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# "OPTIMIZATION OF IN- PLANTA METHOD OF GENETIC TRANSFORMATION IN PIGEON PEA *(Cajanus cajan L. Millsp.)*"


***SUBMITTED BY***

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***DISSERTATION***

*Submitted to the*
*Vasantrao Naik Marathwada Krishi Vidyapeeth*
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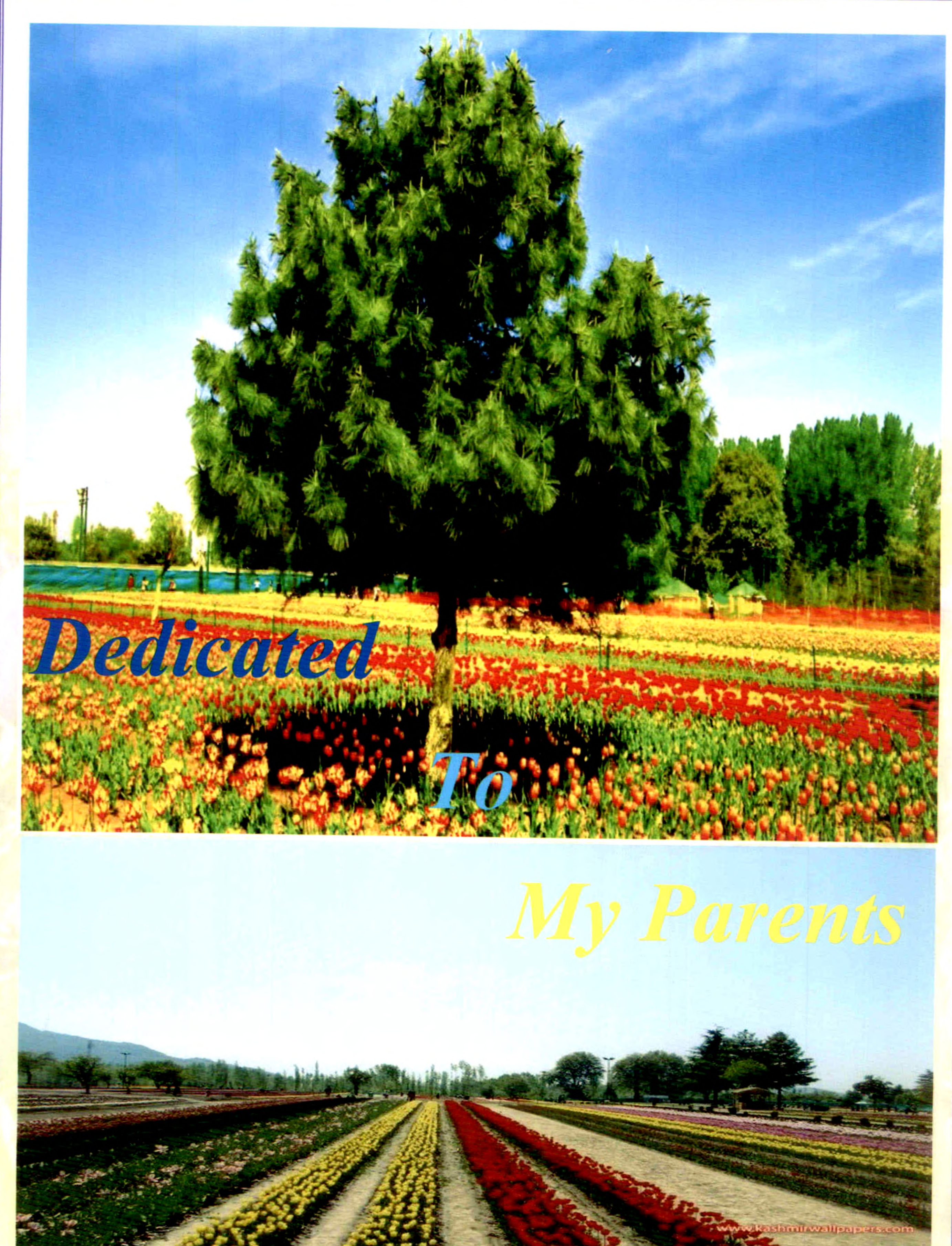
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This is to certify that Shri. **ROUF AHMAD PARRAY** has satisfactorily prosecuted his course and research for a period of not less than four semesters and that the dissertation entitled **"OPTIMIZATION OF *IN-PLANTA* METHOD OF GENETIC TRANSFORMATION IN PIGEON PEA (*Cajanus cajan* L. Millsp.)"** submitted by him is the result of original research work and is of sufficiently high standard to warrant its presentation to the examination.

I also certify that the thesis or a part thereof has not been previously submitted by him for a degree of any university.

PLACE: LATUR

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**( R. L. CHAVAN)**
Research Guide

# CERTIFICATE–II

This is to certify that the dissertation entitled **"OPTIMIZATION OF *IN-PLANTA* METHOD OF GENETIC TRANSFORMATION IN PIGEONPEA (*Cajanus cajan* L. Millsp.)"** submitted by Shri. **ROUF AHMAD PARRAY** to Vilasrao Deshmukh College of Agricultural Biotechnology, Latur in partial fulfilment of the requirements for the degree of **MASTER OF SCIENCE (Agriculture)** in the subject of **AGRICULTURAL BIOTECHNOLOGY** has been approved by the student's advisory committee after oral examination in collaboration with the external examiner.

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# List of Abbreviations

| | | |
|---|---|---|
| CaMV | : | Cauliflower Mosaic Virus |
| CN | : | Cotyledonary node |
| CV | : | Coefficient of variation |
| *cv* | : | Cultivars |
| °C | : | Degree celcius |
| DCMEA | : | Decapitated Mature Embryonic Axes |
| DNA | : | Deoxyribose Nucleic Acid |
| *et al.* | : | etalia (and associate) |
| FAO | : | Food and Agricultural Organization |
| FYM | : | Farm Yard Manure |
| GA | : | Gibbrelic Acid |
| gm | : | Gram |
| GUS | : | Glucuronidase |
| Ha | : | Hectare |
| HCl | : | Hydro chloric Acid |
| IAA | : | Indole Acetic Acid |
| IBA | : | Indole Butyric Acid |
| Kan | : | Kanamycin |
| Kin | : | Kinetin |
| Kg | : | Kilo gram |
| LB | : | Luria Bertani |
| MEA | : | Mature Embryonic Axes |
| mg | : | Miligram |
| mm | : | Milimeter |
| mM | : | Milimolar |
| µM | : | Micromolar |
| Max | : | Maximum |
| Min | : | Minimum |
| ml | : | Mililiter |

| | | |
|---|---|---|
| MS | : | Murashige and Skoog media |
| NAA | : | Napthalene Acetic Acid |
| Ng | : | Nano gram |
| OD | : | Optimal Density |
| PCR | : | Polymerase Chain Reaction |
| pH | : | log [H] ion concentration |
| rpm | : | Revolution Per Minute |
| SD | : | Standard deviation |
| SE | : | Standard error |
| SIM | : | Shoot Induction Media |
| SM | : | Selection media |
| T-DNA | : | Transferred DNA |
| Ti | : | Tumor inducing |
| *viz* | : | Namely |

# Introduction

**CHAPTER I**

# INTRODUCTION

Pigeon pea (*Cajanus cajan* L.) is an important grain legume of the semiarid tropics and form's a significant component of the diet of vegetarians. Pigeon pea is also known as Red gram, Tur, Arhar or Dal in India. Pigeon pea is member of family fabaceae, order fabales and genus *Cajanus*. It is often cross pollinated crop (20-70 per cent) having diploid chromosome number (2n = 22) with an estimated genome size of 833.07 Mb (Varshney *et al.,* 2011). It is short lived perennial, but traditionally, it is cultivated as an annual crop in Asia, Africa, Carribean region and Latin America .Thus it is becoming one of the major grain legume crops of tropics and subtropics. Considering natural genetic variability in pigeon pea and presence of its wild relatives in the region, it has been postulated that, India is the primary centre of origin of pigeon pea (Saxena *et al.*, 2008). Globally, it is cultivated on 4.92 mha with annual production of 3.65 mt and productivity is of 898 kg / ha. About 90% of the global pigeon pea area falls in India corresponding to 93% of global production/ (http://www.icrisat.org). Pigeon pea is second most important leguminous crop grown in India followed by chickpea. The area, Production and yield of pigeon pea during year 2012-13 in India is 3.38 mha, 2.27 mt and 671 kg/ha respectively (Kaur *et al.,* 2012).

It is being cultivated in more than 25 tropical and subtropical countries either as a sole crop or inter mixed with cereals such as sorghum (*Sorghum bicolour*), pearl millet (*Pennisetum glaucum*), maize (*Zea mays*) or with legumes e.g. peanut (*Arachis hypogea*). Pigeon pea seeds contain about 20-22% protein and appreciable amounts of essential amino acids viz., Methionine and Cysteine and minerals (Saxena *et al.,* 2008). It is a favorite crop of small holder dryland farmers, as it can grow well under subsistence level of agriculture and provides nutritive food, fodder and fuel wood. It is also a good source of fibres, vitamins and minerals. Pigeon pea is an excellent source of vitamin D and it also improves soil by fixing atmospheric nitrogen. Its foliage is used as fodder and milling by-products form an excellent feed for domestic animals.

The production of pigeon pea is constrained by use of unfertile land, water logging or dry spells during critical stages of crop growth, pest and diseases problems, narrow genetic base and lack of drought-resistant, high-yielding genotypes. The conventional plant breeding approach with improved Agricultural practices are not found sufficient to improve the pigeon pea production over last 50 years. While the application of various advancements in molecular biology, genetic transformation and *in-vitro* techniques have significantly contributed to improve the production and quality of several crops. However, these modern tools have not been commercialised in pigeon pea to combat the severe losses caused by several biotic (i.e. pest and diseases like Pod borers, Root knot nematodes, *Fusarium* wilt, Sterility Mosaic etc.) and abiotic (i.e. drought, salinity, water logging etc.) stresses. The chief factor among them is pod borer (*Helicoverpa armigera*). Pod borer is a lepidopteron insect pest becoming most serious and being infectious to all cultivated species of pigeon pea. Its larvae attack the flowers and pods of the pigeon pea, resulting in substantial damage and yield losses of over $ 300 million annually worldwide. (Shanower *et al.*, 1999). Pod borer problems is complex and intractable, no single control strategy is successful in keeping its population below economic threshold level (ETL). On the other side, indiscriminate use of pesticides to control pests has lead to series of consequences like insect resistance, pest resurgence, outbreak of secondary pest, harmful residual effects, imbalances in natural ecosystem and higher production costs which have been a concern in India and elsewhere. The wild relatives are available in pigeon pea, but possess very narrow genetic base towards their improvement of this crop through conventional plant breeding techniques. Therefore it is becoming important to develop a rapid transformation system for improvement of pigeon pea.

The advancement in Agricultural Biotechnology facilitates the transfer of cloned and well defined genes across the plant species through methods of genetic transformation viz., microprojectile bombardment, viral vectors, electroporation, sonication, *Agrobacterium*-mediated gene transfer etc. Among these, the most widely used method for genetic transformation in re-calcitrant crop like pigeon pea is the *Agrobacterium* mediated gene transformation (Horch *et al.*, 1985) although it is time

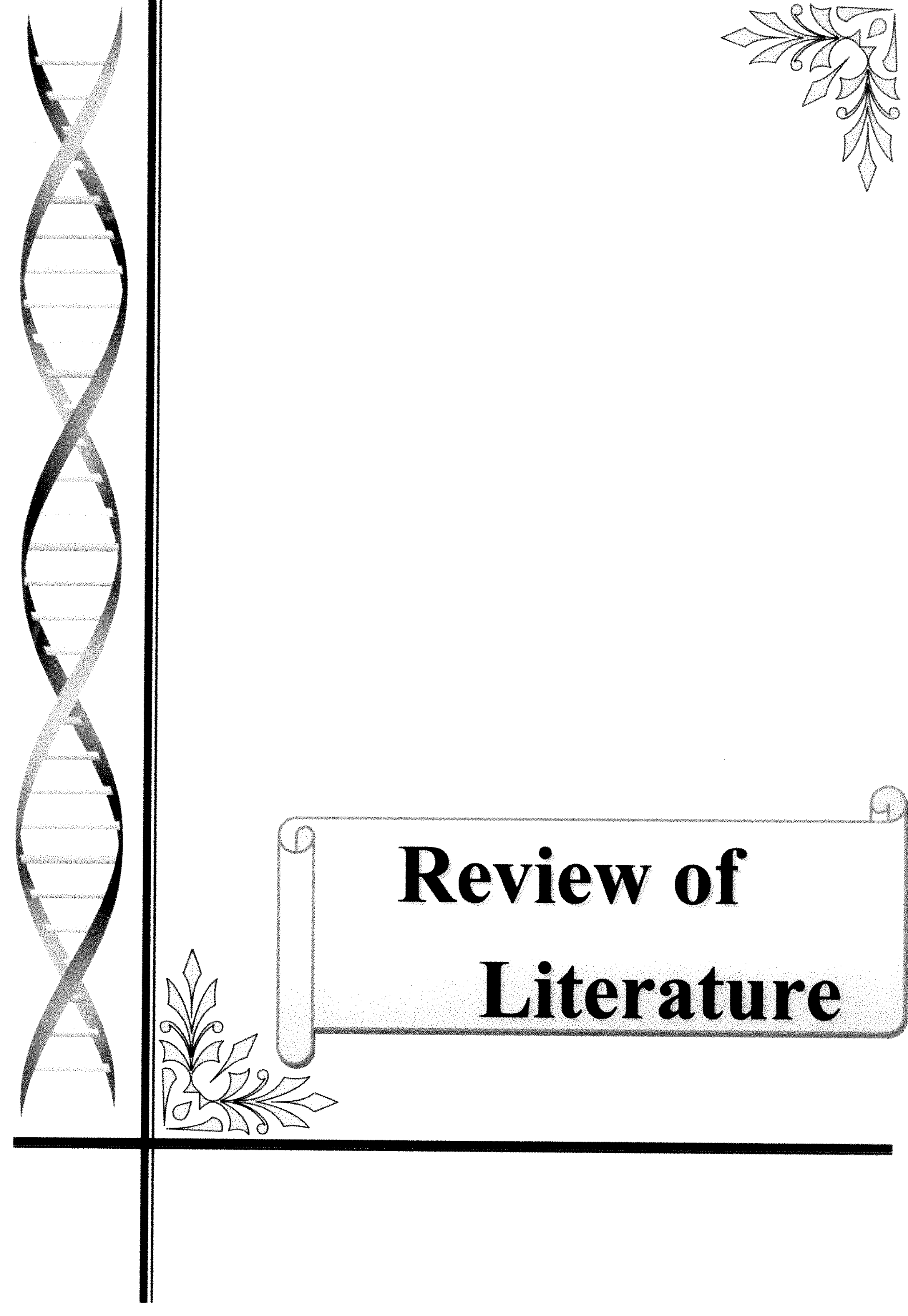
consuming, regeneration system dependant and has difficulty while controlling the overgrowth of *Agrobacterium* etc. Therefore, it is becoming an vital to develop and optimize *In-planta* transformation system in pigeon pea which enables rapid gene transformation in pigeon pea.

