minutes.

Table 4:- Effect of infection time on transient Gus expression in leaf and internodal explants of potato cultivar 'Kufri Pukhraj' using *A. tumefaciens* strains.

TIME OF INFECTION	STRAIN of *A. tumefaciens*	% EXPLANTS SHOWING TRANSIENT EXPRESSION (Mean+S.E)	
		LEAVES	INTERNODES
5 MINUTES	EHA 105	50.8±4.17	31.14±14.48
	LBA 4404	23.52±10.19	45.41±2.08
10 MINUTES	EHA 105	61.07±6.07	55.95±5.95
	LBA 4404	52.14±2.14	49.44±10.56
15 MINUTES	EHA 105	78.60±4.724	85.35±0.35
	LBA 4404	63.61±3.61	75.27±3.05
20 MINUTES	EHA 105	49.58±21.25	29.16±4.16
	LBA 4404	22.22±2.78	34.16±17.50
30 MINUTES	EHA 105	40.83±0.83	27.76±11.11
	LBA 4404	34.84±6.27	40.55±1.11

Gus expression was analysed after 7 days of co-cultivated explants with two strains of *Agrobacterium tumefaciens* (EHA105 and LBA4404) harbouring plasmid vector pBI121. Experiment was repeated two times with fifty explants in each experiment.

5.6) Effect of mode of injury on transient Gus expression

Infection carried out using scalpel, needle and sand paper was also found to affect transient Gus expression. Scalpel was found to be the most suitable for infecting leaf and internodal explants.

Table 5:- Effect of injury mode on transient Gus expression in leaf and internodal explants of potato cultivar 'Kufri Pukhraj' using *A. tumefaciens* strains.

MODE OF INFECTION	STRAIN of *A. tumefaciens*	% EXPLANTS SHOWING TRANSIENT EXPRESSION (Mean+S.E)	
		LEAVES	INTERNODES
SCALPEL	EHA 105	66.55±5.44	75±5
	LBA 4404	91.55±6.22	98.33±1.67
NEEDLE	EHA 105	64.33±16	75.32±7.0
	LBA 4404	47.45±9.54	86±2
SAND PAPER	EHA 105	34.27±15.83	33.33±0
	LBA 4404	19.44±0	51.38±6.94

Gus expression was analysed after 7 days of co-cultivated explants with two strains of *Agrobacterium tumefaciens* (EHA105 and LBA4404) harbouring plasmid vector pBI121. Experiment was repeated two times with fifty explants in each experiment.