Discovery of genetic laws, transformation (Griffith 1928), nature of transforming principle (Avery *et al.,* 1944), elucidation of DNA structure (Watson and Crick,1953), discovery of plasmid, restriction enzymes (Smith, 1970), production of recombinant DNA (Cohen *et al.,* 1973), discovery of Ti plasmid (Schell, 1974) and production of transgenic plants (Schell, 1983) were milestones of scientific and technological developments which are essential to the advancement from the first green revolution to the anticipated second green revolution or gene revolution. Since cloned genes can be transferred directly or indirectly to the plant cells by different methods, there is no limitation with respect to the gene pool available. Additionally, well defined small groups of genes encoding defined and desired traits can be transferred. This enables the elite plant, to keep all its good characteristics intact. Genetic engineering is enabled crop improvements at the molecular level and produces plants with greater ranges of genetic variabilities. Transformation was first attempted in tobacco and Petunia in 1983 (Bevan *et al.,* 1983, Herrera - Estrella *et al.,* 1983; Fraley *et al.,* 1983).

A key component of most of the functional genomics approaches is a high-throughput transformation system which is emerging as an important tool of crop improvement. This becomes an evident in soybean (*Glycine max*) in which Roundup ready soybean cultivars have captured a major stake in market share of soybean planted in USA and Argentina. Transformation technique also offers strategies for over expression or suppression of endogenous genes to generate new phenotypic variation towards investigation of gene function for crop improvement. Thus, it is imperative to have an efficient regeneration and transformation system in order to introduce novel traits in crop like pigeon pea.

Therefore, in view of development of tissue culture independent rapid transformation system towards improvement of pigeon pea, optimization of *In-planta* transformation system was attempted with the following objectives:

1. Optimization of *In-planta* transformation conditions in pigeon pea.
2. Molecular analysis of pigeon pea transformants.


Review of
Literature

CHAPTER II

# REVIEW OF LITERATURE

Pigeon pea is the important crop in semi-arid tropics because of its draught tolerance and production of high yield in years when other crops result in faliure. Pigeon pea ranks fifth among the most important legume crops of the world after beans, peas, chick peas, broad beans and is second most important food legume in India after chickpea. It produces heavy protein rich seeds even on low fertility soils and offers a vast potential over a wide range of climatic and soil conditions in the tropics. It performs best on areas where annual rainfall ranges from 500-1500 mm/yr and where the soils are drained. It is a drought tolerant crop having deep and extensive root system, which provides easy access to water, stored deep in the soil profile (Sheldrake *et al.,* 1979). It can also endure within periods of water stress as a result of relatively high levels of desiccation to the tolerance and osmotic adjustment (Flower *et al.,* 1987). Pigeon pea is grown widely in a number of traditional cropping systems throughout India viz., intercrop system with a cereal (sorghum or millet) and as a long season crop with or without inter-cropping. Pigeon pea contains 17-28% protein and therefore plays an important role in supplementing the diets of the majority of the population in rural areas where low protein roots and cereal crops constitute the major part of the diet. Pigeon pea improves soil fertility by fixing atmospheric nitrogen and recycles, nutrients contributing to sustainability. Dry seeds are being used widely in India as dry split pea for dhal preparation for human consumption. In the Caribbean region, the pigeon pea is consumed as green vegetable (Nene and Sheila, 1990).

Genetic transformation systems have been described for many plant species which involves the delivery of defined foreign genes into plant cells and their expression in the regenerated plants. In present study, an attempt has been made to optimize *in planta* transformation protocols by using GUS reporter gene in pigeon pea genotype, BSMR 853. This genotype was exploited to estimate transformation efficiency, based on number of regenerated plantets. Very few literatures are available on *in planta* transformation of pigeon pea, hence the attempts have been made on *in planta* transformations in some other crops are also reviewed here.

Jaganath *et al.,* (2014) devised an efficient and reproducible *Agrobacterium*-mediated *in planta* transformation method in *Jatropha curcas*. They optimized a number of factors viz., decapitation, *Agrobacterium* strain, pin-pricking, vacuum infiltration duration and vacuum pressure for *in planta* transformation. They have adopted simple vegetative *in vivo* cleft graft method for multiplication of transformants without the aid of tissue culture. Among the various parameters evaluated, decapitated plants on pin-pricking and vacuum infiltration at 250 mm Hg for 3 min along with *Agrobacterium* strain EHA 105 harboring binary vector pGA-492 reported an efficient in all terms with a transformation efficiency of 62.66 %. Transgene integration was detected by the GUS histochemical assay and the GUS positive plants were subjected to grafting. Putatively transformed *J. curcas* served as "Scion" and the wild type *J. curcas* plant severed as "Root Stock". There was no occurrence of graft rejection and the plants were then confirmed by GUS histochemical assay, polymerase chain reaction (PCR) and Southern hybridization. Genetic stability of the grafted plants was evaluated by using randomly amplified polymorphic DNA (RAPD) marker which showed 100 % genetic stability between mother and grafted plants. This resulted into establishment of an efficient *in planta* transformation and grafting based multiplication of *J. curcas*.

The transient expression of recombinant proteins in plant tissues following *in planta Agrobacterium* mediated gene transfer as a promising technique for rapid protein production was studied by Simmons *et al.,* (2014). Attachment of *Agrobacterium tumefaciens* to lettuce leaf tissue was investigated in response to varying infiltration conditions, bacterial density, surfactant concentration and applied vacuum level. Bacterial density was found to most influence attachment levels among the levels tested ($10^8$, $10^9$, and $10^{10}$ CFU/ml). The surfactant levels tested (Break-Thru S240: 1, 10, 100 and 1,000 µl/L) also had a significant positive effect on bacterial attachment while vacuum level (5, 25 and 45 kPa) did not significantly affect on attachment in areas exposed to bacteria. They measured *in planta* transgene transient expression levels with various infiltration levels of bacterial suspension. The highest attachment level tested showed decrease in transient expression and suggested a potential link between bacterial attachment levels and downstream phenomena that may induce gene silencing. They explored the result that attachment can be controlled

by adjusting infiltration conditions and such attachment levels had influence of transient expression in leaf tissue.

Amar *et al.,* (2013) studied transient expression of foreign genes by *Agrobacterium* infiltration as a versatile technique that can be used as a rapid tool for functional analysis and gene silencing studies in grapevines. A reproducible protocol of *Agrobacterium* mediated transient gene transfer was developed for gene expression analysis in greenhouse grown grapevines. This became a complementary approach towards functional genomics and alternative to transgenic. Non-detached leaves from green cuttings were used as the target organ. The explant was subjected to vacuum infiltration for *in planta* inoculation with *Agrobacterium tumefaciens* harboring m*gfp* $_5$-ER gene construct as visual reporter gene. They revealed quality of greenhouse material as well as Agro-infiltration conditions were the major factors which influences successful gene expression assays. Following the optimized protocol, half of the infiltrated leaf surface displayed green fluorescent foci found in the inter-coastal areas. Daily monitoring of transient Green Fluorescent Protein (GFP) expression was achieved for 2 weeks post-infiltration and the highest expression level was recorded on day six. Evidence of GFP silencing in transgenic GFP expressing grapevine *via* Agro-infiltration was reported for the first time. Thus *in planta* infiltration system described here provides a powerful tool to explore easily gene function in grapevine.

*In planta* transformation protocol for large scale transgenic sugarcane production through *Agrobacterium* infection was developed by Mayavan *et al.,* (2013). Sugarcane seeds were infected with *Agrobacterium tumefaciens* strain EHA 105 harboring pCAMBIA 1304-*bar* and transformed plants were successfully developed without undergoing *in vitro* regeneration. They optimized various factors influencing sugarcane *in planta* transformation especially, pre-culture duration, acetosyringone concentration, surfactants, co-cultivation, sonication and vacuum infiltration duration etc. The transformed sugarcane plants were selected against BASTA® and screened by GUS, GFP assay, PCR and Southern hybridization. Among the different combinations of treatments tested, 12 h pre-cultured seeds sonication for 10 min followed by 3 min vacuum infiltration in 100 µM acetosyringone suspended in 0.1 % Silwett L-77 containing *Agrobacterium* suspension and further co-cultivation for 72h revealed, highest transformation efficiency. The amenability of the

standardized protocol was tested on five genotypes. It was found that, all the tested genotypes responded favorably, among which COC671 proved to be the best responding cultivar with 45.4 % transformation efficiency. The developed protocol was cost-effective, efficient, genotype as well as tissue culture independent and could generate relatively a large number of transgenic plants within 2 months.

Andrieu *et al.*, (2012) developed a rapid method for functional genomics study in rice based on the triggering of RNA interference (RNAi) through *Agrobacterium*-mediated transient *in planta* transformation of leaves. Using Agroinfection protocol combining a wound treatment and a surfactant they were able to obtain transient expression of T-DNA-borne *uidA* gene in leaf cells of Japonica and Indica rice. They inhibited gene expression in leaf cells by introducing hairpin RNA (hpRNA) T-DNA constructs containing gene specific tags of the phytoene desaturase (OsPDS) and slender 1 (OsSLR1) genes. SiRNA accumulation was observed in the Agroinfected leaf area for both constructs and indicated successful triggering of the silencing signal. Accumulation of secondary siRNA was observed in both stably and transiently transformed leaf tissues expressing the hpRNA-OSPDS construct.

Naseri *et al.*, (2012) used *in planta* method of genetic transformation to enhance the resistance to the sheath blight fungus of rice through expression of TLP gene under control of CaMV35S promoter. *Agrobacterium* strain harbouring desired gene was cultured in B medium and inoculated into embryonic apical meristem of soaked seeds by piercing up to a depth of 1-1.5 mm with a needle dipped in *Agrobacterium* inoculums. The transformed plants were confirmed by using polymerase chain Reaction (PCR) amplifying 710 bp fragment.

An attempt of *Agrobacterium tumefaciens* mediated transformation of Sunhemp was accomplished by Rao *et al.*, (2012). The protocol optimized could generate whole plant transformants with relative ease and found compatible to all genotypes of Sunhemp, wherein the entire embryo axis of the germinating seed was used as the target tissue for transformation. They standardized transformation conditions and further cotyledonary node of the embryo axis was infected with *Agrobacterium* host LBA 4404 harbouring the recombinant vector pCAMBIA 2301. The bivalent ID gene of the two major foot and mouth disease virus (FMDV) serotypes 'O' and '$A_{22}$' and the neomycin phosphotransferase *(nptII)* gene were used as the markers for optimization of the protocol. The germlings were then allowed to

grow under standard growth room conditions into mature fertile plants. Sixty $T_0$ plants were established from three separate experiments. Three hundred seeds from the sixty $T_0$ plants were sown and rose $T_1$ generation of which 180 were analysed for integration of bivalent FMDV and *nptII* genes. Eighteen out of these 180 plants amplified both the marker genes after PCR analysis. Two independent transgenic lines 24 and 37 showed elevated levels of expression of 12 µg and 8 µg (per gm of fresh leaf) of the bivalent ID antigen "O" and "$A_{22}$. The results showed that, the transformation efficiency was 3 %. ELISA and Western blot of the total protein of the genomic DNA and RT-PCR of positive plants was done to ascertain the expression and the antigenic nature of the expressed protein.

The *Agrobacterium tumefaciens* mediated *in planta* transformation protocol in castor *(Ricinus communis)* was developed by Kumar *et al.,* (2011). They infected two days old seedlings with *Agrobacterium* strain EHA105/pBinBt8 harbouring *Cry1AcF* and $T_0$ plants established in the green house. The $T_1$ generation seedlings were screened on 300 mg L-1 kanamycin and identified the putative transformants. Molecular and expression analysis were used to confirm the transgenic nature and identified high expressing plants through screening. Further, Western blot analysis was used to confirm the cointegration of the *nptII* gene in the selected transgenic plants. Bioassay against *Spodoptera litura* revealed, high expression and identified five promising lines. The feasibility of this method was adjudged by analysing stability of transgene at $T_2$ generation. Among 30 seedlings subjected to *in planta* transformation using EHA105/pBinBt8, 14 individual could establish well and grew normally, flowered and set seed in the green house. The $T_1$ generation plants were further evaluated for transgenic analysis using PCR, Western blot and ELISA techniques.

Razzaq *et al.,* (2011) developed a cheap, efficient and genotype independent protocol for *in planta* transformation in wheat without involvement of any tissue culture procedures. Seeds were surface sterilized, soaked and incised through apical meristem. The wounding of seed was performed without damaging the embryo of seed. Wounded seed was inoculated in *Agrobacterium* culture harbouring *GUS* and *nptII* genes in plasmid. Further seeds were co-cultivated for three days followed by washing and regeneration. The regenerated seeds were further subjected to kanamycin selection. Among 1850 inoculated seeds, 208 kanamycin resistant plants were obtained. During analysis twenty seven percent of the total plants were shown

positive *GUS* activity. Further polymerase chain reaction (PCR) amplification of *nptII* gene using specific primers also confirmed the integration of transgene in kanamycin resistant plants.

The *in planta* and Agro-infilitration methods of soybean transformation were described by Zia *et al.,* (2011). Overnight grown *Agrobacterium tumefasciens* strain harbouring desired plasmid was injected into soybean pods at three different developmental stages. The seeds derived from pods were assessed for transformation through GUS histochemical analysis and PCR. Three plants of genotype NARC-7 and two plants of genotype NARC-4 showed transformation efficiencies of 14.2% and 6.45% respectively at first stage of development. Highest GUS expression i.e. 39.16% was observed in NARC-7 genotype during third stage of development. They described method of direct production of transformed seeds that can be further analysed at progeny level.

Lie *et al.,* (2010) excised tap roots from four days old seedlings of soybean cultivars 'Jack' and 'KS4704' and hairy roots were induced from hypocotyls via *Agrobacterium rhizogenes* mediated transformation. Inoculated hypocotyls were screened on a MS medium containing 200 mg/L kanamycin or 20 mg/L hygromycin. The *β-glucuronidase (GUS)* assay revealed, highest GUS expression in hypocotyls exposed to a 4 days pre-inoculation time, neutral pH (7.0). Further they cloned a 170 bp of the *Fib1* gene and 292 bp of the *Y25C1A.5* gene fragment, both related to nematode reproduction and fitness. The RNAi strategy was transformed in soybean by using the chimeric hairy root system and evaluated for their effect on soybean cyst nematode (*Heterodera glycines*) fecundity. They have confirmed transgenics by polymerase chain reaction and Southern blot analysis. This method could take on average four week to produce chimeric plants ready for transgene analysis.

Xiaoli *et al.,* (2010) developed a simple and rapid method of transformation of *Brassica napus* by dipping directly into an *Agrobacterium tumefaciens* suspension, supplemented with surfactant. They also reported a method selection of transformant by soaking seeds with antibiotic. *Brassica napus* cv. "Ningyou 16" was used as the transformation recipient plant. Transformation of *Brassica napus* were performed by the floral dip method using *Agrobacterium* strain LBA4404 (Clough, 1998). Binary plasmids pCAMBIA2200 which confers kanamycin resistance via *npt II* gene and pCAMBIA1300 confirming hygromycin B resistance via *hpt* gene were used in transformation. The initial bacterial cultures were grown in 5ml of LB liquid medium

with corresponding antibiotic at 28°C and 220 rpm, later shifted to 500ml LB liquid medium at 28°C and 220 rpm for two more days. The rapeseed plants were transformed at bud stages (the initial blossom stage). The plant racemes were submerged in a 20 ml beaker with *Agrobacterium* medium containing 3% sucrose, 0.1% Silwet L-77, 2 ng/mL 6-BA and 8 mg/L acetosyringone. Further plant selection was made by soaking seeds with kanamycin monosulphate solution at concentration of 300 mg/L or hygromycin B at a concentration of 100 mg/L. The seeds subjected to 24-36 h incubation were sown in the soil. Transformants were confirmed through PCR based detection. The PCR analysis predicted that, *Agrobacterium* medium containing Silwet L-77, sucrose, 6-BA and acetosyringone found efficient in transformation by amplifying transformed DNA with target gene.

Agarwal *et al.*, (2009) described a method for the floral dip transformation of the wheat. The method involves pre-anthesis spikes with clipped florets at the early, mid to late uninucleate microspore stage were dipped in *Agrobacterium* infiltration media harbouring a vector carrying anthocyanin reporters and *npt II* selectable marker. Seeds from $T_1$ progeny were examined for colour changes induced in the embryo by the anthocyanin reporters. Putatively transformed seeds were germinated and the seedlings were screened for the presence of the *npt* II gene based on resistance to paramomycin spray and assayed with *npt* II ELISA. Further transgenics were confirmed by Southern hybridization. The transformation efficiencies were ranged between 0.3 – 0.6 % under un-optimized conditions.

Smagur *et al.*, (2009) evaluated two methods viz., floral dip, vacuum infiltration of *in planta* transformation in *Arabidopsis thaliana*. The work was primarily conducted to identify the best developmental stage of *A. thaliana* based upon the identification of the developmental stages of *A. thaliana* for successful transformation. They identified flower developmental stage is the most appropriate and efficient stage of transformation in *A. thaliana*. They used two plasmids viz., CAMBIA 1305.1 and CAMBIA 2301 in transformation experiment and expression of reporter *GUS* gene was analysed by molecular and histochemical methods. A most efficient treatment of floral dip transformation was revealed, 2 min long flower bud inoculation and 400 µl/L surfactant while in case of vacuum infiltration method gave 1.73% and 2.01% transformation efficiency with vector pCAMBIA 1305.1 and pCAMBIA 2301 respectively. The highest efficiency of the transformation was reported when the inoculation time was 4 min.

The transformation of *indica* rice varieties using traditional transformation methods due to their poor regeneration is very difficult (Lin *et al.,* 2009). In this regard, they developed a simple *in planta* method of transformation of *indica* rice. In this method, the mature embryos of soaked seeds were pierced by a needle and soaked in the *Agrobacterium* inoculum under vacuum infiltration. The inoculated seeds were germinated and grown to $T_0$ maturation stage under non sterile conditions. The transgenics were further confirmed by antibiotic and molecular analysis of putative $T_0$ plants. Transformation efficiency was reported up to 6.0%. Further, they revealed this method of transformation of *indica* rice was significantly short and easier.

Yasmeen *et al.,* (2009) reported *in planta* transformation of tomato (*Solanum lycopersicum* L.), using fruit injection and floral dip method. *Agrobacterium tumefaciens* strain EHA 105 containing three constructs, i.e. pROKIIAP1-GUSint (carrying *Apetala 1* [*AP1*] gene), pROKIILFY-GUSint (carrying *LEAFY* [*LFY*] gene) and p35SGUSint (carrying *β-glucuronidase* [*GUS*] gene) was used for plant transformation. In case of injection transformation method, no significant effects of the construct were observed. However, the highest frequency of transformation was obtained following 48h incubation of tomato fruit with bacterial cells harbouring either one of the three constructs. Transformation frequencies for *AP1*, *LFY*, and *GUS* gene constructs were 17%, 19% and 21% respectively. While in fruit injection experiments, mature red fruit resulted in higher frequency of transformants than immature green fruit with 40%, 35% and 42% for *AP1*, *LFY* and *GUS* gene constructs, respectively.

Keshamma *et al.,* (2008) developed transgenic cotton plants by tissue culture independent *Agrobacterium tumefaciens* mediated transformation procedure. *Agrobacterium* strain LBA 4404 harbouring binary vector pKIWI105 possessing *beta-glucuronidase* (GUS) and *neomycin phosphotransferase* (*npt*) *II* genes were used for transformation. The seeds with emerging plumule were infected by pricking with a sterile sewing needle at the meristem and subsequently dunked into *Agrobacterium* culture for 60 minutes. Following infection, the seedlings were transferred to autoclaved soil moistened with water for further development. Out of 50 seedlings subjected to *Agrobacterium* infection, 37 plants survived and were transferred to green house. Among 37 plants, 25 plants survived, attained maturity, flowered and set seed. However, the growth of transformed plants was slow compared to untransformed controls. Among $T_3$ generation, eleven plants were selected for

flourimetric analysis of GUS activity. Ten plants continued to retain inheritance and expression of the gene *uid* A.

Rao *et al.*, (2008) described a non tissue culture based method of generating transgenic pigeon pea using Ti plasmid mediated transformation system. They raised $T_0$ plants directly from *Agrobacterium* infected young seedlings. Out of the 22-25 primary transformants, 15 plants on the average established on the soil under greenhouse conditions, showed slow growth initially, flowered and set seed eventually. Of the several seeds harvested from all the $T_0$ plants, six hundred seeds were sown and obtained $T_1$ plants. Among these $T_1$, 350 plants were randomly analysed in PCR with GUS (*uid* A) and *npt II* gene towards confirmation of transgenic nature. PCR was performed for both *GUS* (*uid*A) and *nptII* genes. Forty eight out of 350 $T_1$ plants were amplified for both transgenes. Southern blot analysis also reported successful integration and transmission of these genes.

Seol *et al.,* (2008) developed *in planta Agrobacterium tumefaciens* mediated transformation with vacuum infiltration, pin-pricking and a combination of these two methods in *Notocactus scopa* cv. *Soonjung.* The pin-pricking combined with vacuum infiltration method (20–30 cm Hg for 15 min) resulted in transformation efficiency of 67–100% and the expression of the *uid* A and *nptII* genes was detected in transformed cactus. The established *in planta* transformation technique generated a transgenic cactus with higher transformation efficiency, shortened selection process and stable gene expression via asexual reproduction. They reported that, the *in planta* transformation method used in the transformation study proved an efficient and time saving procedure for the delivery of genes into the cactus genome and the technique could be useful in other asexually reproducing succulent plant species.

Ana *et al.,* (2007) analysed two different *in planta* transformation methods viz., vacuum infiltration and infiltration by syringe to develop a reliable and rapid transgenic system for functional study of specific buckwheat gene constructs. An overnight grown culture of *A. tumefaciens* (10 ml) was inoculated in 1 L of LB medium supplemented with 150 µM acetosyringone (AS), 50 µg/ml kanamycin and 10 µg/ml rifampicin. Bacterial cultures were grown overnight at 28°C to OD 600 ranging from 0.8 to 2.1, centrifuged and resuspended in ½ MS infiltration medium containing 5% sucrose, 2.6 µM MES, 44 nM 6-BAP, 150 µM AS, 0.02% Silwett L-77, pH 5.7. Plants were removed from pots and immersed in 1L of infiltration medium an hour before vacuum infiltration. Two different vacuum conditions were

used for infiltration [pressure of 104 Pa for 5 min and 102 Pa for 20 min]. They revealed that, vacuum infiltration method was the method of choice for transient buckwheat transformation.

A simple and an efficient method of *in planta* transformation for wheat using *Agrobacterium tumifaciens* was developed by Supartana *et al.,* (2006). The transformation procedure involved soaking of wheat seeds at $22^0$C for one day. The embryos of the soaked seeds were inoculated with *Agrobacterium tumificiens* by piercing a region of embryonic apical meristem through needle already dipped in *A.tumifacians* inoculum. The inoculated seeds were incubated at $22^0$C for two days and sterilized by cefotocxime (1000 ppm water solution) treatment and vernalized at $5^0$C for 25 days. The seedlings were grown to maturation ($T_o$ plants) and allowed to pollinate naturally for seed setting ($T_1$ progeny) in pots under non sterile conditions. The transformation efficiency of $T_1$ plants was detected 33% by PCR analysis, 75% by Southern hybridization and 40% by plasmid rescue methods.

*In planta* transformation of narrow leafed lupin (*Lupinus angustifolius*) by vacuum infiltration with *Agrobacterium* was attempted by Ratanasanobon *et al.,* (2004). They wounded apical area of young shoots by sonication prior to infiltration with *Agrobacterium* cells containing a reporter gene (35S *Gus*) and a selectable marker gene (35S *bar*) for herbicide tolerance. Sonication time and infiltration time were standardized towards optimization of transformation conditions. Thirteen minutes of sonication and 10 minutes of infiltration with *Agrobacterium tumefaciens* AGL0 were the best conditions for transformation. However, longer times increased the number of fatalities of seedlings. Similarly, they investigated other important parameters, namely bacterial growth phase and the composition of infiltration solution, in order to increase stable transformation as determined by the number of blue spots in the tissue. They have screened 1720 treated plants towards analysis of expression of the *bar* gene through seed derived from application of 50 mg/L phosphinothicin (PPT). All the seedlings were died in a manner, similar to the controls. They reported that, on explants *in vitro* conditions, T-DNA transfer began at day 3 of the co-cultivation period, then increased rapidly to a maximum by day 5. They reported that, *A. tumefaciens* had a very low rate of survival on the seedlings. The survival of *A. tumefaciens* cells on the plant was very low, about 103 times less than required for successful transformation of lupins via the routine *in vitro* route

(CLIMA lupin transformation protocol). Thus, they concluded that, *in planta* transformation has not yet been possible in lupin.

Curtis *et al.,* (2001) successfully produced transgenic radish (*Raphanus sativus* L. *longipinnatus* Bailey) plants through *in planta* transformation method carrying *GUSA* and *bar* genes. They evaluated the importance of development of the floral dipped transgenic plant with presence of surfactant in the inoculation medium. Plants dipped at the primary bolt stage of growth into a suspension of *Agrobacterium* containing 0.05% (v/v) Silwet L-77 resulted in optimum transformation efficiency of 1.4%. They reported that, the presence of surfactant, Pluronic F-68 or Tween 20 in the inoculation medium was found significant treatment compared to treatments without surfactant. Putative transformed $T_1$ plants were tested positive for gus activity when analysed both histochemically and fluorometrically. Southern analysis revealed that, both the *gus*A and *bar* genes integrated into the genome of transformed plants and segregated as dominant Mendelian traits. Based on these results, they demonstrated that, radish can be genetically modified for the improvement of important vegetable crop.

Buckwheat Agro-infiltration by syringe was performed by Yang *et al.*, (2000). *Agrobacterium* cultures were grown overnight at 28°C and 150 rpm to OD 600 ranging between 0.8 to 2.1. Cells were collected by centrifugation and resuspended in infiltration medium [10 mM $MgSO_4$, 10 mM MES (pH 5.5), 150 µM AS]. The bacterial suspension was infiltrated by applying pressure against the lower side of a young leaf with a needle less syringe. After infiltration, plants were kept covered with transparent plastic bags for two days without watering. The level of transient GUS activity monitored by fluorescence assay (Jefferson, 1987) was used as a measure of transformation efficiency. The results were obtained under defined optimal conditions namely *Agrobacterium* culture density, vacuum conditions and leaf maturity. The median value obtained for GUS activity during vacuum infiltration was 57.3 times higher than that obtained with normal infiltration by syringe. The results indicated that the vacuum infiltration method was much more efficient and can therefore be considered the method of choice for buckwheat transformation.

Ching and Wang (1999) developed two non tissue culture based soybean transformation procedures to overcome the recalcitrancy in soybean viz., pollen tube pathway, ovarian injection etc. In pollen tube pathway, introduction of exogenous total genomic DNA of *Glycine gracilis* and ovarian injection with exogenous plasmid

DNA containing atrazine resistant gene. Ovarian injection resulted in the production of atrazine resistant $F_1$, $F_2$ and $F_3$ plants. They revealed both exogenous single genic Mendelian traits and multi-genic quantitative traits were transferable with these simple and inexpensive procedures.