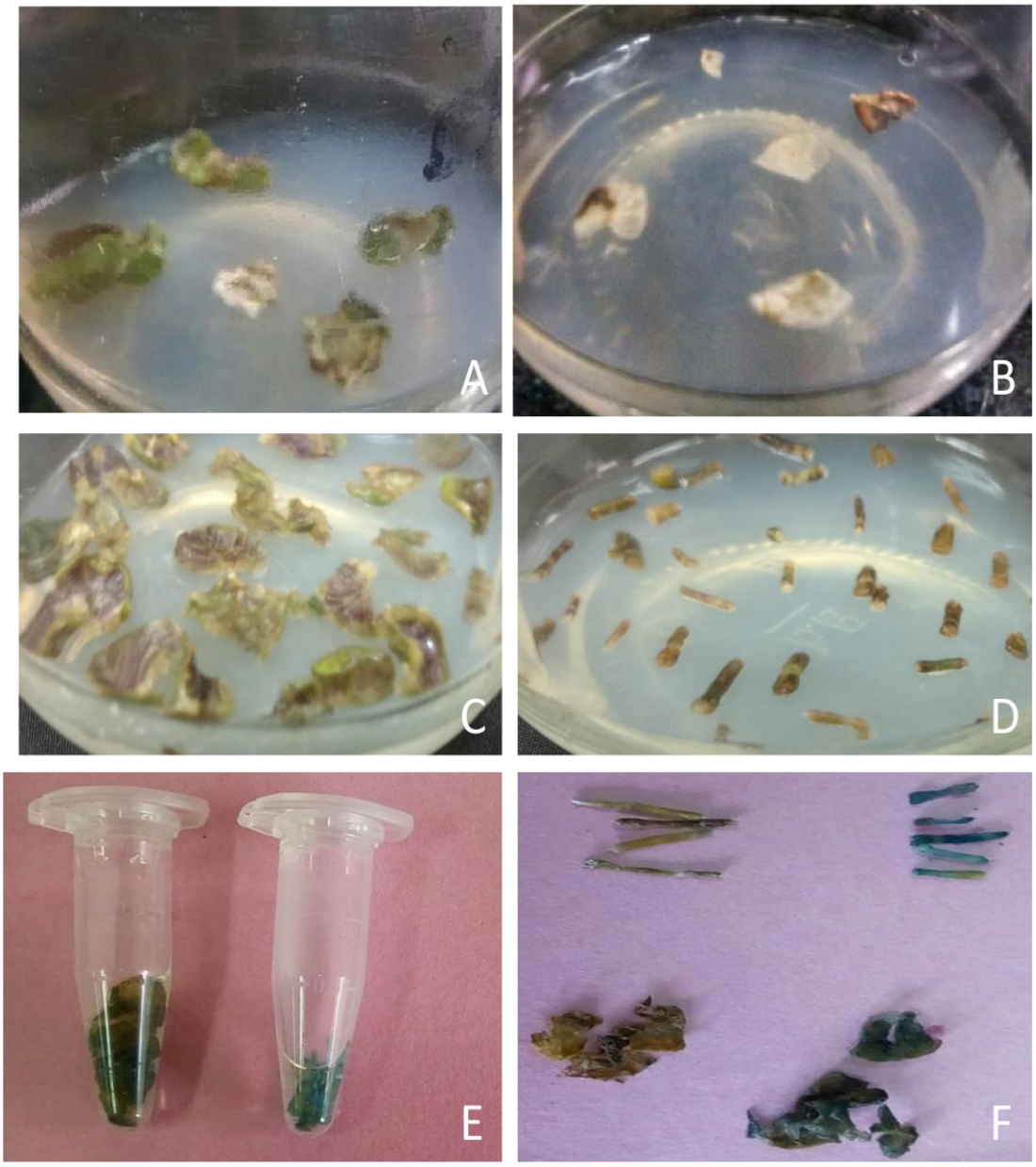

Figure 4: Explants of *Solanum tuberosum* cv 'Kufri Pukhraj' showing necrosis on selection medium containing A) 50 mg/l B) 100 mg/l kanamycin. Callus was observed from C) leaves D) internodal explants after 30 days of culture on selection cum regeneration medium supplemented with 2.5 µM 6-Benzylaminopurine (BA) and 5µM Gibberellic acid (GA_3). E-F) Leaf and internodal explants showing transient Gus expression.

Chapter 6: DISCUSSION

Present study was focused on optimization of transformation protocol for potato cultivar **'Kufri Pukhraj'**. Transformation efficiency is dependent on various factors responsible for T-DNA delivery using *Agrobacterium tumefaciens.* As shoot organogenesis is a pre-requisite to carry out transformation experiments, previously optimized regeneration protocol for potato was also re-tested. Shoot organogenesis was observed in MS medium supplemented with 2.5 µM 6-Benzylaminopurine (BA) and 5µM Gibberellic acid (GA_3). Investigation of kanamycin sensitivity to leaf explants of potato cultivar 'Kufri Pukhraj' showed that addition of kanamycin causes toxicity to explants and finally leads to necrosis. Survival rate was found 100% on medium lacking kanamycin, whereas at 100 mg/l Kanamycin concentration, no survival was observed (Fig. 2). Therefore all the transformation experiments were performed using 100 mg/l concentration of kanamycin in selection medium. The tocxicity of kanamycin to explants of potato observed was similar to previous reports on different plants (Mante *et al.,* 1991; Laparra *et al.,* 1995; Prakash and Gurumurthi, 2009).

Transformation efficiencies are also known to be dependent on the bacterial strain used. Therefore, two strains of *Agrobacterium tumefaciens* EHA105 and LBA4404 were tested. Both the strains were found to be equally effective in potato (Fig. 3). EHA105 induced higher Gus transient expression (75%) in leaf explants whereas LBA4404 induced about 90% Gus activity in internodal explants in comparison to 85% Gus activity induced by EHA105. Virulence of EHA has already been demonstrated by Hood *et al.,* 1993. In reports on leaf transformation, EHA has been reported as an efficient strain in comparison to LBA4404 (Aggarwal *et al.,* 2011). Moreover, problem with elimination of bacteria from transformed cultures cannot be neglected. Thus cefotaxime with concentration 200 mg/l was added in selection medium. Cefotaxime is also reported to have no effect on decrease of shoot organogenic potential im many plants. (Aggarwal *et al.,* 2011).