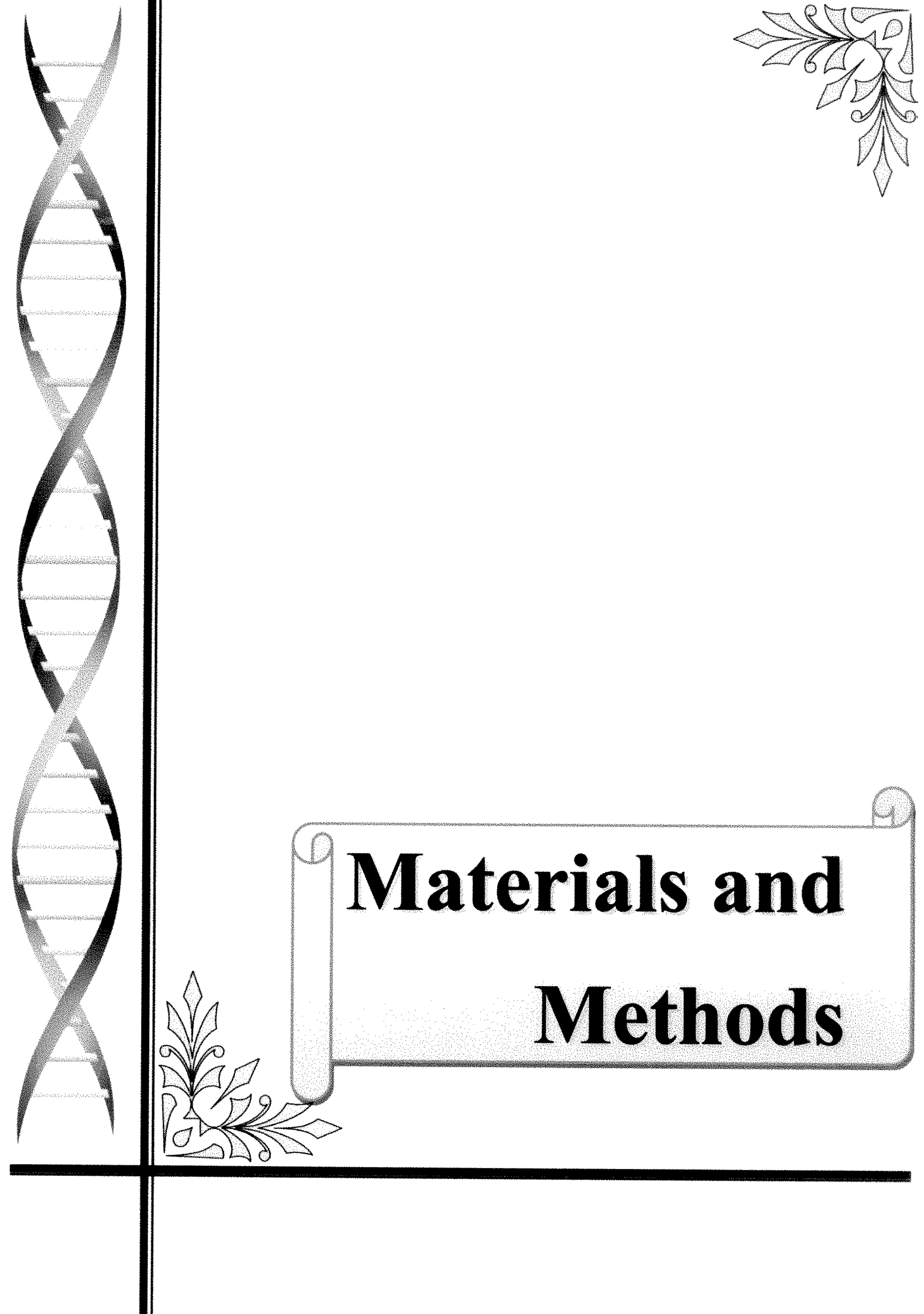
Clough and Bent (1998) used simple dipping of floral tissues and produced transgenic Arabidopsis plants without involvement of tissue culture and regeneration system. Floral tissues of *Arabidopsis thaliana* were dipped into a solution containing *Agrobacterium tumefaciens* culture containing 5% sucrose and 500 µl/L of surfactant, Silwet L-77. They revealed that, repeated application of *Agrobacterium,* improves transformation rates and over all yield of transformants approximately two fold. Further they covered inoculated plants for one day to retain humidity and reported increase in transformation efficiency twice. They used kanamycin selection pressure towards detection of the transformed progeny seedlings and the validity of this method was confirmed by DNA hybridization experiments.

Feldmann and Mork (1987) developed seed transformation procedure for the development of an insertion mutagenesis system in *Arabidopsis thaliana*. The aim of this experiment was to develop a system which would be less labour intensive than traditional tissue culture methods. They co-cultivated imbibed $T_1$ seeds with *Agrobacterium* and the plants were grown to maturity. Transformants were selected from the $T_2$ progeny of these infected plants. Further selected $T_2$ plants were found heterozygous for the insert(s) and their progeny ($T_3$) were screened for insertional mutants.

Chee *et al.,* (1989) transformed soybean by using an avirulant strain of *A. tumefaciens* containing the binary vector pGA482. They used plumule, cotyledonary node and adjacent cotyledonary tissue of germinating seeds for agroinfection. They identified neomycin phosphotransferase (*npt-II*) enzyme activity in tissues of 16 ($R_0$) soybean plants and reported that transformation of *npt II* gene into somatic tissue. They concluded that, the advanced $R_0$ soybean plants produced $R_1$ plants and the transformation efficiency during this investigation was reported up to 0.1%.

Jefferson *et al.,* (1987) used the *E. coli* β-*glucuronidase* gene (GUS) as a fusion marker for the analysis of gene expression in transformed plants. They have designed gene fusion experiment by using cauliflower mosaic virus (CaMV) 35S promoter and directed the expression of β-*glucuronidase* in transformed plants. Expression of GUS was measured accurately by using fluorometric assays of small

amounts of transformed plant tissue. They reveled that, plants expressing GUS were found normal, healthy and fertile. They also reported that, the GUS is very stable and tissue extracts continued to show high levels of GUS activity after prolonged storage.


Materials and
Methods

CHAPTER III

# MATERIALS AND METHODS

The materials required and methodology utilized towards the optimization of *in planta* transformation parameters in pigeon pea were described in this chapter.

## 3.1 Surface sterilization and seedling development

### 3.1.1 Material requirement

Following equipments and glassware were used for the completion of this investigation.

**a) Equipments**

- Laminar air flow cabinet (LAF)
- Autoclave
- Hot air oven
- pH Meter
- Photometer (eppendorf)
- Thermocycler (eppendorf)
- Gel documentation system (Alpha Innotech)
- Orbital shaker (REMI)
- Electrophoresis Unit (SCI-PLUS)
- Centrifuge machine (eppendorf)
- Weighing Balance (Sartorius)

**b) Glasswares**

- Culture bottles
- Culture tube
- Reagent Bottles (250 ml, 500 ml, 1000 ml)
- Beakers
- Measuring cylinder
- Petridishes
- Micropippets
- Conical Flask

### 3.1.2 Selection of Genotype

Seeds of pigeon pea cultivar BSMR 853 procured from Agricultural Research Station (ARS), Badnapur was used during course of this investigation. Following overnight soaking in distilled water, seeds were surface sterilized with 1% (w/v) Bavistin for 10 min and later with 0.1% (w/v) $HgCl_2$ for 5 seconds, rinsed with distilled water following treatment with each sterilant. Seeds were later germinated in ½ MS germination medium for 2-3 days old stage at which infection to be carried out was determined.

**Table 3.1: The varietal characteristics of genotype BSMR 853**

| Sr. No. | Characteristic features of selected cultivar | BSMR-853 |
|---|---|---|
| 1 | Plant Branching Pattern | Semi spreading (30-60) |
| 2 | Plant Growth Habit | Indeterminate |
| 3 | Stem Colour | Sunred |
| 4 | Flower Colour | Red |
| 5 | Plant Height (cm) | 145-150 |
| 6 | Seed Colour | White |
| 7 | Maturity (time of duration) | 178-180 days |
| 8 | Resistance | Sterility Mosaic & Wilt |
| 9 | Yield | 14-15 q/ha |
| 10 | Protein (%) | 21% |

### 3.1.3 Preparation of Stock solution

Stock solutions of major and minor elements (8X) and vitamins (50X) were prepared as per composition given by Murashige and Skoog (1962).

**Table 3.2: Components of stock solution of 8X and 50X**

| Major & Minor Elements | Chemical Formula | Qty (mg/L) | Volume taken for one litre medium |
|---|---|---|---|
| **Stock A: Major nutrients (8X)** | | | |
| Ammonium nitrate | $NH_4NO_3$ | 13200 | 125 ml |
| Potassium nitrate | $KNO_3$ | 15200 | |
| Calcium chloride | $CaCl_2 . 2H_2O$ | 3520 | |
| Magnesium sulphate | $MgSO_4 . 7H_2O$ | 2960 | |
| Potassium dihydrogen Orthophosphate | $KH_2PO_4$ | 1360 | |
| Potassium iodide | KI | 06.64 | |
| Boric acid | $H_3BO_4$ | 49.60 | |
| Manganese sulphate | $MnSO_4 . 4H_2O$ | 178.40 | |
| Zinc sulphate | $ZnSO_4 . 7H_2O$ | 68.80 | |
| Sodium molybdate | $Na_2MoO_4 . 6H_2O$ | 02.00 | |
| Copper sulphate | $CuSO_4 . 5H_2O$ | 00.20 | |
| Cobalt chloride | $CoCl_2 . 6H_2O$ | 00.20 | |
| Disodium EDTA | $Na_2$ EDTA | 298.40 | |
| Ferrous sulphate | $FeSO_4 . 7H_2O$ | 222.40 | |
| **Stock B: Vitamins and others (50X)** | | | |
| Inositol | $C_6H_{12}O_6$ | 5000 | 20 ml |
| Glycine | $C_2H_5NO_2$ | 100 | |
| Thiamine HCl | $C_{12}H_{17}ClN_4OS.HCl$ | 5 | |
| Pyridoxine HCl | $C_8H_{11}ClNO_3HCl$ | 25 | |
| Nicotinic acid | $C_6H_5NO_2$ | 25 | |

**Note:** Dissolve ferrous sulphate ($FeSO_4 . 7H_2O$) and disodium EDTA ($Na_2EDTA$) separately. Dissolve disodium EDTA into warm sterile water and then add ferrous sulphate to the same solution and dissolved under continuous stirring

### 3.1.4 *In planta* transformation of pigeon pea

Following transforming parameters and treatment conditions were used for *in planta* transformation of pigeon pea cv. BSMR 853

**Material requirement:**

1. YEM medium
2. Inoculating needle
3. Acetosyringone (150-250μM)

**Table 3.3: Treatment parameters used for *in planta* transformation of pigeon pea.**

| Sr. No. | Optimizing parameter | Treatment | No. of seedlings inoculated |
|---|---|---|---|
| 1. | Bacterial O. D. | 0.5, 1.0 and 1.5 | 15 |
| 2. | Acetosyringone (μM) | 150, 200 and 250 | 15 |
| 3. | Infection time (min) | 0.5, 1.0 and 1.5 | 15 |

**Table 3.4: Treatment combinations used for *in planta* transformation experiment.**

| Sr. No. | Treatments $OD_{600}$ + Virulence inducer (μM) + Inoculation time (min) | No. of seedlings inoculated |
|---|---|---|
| 1. | 0.6+150+0.5 | 15 |
| 2. | 0.6+150+1.0 | 15 |
| 3. | 0.6+200+1.5 | 15 |
| 4. | 0.6+200+0.5 | 15 |
| 5. | 0.6+250+1.0 | 15 |
| 6. | 0.6+250+1.5 | 15 |
| 7. | 1.0+150+0.5 | 15 |
| 8. | 1.0+150+1.0 | 15 |
| 9. | 1.0+200+1.5 | 15 |
| 10. | 1.0+200+0.5 | 15 |
| 11. | 1.0+250+1.0 | 15 |
| 12. | 1.0+250+1.5 | 15 |
| 13. | 1.5+150+0.5 | 15 |
| 14. | 1.5+150+1.0 | 15 |
| 15. | 1.5+200+1.5 | 15 |
| 16. | 1.5+200+0.5 | 15 |
| 17. | 1.5+250+1.0 | 15 |
| 18. | 1.5+250+1.5 | 15 |

**Note:** 15 plants were inoculated under each treatment in triplicates.

### 3.1.5 Antibiotics

- Kanamycin 50 mg/l

### 3.1.6 Histochemical *GUS* analysis

The histochemical *GUS* analysis was carried out to determine the β-glucuronidase activity in the putatively transformed plantlets.

**Reagents:**

**A) Phosphate Buffer**

1. 1M Monobasic Sodium Phosphate ($KH_2PO_4$)
2. 1M Dibasic Sodium Phosphate ($K_2HPO_4$)
3. Phosphate buffer solution, pH 7.0 stored at 4°C.

**B) Triton/Ethanol Stock**

Prepared 100 ml stock by adding 10 ml of Triton X-100, 40 ml ethanol and 50 ml sterile water and kept at room temperature.

**C) Staining solution**

Prepared 100 ml staining solution by taking 94 ml phosphate buffer, 1.0 ml Triton/Ethanol and 5 ml X-gluc solution.

### 3.1.7. Reagent for DNA isolation and PCR based confirmation of putative transformants

- Leaf sample
- Mortar and pestle
- TE buffer (pH 8.0):
  1) 100 mM Tris-HCl
  2) 1.0 mM disodium EDTA
- DNA Extraction buffer:
  1) 100 mM Tris-HCl (pH 8.0)
  2) 20 mM disodium EDTA
  3) 0.5 M NaCl
  4) 1 % SDS
- 7.5M Ammonium acetate
- 99 % ethanol
- 70 % ethanol

- *RNase*
- Agarose
- 6X gel loading dye
- Electrophoresis buffer
- Ethidium bromide
- Sterile water

## 3.2 Methodology:

### 3.2.1 Procedure for preparation of ½ MS media

- One litre of MS basal media was prepared by taking 62.5 ml of stock of major nutrient (8X) and 10 ml of stock of minor nutrient (50X) to the clean and sterile beaker and stirred well with help of magnetic stirrer.
- The major source of carbon (Sucrose) was added at a concentration of 3% and dissolved to the medium while continuous stirring, the volume of solution was made up to 900 ml with double distilled water.
- The pH of the medium was adjusted to 5.8 by using 0.1N HCl and 0.1 N NaOH before sterilization.
- Final volume of solution was made up to one litre by using double distilled water.
- Solidifying agent, Agar-agar was added to the medium at 0.8% concentration and mixed thoroughly to the medium by gently heating of the media.
- The required quantity of media approximately 30 ml per culture bottle was added and labelled properly.
- Culture bottle containing media were autoclaved at $121^0$C at 15 lbs for 20 min.
- After sterilization the media was allowed to cool and solidify till further inoculation of seed of pigeon pea.
- The bottles were sealed with parafilm and stored at $25^0$C until use.

### 3.2.2 Surface sterilization and seed germination

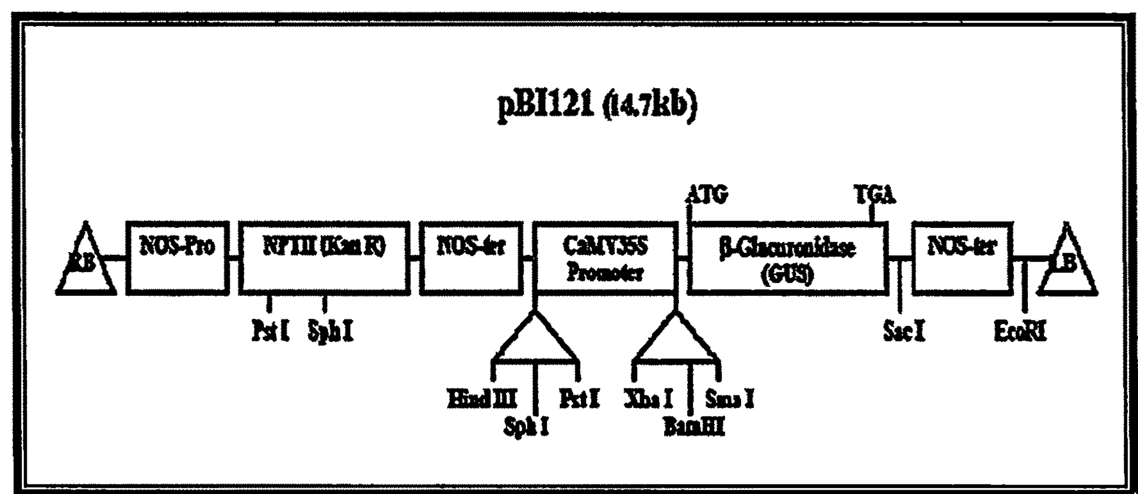
The pigeon pea seeds of BSMR-853 were washed in distilled water initially and then rinsed in 70% ethanol for 5 minutes. This step was repeated twice. Then the seeds were treated with 0.1% $HgCl_2$ solution for 2 minutes followed by washing in double

distilled water for 5 minutes to remove the traces of surface sterilent. The sterilized seeds were placed in ½ MS seed germination medium for 3-5 days.

### 3.3 *In planta* tansformation of pigeon pea

#### 3.3.1 Gene constructs

The *Agrobacterium* strain EHA101 harbouring the *pBI121* gene construct containing *GUS* gene procured from NRCPB, New Delhi, India were used in this study.




Gene construct *pBI121* made up from engineered reporter gene, β-glucuronidase (*GUS*) and selectable marker gene, kanamycin monophosphate resistance (*kan*[R]) driven by promoter CaMV 35S. The gene construct had number of restriction sites such as *Pme*I, *Bsu*36I, *Apa* I, *Cla* I, *Hind* III, *Bam* HI and *Eco*RI.

Figure 1 (a): Schematic representation of gene construct *pBI*121 harbouring GUS gene.

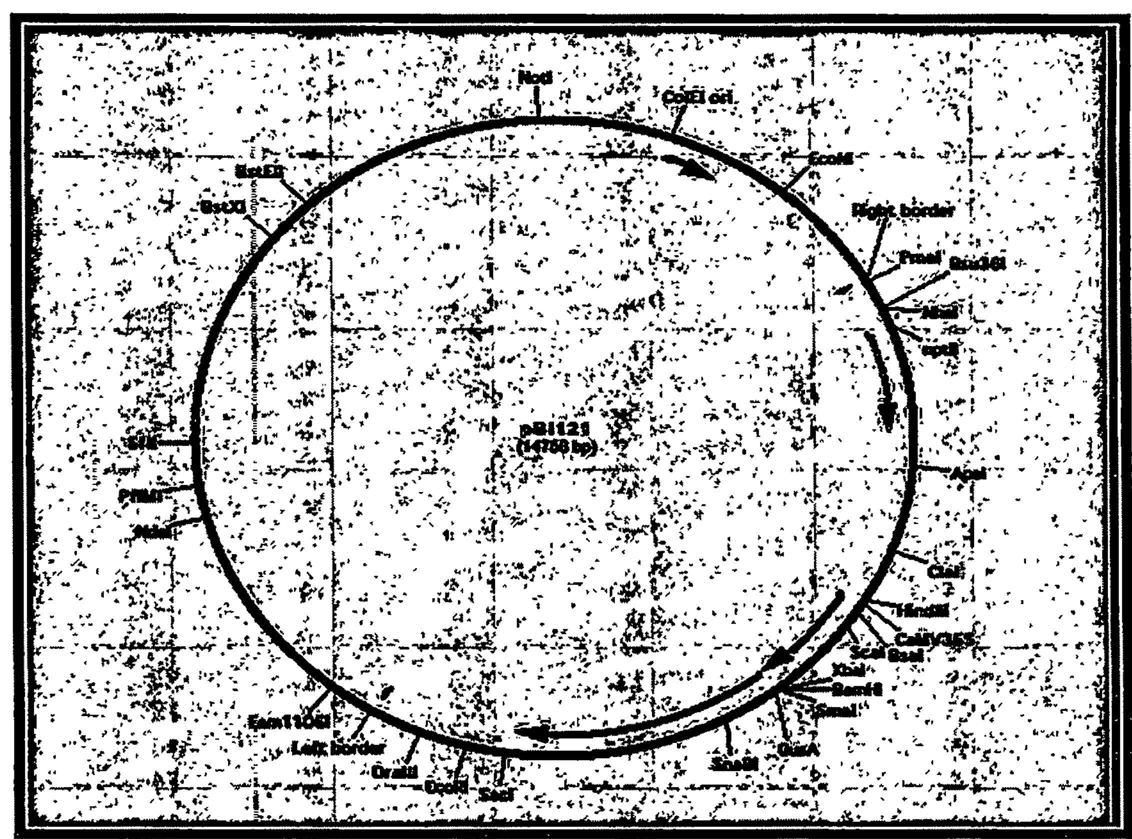


Figure 1 (b): The circular map of gene construct *pBI*121 showing multiple cloning sites and recognition sequences.

### 3.3.2 Preparation of *Agrobacterium* culture

A single colony of *Agrobacterium tumefaciens* strain, EHA101 harbouring the binary plasmid *pBI121* containing *GUS* gene was inoculated in 25 ml of YEM liquid medium supplemented with 50 mg/L kanamycin and incubated at $28^0$C on shaker at 200 rpm for 18 h. The bacterial growth of 0.6 O.D. values was taken for transformation. The suspension culture was pelleted at 5000 rpm at $4^0$C for 10 min. The pellet was resuspended in an equivalent volume of MS basal liquid medium and stored at $4^0$C. This bacterial suspension of different treatments was further used for *in planta* transformation of pigeon pea.

### 3.3.3 Procedure for *in planta* transformation of pigeon pea

*Agrobacterium tumefaciens* strain, EHA101 harbouring the binary plasmid *pBI121* containing *GUS* gene was used for transformation. The vector contains the *neomycin phosphotransferase II* (*nptII*) gene driven by the nopaline synthase promoter.

*Agrobacterium* was grown overnight at $28^0$C in 25 ml of YEM medium (pH 7.0) containing 50 µg/ml kanamycin. The bacterial culture was later pelleted at 6000 rpm for 5 min. The *Agracterium* pellets were resuspended in 50ml liquid ½ MS medium and stored at $4^0$C till further use. The suspension culture approximately 15 ml of *Agrobacterium* strain EHA101 harbouring *GUS* gene was taken separately in sterile Petridish. Further Acetosyringone was added at different concentrations (150, 200 and 250µM) to increase efficiency of transformation. The needle incised germinated seedlings were dipped into the suspension culture containing *GUS* gene in ½ MS media for 5-10 seconds and shake at 50 rpm for 5 minutes. The seedlings were removed from the suspension culture and dried on sterile filter paper. Further the inoculated seedlings/ germlings were sown in plastic cup containing sterile cocopeat. Further these plants were transferred into plastic pot containing sand, soil and FYM and grown upto the maturity in green house.

### 3.4 Molecular analysis of the putative transgenic plants

The Putatively transformed pigeon pea plantlets grown under greenhouse conditions were subjected to histochemical *GUS* assay and PCR analysis to check integration of the *GUS* gene.

#### 3.4.1 Histochemical *GUS* analysis

The histochemical *GUS* assay was performed at shoot initiation and developmental stage of transformed pigeon pea plants. This assay was used to check the presence of *GUS* gene incorporation into transformed pigeon pea plants. Shoots of putative pigeon pea transformants were dipped into 20 ml assay solution and incubated at $37^0$C overnight in the dark chamber through wrapping with aluminium foil. Based on appearance of blue colour precipitate into pigeon cells the qualitative analysis of pigeon pea transformation was performed.

#### 3.4.2 PCR based detection of putative pigeon pea transformants

Genomic DNA isolated from the putatively transformed plantlets was subjected to PCR analysis. A primer specific to *GUS* gene amplifying an expected fragment of 750 bp was used for confirmation of transformed pigeon pea plantlets.

#### 3.4.2.1 Extraction of genomic DNA for PCR analysis

The protocol standardized by Lie *et al.*, (2007) was utilized for DNA isolation from putative transgenic pigeon pea plants. Leaf sample of transformed pigeon pea plant (600-700 mg) was ground in mortar and pestle at room temperature with 1.5 ml washing buffer. The mixture was transferred into 1.5 ml microfuge tubes and centrifuged at 12000 rpm for 10 min at 4°C. The upper aqueous layer was removed and added 700 µl preheated high-salt CTAB buffer (65°C) to the supernant and incubated on ice for 5 min. Further sample was incubated at 65°C for 60 min in a water bath with 4-6 times inversion during incubation. After incubation 500 µl chloroform/iso-amyl alcohol (24:1) was added to each tube, mixed by inverting 4-5 times and kept for 5 min at room temperature. Tubes were centrifuged at 12000 rpm for 10 min at 4°C. Aqueous layer of 750 µl was transferred to a fresh tube. This step was repeated twice. Again aqueous phase was carefully transferred to a fresh tube and precipitated by adding 2 volumes of absolute alcohol followed by centrifugation at 12000 rpm for 10 min. The supernatant was discarded. Pellet was washed with 70% ethanol, air dried and dissolved in 100 µl TE. The DNA was stored at -20°C until further use. The quality as well as quantity of DNA was assessed by gel electrophoresis and spectrophotometric analysis.

#### 3.4.2.2 Quantification of DNA

Spectrophotometer was used for qualitative and quantitative analysis of DNA. One µl of DNA sample was added in Cuvette carrying 49 µl of sterile $H_2O$ and absorbance was measured at 280 nm wavelengths. Similarly the purity of DNA was checked by measuring the ratio of OD at A260/A280 nm. Quantification of DNA was calculated by using following formula,

$$\text{DNA } (\mu g/\mu l) = \frac{\text{OD at 260 nm X dilution factor X 50}}{1000}$$

#### 3.4.2.3 Confirmation of *GUS* gene by Polymerase Chain Reaction (PCR)

The PCR protocol for *GUS* gene confirmation was optimized by using various PCR components (Table 3.5) and thermal cycler programme (Table 3.6).

**Table 3.5: PCR Components used for detection of *GUS* gene in pigeon pea**

| PCR Components | Required Concentration | Volume/reaction |
|---|---|---|
| PCR Buffer (10X) | 1X | 2.5 µl |
| $MgCl_2$ (25 mM) | 1.5 mM | 1.5 µl |
| dNTP mix (2.5 mM each) | 0.25 mM | 2.0 µl |
| Primer (10 µM) | 0.4 µM | 2.0 µl |
| *Taq* DNA Polymerase (5 U/µl) | 1.25 U | 0.3 µl |
| Sterile water | - | 16.7 µl |
| Template DNA | 30 ng | 1.0 µl |
| **Total** | ---- | **25 µl** |

A 24 µl master mixture containing all the above reactants, except template DNA were dispensed in autoclaved PCR tubes (0.2 ml). Further 2 µl genomic DNA of each of putative transformed pigeon pea plants was added to the individual tubes containing the master mixture. The contents of each tube were mixed by tapping with fingers followed by a brief spin to collect contents at the bottom of the tube. The tubes were placed in thermocycler and subjected to PCR according to the protocol standardized below:

**Table 3.6: Standardized PCR protocol for amplification of GUS gene**

| Step No. | Steps | Temperature | | Time |
|---|---|---|---|---|
| 1 | Initial Denaturation | $94^0C$ | | 4 min |
| 2 | Denaturation | $94^0C$ | 30 cycles | 1 min |
| 3 | Annealing | $56^0C$ | | 1 min |
| 4 | Extension | $72^0C$ | | 1.5 min |
| 5 | Final Extension | $72^0C$ | | 10 min |

The amplified PCR product was separated on 1.2 % agarose gel, stained with ethidium bromide and visualized under Gel documentation System. Similarly the PCR

was also performed with DNA of non-transformed pigeon pea plant as a negative control. Based on number of plants recovered and *GUS* positive plant derived after PCR confirmation, transformation efficiency was calculated.

# Results and Discussion

CHAPTER IV

# RESULTS AND DISCUSSION

The present investigation entitled "Optimization of *in planta* method of genetic transformation in pigeon pea (*Cajanus cajan* L. millsp.) genotype: assessment and optimization of important parameters" was conducted for genotype BSMR 853. The germinated seedlings were used for *Agrobacterium* transformation experiment. The *in planta* transformation protocol was optimized through testing of various parameters viz., different levels of virulence inducer i.e. acetosyringone, optical densities of *Agrobacterium* inoculum harboring *β-glucoronidase (GUS)* gene, inoculation time etc.

The observations of each treatment combinations were recorded and results are described in this chapter.

**4.1 Preparation of *Agrobacterium* inoculum:**

The *Agrobacterium* strain EHA 101 harboring *β Glucoronidase (GUS)* gene was streaked on petridish containing LB Agar medium supplemented with kanamycin 50 mg/ml. The culture of *Agrobacterium* was started by inoculating 50 mg/ml LB in conical flask with single colony of *Agrobacterium* EHA 101 harboring *GUS* gene Further inoculated flask was kept in orbital shaker at 180 rpm at $28^0$C to multiply the *Agrobacterium* cells. The growth of *Agrobacterium* was measured by reading absorbance at 600 nm wavelength. Further O.D. of *Agrobacterium* culture viz., 0.6, 1.0 and 1.5 as per mentioned in different treatment combinations were measured. The respective cultures of *Agrobacterium* were pelleted through centrifugation at 6000 rpm for 5 min. *Agrobacterium* pellets of O.D, 0.6, 1.0 and 1.5 were resuspended into ½ MS liquid medium and stored at $4^0$C till to the infection of pigeon pea seedlings.

**4.2 Seedling development and infection:**

Five days old seedlings (Figure 4.1a) grown on ½ MS medium were isolated aseptically under Laminar Air Flow cabinet. Each of *Agrobacterium* suspension of different treatment combinations, acetosyringone was taken into separate sterile petri-dish. Wherein, eighteen different treatment combinations were adopted towards optimization of three important transformation parameters *viz.,* O.D. of *Agrobacterium*, concentrations of virulence inducer, inoculation time etc. during *in planta* pigeon pea transformation. Fifteen plants were infected under each treatment.