Several factors such as preculture days, co-cultivation days, infection time and mode of injury are known to influence the transformational efficiencies. These factors were also studied during the present research. Preculture of explants on shoot organogenesis medium prior to transformation was found to have an important role in deciding Gus transient expression. Maximum 82 to 98% transient expression was induced in leaf and internodal explants on one to two days of preculture (Table 2). Increase in preculture days resulted in decrease in Gus expression. However, in potato different results reporting 4 days preculture as optimal factor for transformation has also been reported (Wenzler *et al.,* 1989). Reason behind the importance of preculture can be that the cells of the plant undergo a physiological adaptation for becoming competent during the pre-conditioning step of transformation (Sunilkumar *et al.,* 1999). Further, it has also been reported that plant stress is reduced during pre-cultivation (Venkatachalam *et al.,* 1998).

Co-cultivation of infected explants for two days is reported to be a convenient period, resulting in the production of transient Gus expression. Whereas, when co-cultivation period is allowed beyond two days reduced percentage of explants showing transient Gus activity were seen (Table 3). Our result was similar to the report of Li *et al.,* 2003 where two days co-cultivation period were found to be optimal for the transformation of hypocotyls and pepper cotyledons. The results were also in accordance with results cited by Ducreux *et al.,* (2005). Controlling the overgrowth of *A. tumefaciencs* in later stages is difficult and results in complete explants wilting due to infection. Therefore, co-cultivation period of 2 days was reflected to be the standard for all the subsequent experiments that were performed. Some cultivars have also reported a co-cultivation period of three days or more to be more effective in different explants of potato (Wenzler *et al.,* 1989). The reason for difference in co-cultivation duration can be the combined effect of different parameters like injury mode, explants type, using specific strain of *Agrobacterium tumefaciens*

Time of infection is another important parameter affecting the transformation efficiency in plants. Incubation for fifteen minutes was found to be optimal for transformation (Table 4). The reason for less transformation frequency after infection time of 15 minutes might be due to excessive bacterial growth on the surface of

explants. The result was found to be in accordance with Banerjee *et al.*, (2006). Whereas, Trujillo *et al.*, 2011 found ten minutes to be the most effective time for carrying out transformation in leaf explants. Sarker and Mustafa, 2002 found that maximum Gus expression was shown in a time of fifty minutes by potato leaves. The reason behind different transformation efficiencies and time of infection may be associated with the difference in genotypes explants used.

Different modes of injury for wounding the explants resulted in significant transformation differences (Table 5). Wounding the explants with scalpel was easier and less time consuming than other modes of injury and was determined to be the best method for injuring the explans. Results were found to be in line with previous reports by Banerjee *et al.*, (2006).

In conclusion, various factors affecting genetic transformation efficiencies have been optimized for potato cultivar **'Kufri Pukhraj'.** The factors can be used as a platform to carry out genetic modification in Indian cultivar of potato.

Chapter 6: CONCLUSION

Factors affecting *Agrobacterium tumefaciens* mediated genetic transformation of Indian potato cultivar 'Kufri Pukhraj' were studied.

- *A. tumefaciens* strain EHA105 was found to be more efficient for inducing transient Gus expression in leaf explants. Whereas strain LBA4404 resulted in higher Gus activity in internodal explants.
- Pre-culture and co-cultivation of two days was found to be optimum for genetic transformation.
- Explants infected with both the strains of *A. tumefaciens* (EHA105 and LBA4404) for fifteen minutes resulted in higher transient Gus expression in comparison to lower and higher incubation times.
- Explants injury with scalpel yielded better results than needle and scalpel.

Chapter 7: APENDIX 1

1} MEDIA USED

- **Luria Bertani (LB) medium**

Yeast extract	0.5% (w/v)
Tryptone	1.0% (w/v)
NaCl	1.0% (w/v)
Agar	1.5% (w/v)

- **Murashige and Skoog (MS) Media (1962)**

Components:

Macro nutrients	**Concentration (mg/L)**
NH_4NO_3	1650
KNO_3	1900
$CaCl_2.2H_2O$	440
$MgSO_4.7H_2O$	370
KH_2PO_4	170
Micro nutrients	**Concentration (mg/L)**
$MnSO_4.H_2O$	16.9
$ZnSO_4.7H_2O$	8.6
H_3BO_3	6.2
KI	0.83
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025

Vitamins	**Concentration (mg/L)**
Nicotinic Acid	0.5
Pyridoxine HCl	0.1
Thiamine HCl	0.5
Glycine	2
Freshly Added Components	
Myoinositol	100 g/l
Sucrose	30.0 g/l
Na_2Fe-EDTA	30.0 g/l
PH	5.8

Chapter 8: REFERENCES

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