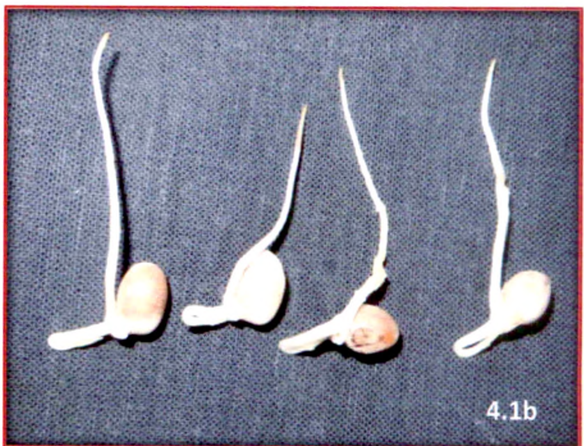



**Figure 4.1a-b:** a, Five days old seedlings of pigeon pea cv. BSMR 853 germinated on ½ MS medium; b, Washed seedlings used for Agroinfection.

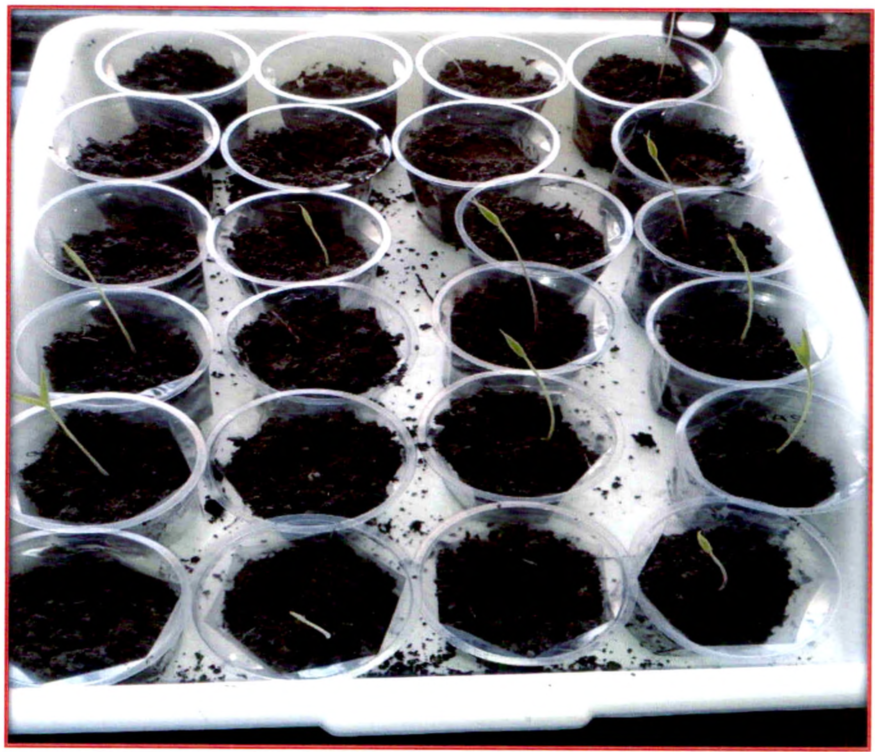


**Figure 4.2:** Recovery of Agroinfected seedlings of each treatment grown in plastic cup.

Seedlings with plumule just emerging were pierced at the apical meristem of axis and at the intercotyledonary region with a sterile needle and infected by immersion in the suspension of *Agrobacterium* for 0.5 to1.5 min. After infection, seedlings were washed briefly with sterile water and planted in plastic cup containing sterilized cocopit (Figure 4.2). The planted seedlings were maintained at control conditions in greenhouse. During this experiment the explant comprising axis of apical meristem and intercotyledonary region gave better response for *Agrobacterium* co-cultivation and further recovery of seedlings post co-cultivation. Similar procedure of Agroinfection was adopted by Rao *et al.,* (2008) for Agroinfection of pigeon pea. In addition, Keshamma *et al.,* (2008) observed that, embryonic axes showed better response towards agro-infection and recovery of seedlings compared to other explants. While, in tomato embryonic apical meristem was found efficient during *in planta* transformation experiment (Supratna *et al.,* 2006).

**4.3 Green house maintenance of transformed plants:**

Initially, a total of 270 plants of 18 different treatment combinations were grown in plastic cup containing sterilized cocopit at green house conditions (Figure 4.3a). These plants were covered with polythene bags for 2-3 days to maintain high humidity. Further fully established seedlings were transferred into plastic pots containing sand, soil and FYM (2:1:1) at containment type green house (Figure 4.3b). These plants were allowed to grow upto the maturity stage and further covered with muslin cloth in order to harvest seed for next progeny (Figure 4.8). The survival rate of the plant at green house condition was calculated. On an average the maximum recovery of plantlets was observed upto 75.60% (Table 4.2)

It was revealed that, the germination process of pigeonpea after *in planta* transformation remains unaffected. Similar type of results were recorded by Rao *et al.,* (2008) and described that, germination percentage and growth process of germinating embryos do not have any adverse effect of *Agrobacterium* during transformation. They reported fresh and healthy seedlings with germination frequency of 50% during prolonged time i.e. 1 h infection period.

**4.4 Optimization of *in planta* method of pigeonpea transformation:**

The *in planta* transformation parameters comprising, bacterial O.D (0.5, 1.0 & 1.5 min.), virulence inducer i.e. acetosyringone (150, 200, & 250 μM/ml) and inoculation period (0.5, 1.0 & 1.5 min) were assessed through 18 different treatment combination. The putative pigeon pea transformants grown at green house condition


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**Figure 4.3a-b:** a, Putatively transformed seedlings of pigeon pea grown in plastic cup containing coco peat; b putatively transformed seedlings of pigeon pea grown in plastic pot containing Sand, Soil and FYM under Greenhouse conditions.

**Figure 4.4:** Putatively transformed plantlets of pigeon pea grown under Greenhouse conditions.

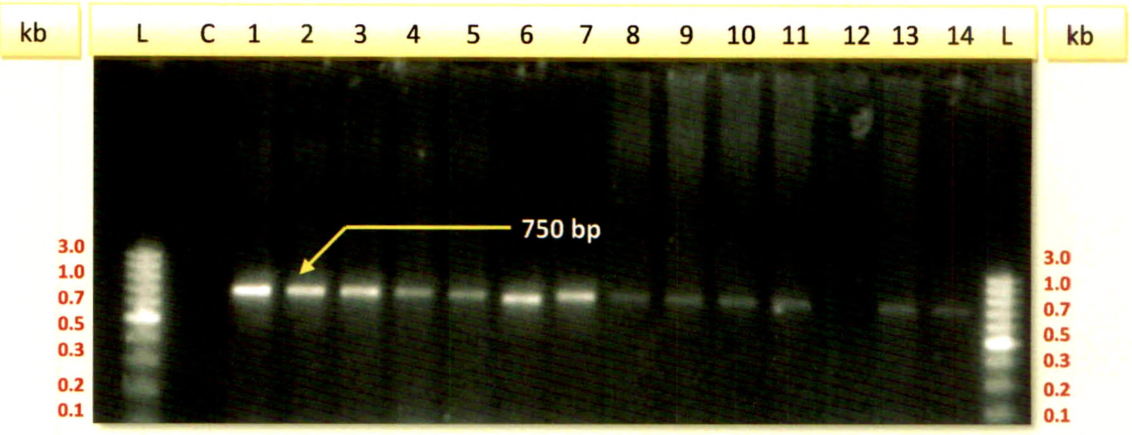



**Figure 4.5:** PCR based confirmation of transformed plantlets of pigeon pea by amplifying 750 bp GUS gene amplicon; L, 100 bp DNA ladder; C, Control plant; 1-14, putatively transformed plantlets under screening.

**Figure 4.6:** Transformed pigeon pea plantlets showing histochemical *GUS* gene expression

were further assessed for confirmation of transgene integration. Further, the best treatment conditions were evaluated by adjudging optimal concentration of transforming parameters in genotype BSMR 853.

### 4.4.1 Confirmation of transformed plants by histochemical GUS assay

Leaf samples of greenhouse grown transformed pigeon pea plants were collected at different developmental stages viz., seedling, branching and and maturity stage and further tested for histochemical GUS analysis. The histochemical GUS assay discriminated the transformed and non-transformed pigeon pea plantlets of cv. BSMR 853. The transformed plantlets shown blue color precipitate at midrib area of leaf, stem and on younger leaves (Figure 4.6). The plantlet developed through each treatment was screened through histochemical GUS assay. However the histochemical *GUS* assay was also adopted with non- transformed /control plantlets of the same cv. BSMR853 wherein, they did not show the blue color precipitates on tested leaf sample (Figure 4.7a). The histochemical *GUS* assay method described by Jefferson *et al.,* (1987) is simple, rapid and require less expertise. Many of researchers used this method for confirmation of transgene as Rao and Rohini (1999) utilized this method for confirmation of pigeon pea transformants, Keshamma *et al.*, (2008) in cotton; Ombori *et al.,* (2013) in maize; Ching *et al.,* (1997) and Razzaq *et al.,* (2011) in wheat; Lee *et al.,* (2011) in soybean crop plants. They revealed that, *GUS* gene expressed plants grew normally and remain fertile. Similarly, *GUS* is very stable and tissue extracts continue to show high levels of *GUS* activity after prolonged stage of harvested samples (Jafferson *et al.,* 1987). It could help to make simplicity in histochemical analysis via. collecting and preserving samples for longer duration. Thus, during this course of investigation an attempt have been made to optimize *in planta* transformation protocol in pigeon pea by using *GUS* reporter gene.

### 4.4.2 Confirmation of transformed plant by PCR analysis

The genomic DNA extracted from putative pigeon pea transformants of genotype BSMR 853 was subjected to PCR amplification with *GUS* gene specific primer. The PCR amplified product was resolved on 1.2 % agarose gel. The 9.25 mean number of putative transformants showing 750 bp amplicon (Figure 4.5) was considered as *GUS* gene positive pigeonpea plants. While those lacking were designated as non-transformed plants. Based on histochemical *GUS* assay and PCR confirmation the transformation efficiency of genotype BSMR 853 was calculated. The *GUS* histochemical based early detection of transformants was not recommended

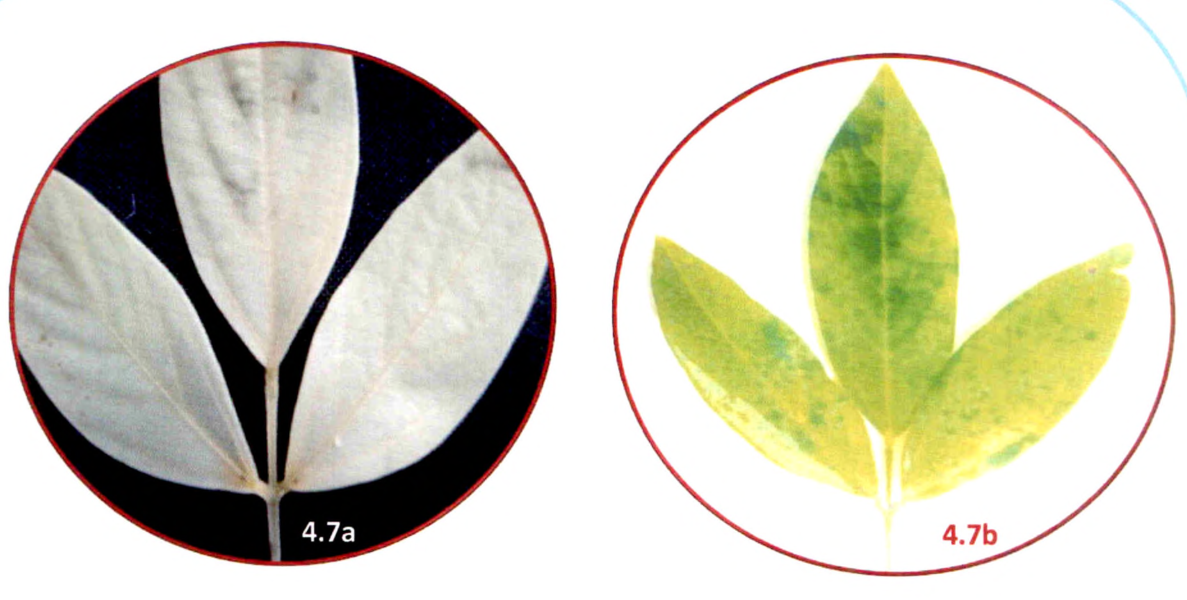



**Figure 4.7a-b:** a, Histochemical *GUS* assay of control plant; b, Histochemical *GUS* assay of transformed plant.

**Figure 4.8:** Transformed pigeon pea plantlets were maintained under green house conditions for further selfing and generation of T1 progeny.

in pigeon pea, as it gave false positive due to endogenous *GUS* like activity exhibited by pigeon pea (Rao *et al.,* 2008) and other crops (Sudan *et al.,* 2006). Hence in present investigation, PCR based confirmation of transformants was adopted. Similarly, this kind of approach was adopted by many of the researchers in different crops namely, Subramaniam *et al.,* (2013) in sugarcane; Lin *et al.,* (2009) in rice; Supartana *et al.,* (2006) and Razzaq *et al.,* (2011) in wheat; Lee *et al.,* (2011) in soybean etc.

**4.4.3 Optimization of transformation parameters**

Efficient transformation systems using readily available explants are in high demand for agronomically important plants. Though fertile transgenic plants have been generated from more number of plants, yet the transformation frequency for most species is still low. *Agrobacterium*-mediated transformation technology has not been routinely applied to pigeon pea because of recalcitrant approach (Rao *et al.,* 2008). However, the *in vitro* regeneration systems available in pigeon pea limited to few genotypes and morphogenetic response of the pigeon pea is known to be a genotype specific phenomenon described by Mohan & Krishnamurty (1998). Hence, further optimization of the transformation parameters such as bacterial OD, inoculation time and virulence inducer would be useful to increase *in planta* transformation efficiency.

During this investigation, the average transformation efficiency was calculated based on mean number of survived plantlets and actual transformed plantlets under each treatment of transformation experiment. The *Agrobacterium* OD, 1.0 was found more effective for transient expression of *GUS* gene. (Table.4.2) The maximum numbers of transformed plants were obtained at OD 1.0 under each treatment compare to OD 0.6 and 1.5. The transformation efficiency of genotype, BSMR 853 was ranged between 68.76 to 90.02%. It was also observed that, increase in concentration of O.D i.e. of 1.5 as well as decrease in concentration of O.D. i.e of 0.6 laid impact on decrease in transformation efficiency of genotype BSMR 853. Moreover, higher density of *A. tumefaciens* could increase the transient *GUS* expression but could not give stable transformation frequency. Similar findings were also reported by Cheng *et al.,* (1997) & Supatana *et al.,* (2006).

The second transformation parameter, i.e. inoculation time of 1.0 min, 3min and 5 min were assessed through different treatment combinations with other parameters. The effect of inoculation time was not much correlated with percent

transformation efficiency. It was highest at lower inoculation time (0.3min). However it was also found highest at higher inoculation time (1.5 min). The influence of lower as well as higher inoculation time was found at par with each other. The range of *GUS* expression and transformation efficiency among transformed pigeon pea plants were ranged between 68.76 to 90.02%. Further, it was noticed that, the hardened seedlings of pigeon pea remained fresh and healthy after infection with Agrobacterium. Thus, it was concluded that, there is no more effect of Agroinfection to the germination of seedlings in pigeon pea. Maximum number of plantlets gets recovered after infected with *Agrobacterium*. Thus, inoculation time had not much influence in-terms of *GUS* expression, seedlings germination and transformation efficiency.

Similar type of observations were reported by Rao *et al.,* (2008), wherein they revealed that, seedlings of pigeon pea cv. TTB7 remained unaffected after prolonged inoculation time. They also stated that, there was no effect of agroinfection on germination frequency and infected seedlings. It remained fresh and healthy even the infection time is prolonged to 1h. In addition, the study in rice by Wagiran *et al.*, (2010) reported that, inoculation time did not have any effect on *GUS* expression and transformation efficiency. They added, the inoculation time was different in different plant species and type of explant dependent, and it might be due to susceptibility of explant to *Agrobacterium* infection.

The addition of virulence inducer i.e. acetosyringone during transformation experiment showed significant influence on transient expression of *GUS* gene. Inclusion of 250 µM/ml acetosyringone in *Agrobacterium* suspension during infection results in the highest *GUS* activity i.e. 90.02% for pigeon pea genotype, BSMR 853. While, the acetosyringone at concentration 150 µM/ml showed minimum transformation efficiency i.e. of 68.76% of the genotype. This result is supported with evidence reported earlier by Wagiran *et al.*, (2010) in rice cultivars wherein, they stated as increase in concentration of acetosyringone beyond 300 µM resulted into decline of percentage of *GUS* activity. In present study, virulence inducer i.e. acetosyringone played a crucial role towards enhancing the transformation of pigeonpea. The addition of acetosyringone in co-cultivation media activates the induction of *vir* genes and also extends the host range of *Agrobacterium* strains (Saharan *et al.*, 2004; Zhao *et al.*, 2000). They have also stated that, the optimum concentration of acetosyringone in view of induction of highest transformation efficiency was varied from genotype to genotype. Thus in present investigation the

treatment combination comprising bacterial O.D 1.0, 250 μm/ml acetosyringone and inoculation time 1.0 min was found optimum for transformation of genotype BSMR 853 (Table 4.2).

Earlier, similar type of experiments on optimization of transformation parameters in different crops have been attempted by several researchers viz., in pigeon pea (Rao *et al.*, 2008); cotton (Keshamma *et al.*, 2008); buckwheat (Kojima *et al.*, 2000); mulberry (Ping *et al.*, 2003); soybean (Chee *et al.*, 1989); rice (Supartana *et al.*, 2005) ; sugarcane (Subramaniam *et al.*, 2013) etc.

Thus the present investigation could result in the standardization of an efficient *in planta* transformation protocol in pigeon pea which gave 68.76-90.02% transformation efficiency in genotype BSMR 853 (Table 4.2). This protocol optimized in this study is found efficient and does not involve any tissue culture regeneration procedure. Also, the protocol could generate relatively large number of $T_0$ transgenic in a short time. Similar findings were also reported earlier by Rao *et al.*, (2008) in pigeon pea and Rohini *et al.*, (1999) by producing 50-76.60% transformation efficiency in sunflower genotype Morden, while Lucas *et al.*, (2000) reported 45-62 % transformation efficiency in sunflower *cv.* LSF 8. Preliminary experiments suggested that, germination and growth processes of the germinating embryos of pigeon pea remained unaffected in the transformation procedure adopted here. Further steps of hardening of transformed seedling also did not adversely affect the development of the seedlings (Figure 4.4). The seedlings remained fresh and healthy and the germination frequency was not affected after inoculation of *Agrobacterium*.

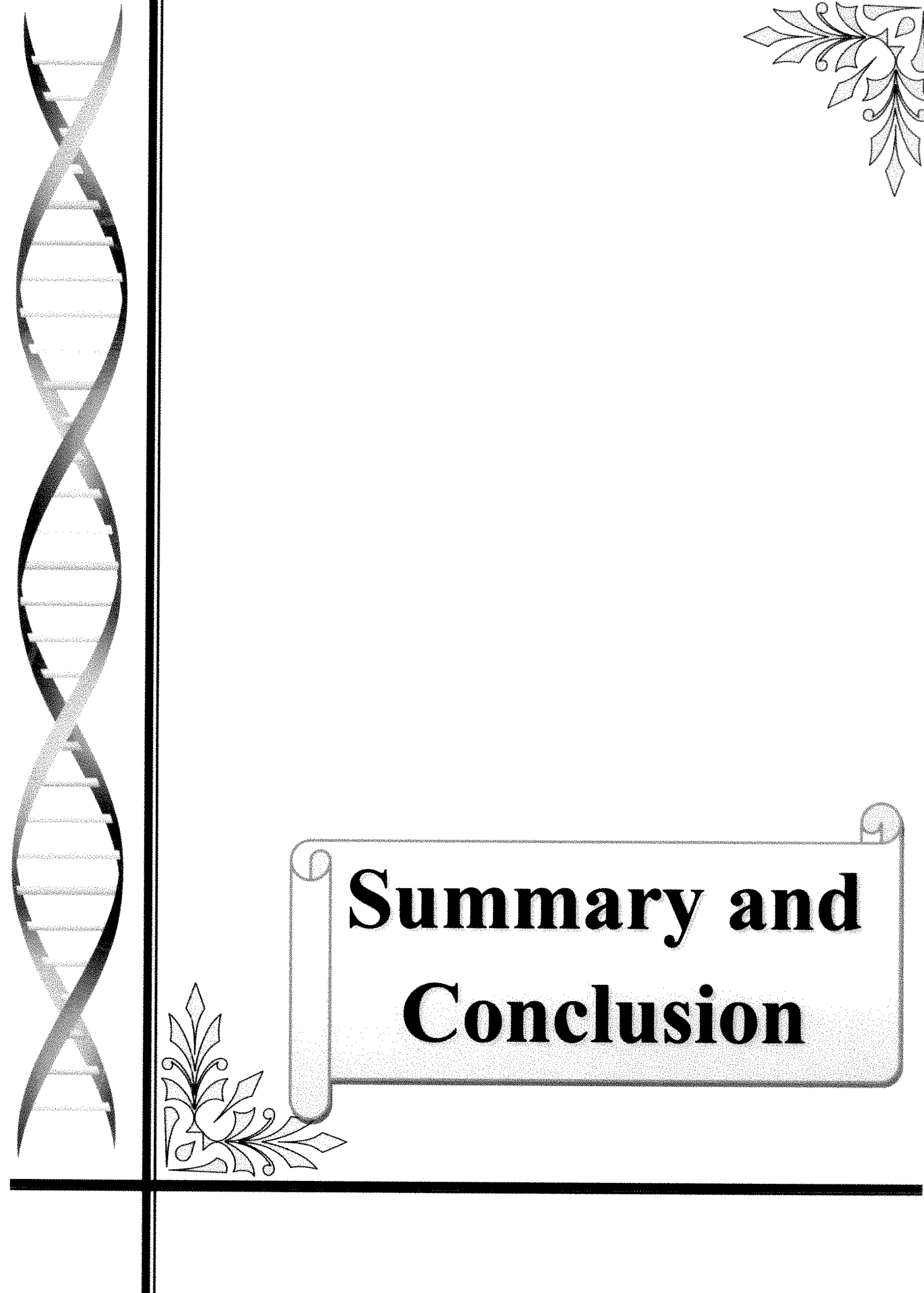
**Table 4.1: Optimized transformation parameters *viz* Bacterial OD, Virulence inducer and Inoculation period.**

| Transformation parameters | Value of parameter taken | Optimized value of parameters |
|---|---|---|
| Bacterial density ($OD_{600}$) | 0.5, 1.0, 1.5 | **0.5** |
| Virulence inducer, Acetosyringone (μM/ml) | 150, 200, 250 | **250** |
| Inoculation period of *Agrobacterium* (min) | 0.5, 1.0, 1. 5. | **1.0** |

**Table 4.2: Transformation efficiency of pigeonpea genotype BSMR 853**

| Treatment combination | Pigeon pea cv. BSMR 853 | | |
|---|---|---|---|
| $OD_{600}$ + Virulence inducer (µM) + Inoculation (min) | Mean No. of plants recovered | Mean No. of plants transformed | Transformation efficiency (%) |
| 0.6+150+0.5 | 12.33 | 9.33 | 75.66 |
| 0.6+150+1.0 | 12.00 | 9.00 | 75.00 |
| 0.6+200+1.5 | 11.30 | 8.66 | 70.40 |
| 0.6+200+0.5 | 12.66 | 10.00 | 78.98 |
| 0.6+250+1.0 | 12.00 | 10.00 | 83.33 |
| 0.6+250+1.5 | 11.66 | 9.66 | 82.84 |
| 1.0+150+0.5 | 10.33 | 9.00 | 87.12 |
| 1.0+150+1.0 | 10.33 | 9.00 | 87.12 |
| 1.0+200+1.5 | 9.00 | 7.00 | 77.77 |
| 1.0+200+0.5 | 13.00 | 11.33 | 87.15 |
| **1.0+250+1.0** | 13.33 | 12.00 | **90.02** |
| 1.0+250+1.5 | 12.66 | 11.33 | 89.49 |
| **1.5+150+0.5** | 10.66 | 7.33 | **68.76** |
| 1.5+150+1.0 | 10.33 | 7.66 | 74.15 |
| 1.5+200+1.5 | 10.66 | 9.00 | 84.42 |
| 1.5+200+0.5 | 10.00 | 8.33 | 83.30 |
| 1.5+250+1.0 | 11.33 | 9.33 | 82.34 |
| 1.5+250+1.5 | 10.66 | 8.66 | 81.23 |
| **Total Mean** | **11.34** | **9.25** | **81.06** |
| **SD** | **1.15** | **1.34** | **6.33** |
| **CV** | **10.15** | **14.49** | **7.8** |
| **SE** | **0.27** | **0.31** | **1.49** |

**Note:** Transformation efficiency was calculated based on mean number of regenerated plantlets and mean number of transformed plantlets. Each treatment was performed in triplicates with 15 number of seedlings treatment.



# Summary and Conclusion

CHAPTER V

# SUMMARY CONCLUSION

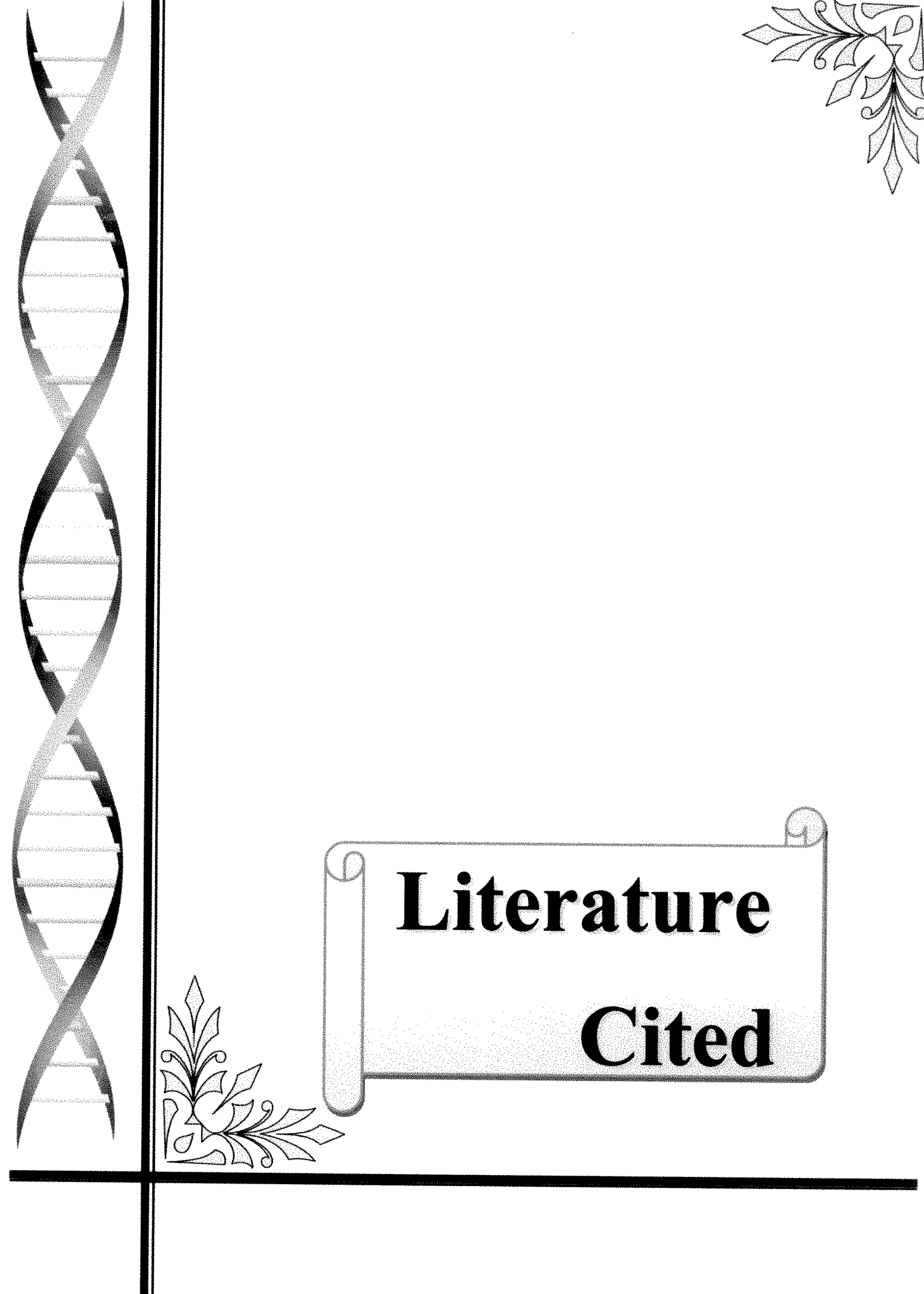
Pigeon pea or red gram (*Cajanus cajan* L. Millisp.) is one of the major grain legumes of tropics or subtropics. The production of pulses in India during (2012-13) is 17.58 Million tonnes/bales (Source: http://indiabudget.nic.in). India is major producer of pigeon pea accounting for 85-90% share in world produce. Though India is a producer of pigeon pea, its productivity has not been improved during the last four decades due to influence of various biotic and abiotic factors, narrow genetic base and incompatibility with wild species. The major impediments on the yield of pigeon pea are, major diseases viz., *Fusarium* wilt, *Phytophthora* stem blight, Sterility mosaic etc. and more than 200 species of insect pest accounting 46.6 to 63.6% losses. Thus to provide solution to the constraints that limit production and quality of pigeonpea, there is a need to widen its genetic base through adapting transgenic technology. However, development of transgenic in pigeon pea remained dogged by poor plant regeneration *in vitro* and low transformation efficiency.

Therefore, in the present investigation, an attempt has been made on development of tissue culture independent *in planta* method of genetic transformation of pigeon pea through optimizing various critical parameters. The protocol involves raising of whole plant transformants ($T_0$ plants) directly from *Agrobacterium*-infected young seedlings. The plumular (embryonic axis) and intercotyledonary meristem axes of the seedling was targeted for transformation. The main aim of this study was to develop a rapid transformation system for pigeon pea to overcome the recalcitrant approach of the explant and the problems of somaclonal variation. During this course of investigation, the transforming parameters of *in planta* transformation of pigeon pea viz., optical density, virulence inducer and inoculation time were optimized. During this study, the optical density, 1.0 of *Agrobacterium tumefaciens* strain EHA101 harbouring *GUS* gene was found optimum. While, virulence inducer i.e. Acetosyringone at concentration 250 µM /ml and infection time of 0.5 min was found optimum among 18 different treatment combinations used in the study. The putative transformants were confirmed either by *GUS* histochemical assay or by PCR based detection method through amplifying

fragment of size 750 bp with *GUS* gene specific flanking primers. The transformation efficiency was calculated based on mean number of recovered pigeon pea seedlings and transformed plantlets. The treatment comprising, *Agrobacterium* suspension of O.D. 1.0 with 250 μM/ml acetosyringone at infection time of 1.0 min gave highest transformation efficiency i.e. 90.02%. While, the treatment of *Agrobacterium* suspension of O.D. 1.5 supplemented with 150 μM of Acetosyringone at 0.5 min infection period revealed lowest transformation efficiency i.e. 68.76% in pigeon pea genotype BSMR853. However, it was also revealed that, the influence of infection time, on transformation efficiency was not found more significant.

The present investigation revealed the suitability of pigeon pea genotype BSMR853 for future transformation and integration of variety of transgens towards insect pest, disease resistance and quality improvement etc. through tissue culture independent method developed in this study. During this study, suitability of growing embryonic axis of cv. BSMR853 towards transformation efficiency was also assessed. The explant growing embryonic axis provided 11.34 mean numbers of recovered plantlets after co-cultivation. The transformation efficiency based on *GUS* and PCR assay was found as high as 90.02% by using embryo as explants for direct Agroinfection.

Thus, this investigation concluded with, optimization of tissue culture independent *in planta* transformation systems in pigeon pea genotype BSMR 853. This protocol would be exploited in genetically as well as qualitatively improvement of pigeon pea through rapid development of transgenic.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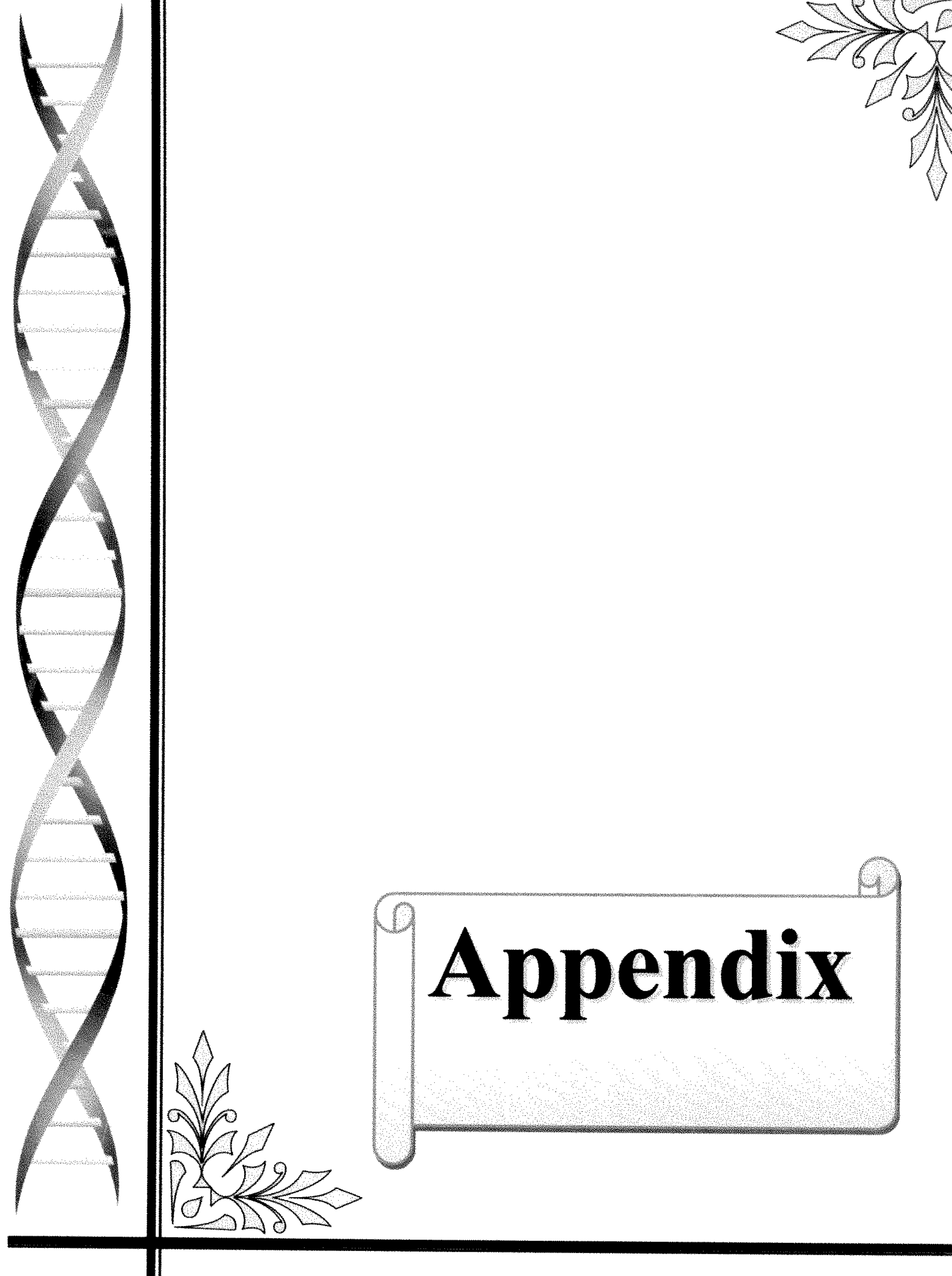


# Appendix

# APPENDIX

**Loading dye (6x):**

0.25% Bromophenol blue

40% (w/v) sucrose in water

Stored at 4°C

**TAE Tris Acetate:**

| | |
|---|---|
| Tris Base | 241 gm |
| Glacial acetic acid | 57.1 gm |
| 0.5 M EDTA (pH 8.0) | 100 ml |
| Distilled water | 1000 ml |

**YEMA medium (Yeast Extract Mannitol medium):**

| | |
|---|---|
| Mannitol | 10 gm |
| Yeast extract | 01 gm |
| $K2HPO_4$ (2%) | 10 ml |
| $MgSO_4.7H_2O$ (1M) | 0.8 ml |
| $CaCl_2.\ 2H_2O$ (1M) | 0.4 ml |
| Agar | 16 gm |
| Water | 1000 ml |

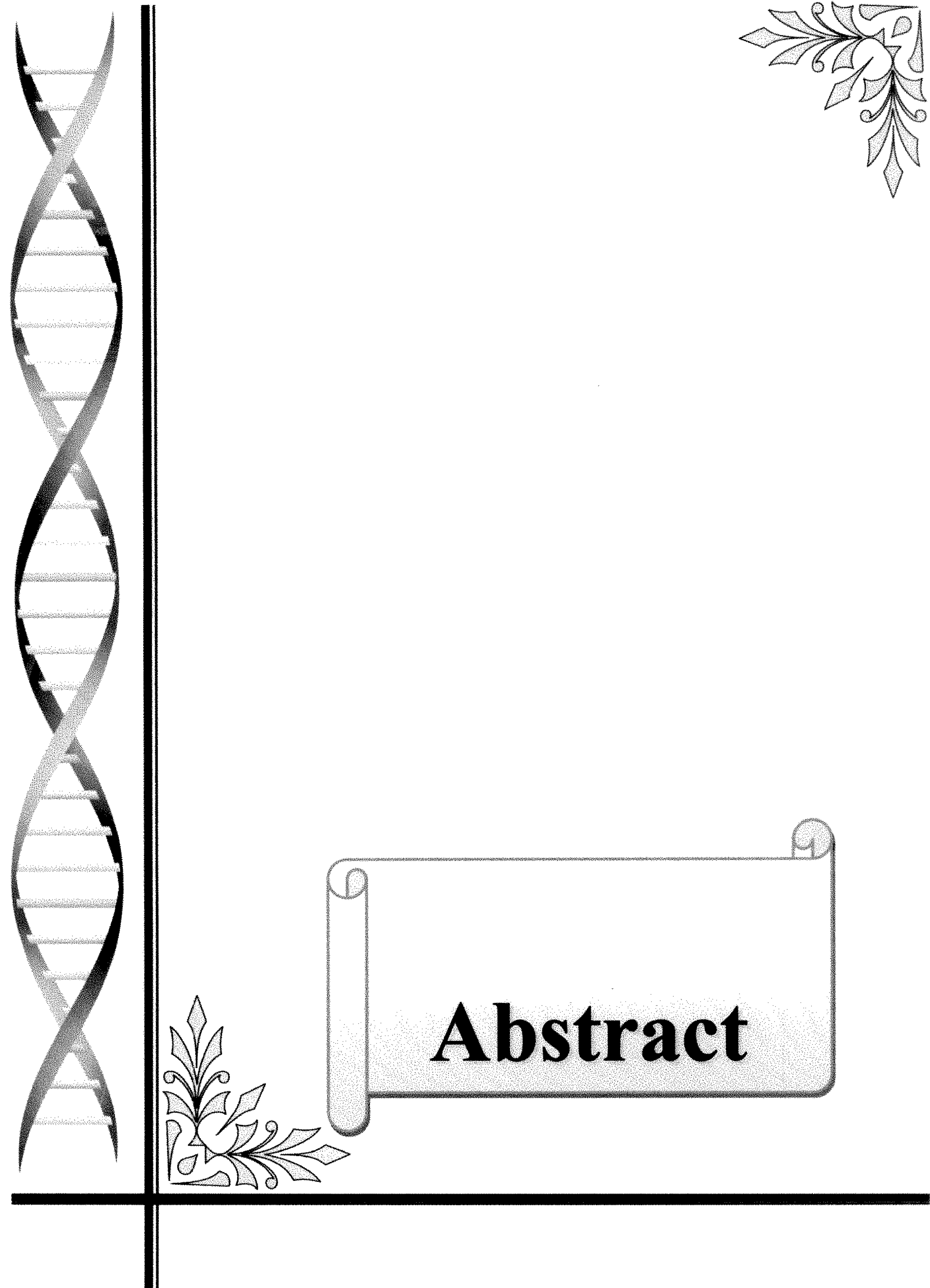
**DNA extraction buffer (Li *et al.*, 2007):**

| | |
|---|---|
| CTAB (20 %) | 10.0 ml |
| 5 M NaCl | 28.4 ml |
| 1 M Tris HCl (pH 8.0) | 10.0 ml |
| 0.5 M EDTA (pH 8.0) | 20.0 ml |
| β-mercaptoethanal | 01.0 ml |
| 20 % PVP | 05.0 ml |
| Double distilled water | 25.6 ml |
| Sub Total | 100 ml |

| **T.E Buffer pH 8.0** | | **Quantity taken** |
|---|---|---|
| 10mM Tris–HCl | - | 0.121 gm |
| 1mM EDTA | - | 0.03 gm |
| Double distilled water | - | 80 ml |
| Final volume | - | 100 ml |

**RNase A stock (10 mg/ml solution):**

| | | |
|---|---|---|
| 10mM Tris HCl (pH 7.5) | - | 10 µl |
| 1.5mM NaCl | - | 03 µl |
| RNase | - | 10 mg |


Abstract

## THESIS ABSTRACT

| | | |
|---|---|---|
| A) Title of research topic | : | **Optimization of *In Planta* method of genetic transformation in pigeon pea (*Cajanus cajan* L. Millsp.)** |
| B) Name of the student | : | ROUF AHMAD PARRAY |
| C) Degree to be awarded | : | M.Sc. Agril. Biotechnology |
| D) Major Subject | | Plant Biotechnology |
| E) Total number of pages in thesis | : | |
| F) Number of words in thesis Abstract | : | 295 |
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## OPTIMIZATION OF *IN-PLANTA* METHOD OF GENETIC TRANSFORMATION IN PIGEON PEA (*Cajanus cajan* L. Millsp.)


### ABSTRACT

Pigeon pea (*Cajanus cajan* L. Millsp.) is one of the major grain legume crops of the tropics and subtropics grown in about 50 countries of Asia, Eastern and Southern Africa and the Caribbean for various uses such as food, fodder & firewood. The present study was conducted to develop and optimize tissue culture independent in planta transformation system in pigeonpea. This system can help in widening genetic base as well as solutions over various biotic and abiotic factors through engineering novel genes across the species. The various transformation parameters viz., Optical density of Agrobacterium suspension, virulence inducer and infection time were optimizad through 18 different treatment combinations.

The plumular and intercotyledonary meristem axes of 2-3 days old germlings of pigeon pea cv. BSMR 853 was exploited for Agroinfection by sewing needles. Out of 270 inoculated germlings 11.34 mean number of plantlets were recovered. The putative transformants were confirmed by GUS histochemical and PCR assay. Transformation efficiency was calculated on mean number of recoverd plantlets and mean number of transformed plantlets. Among 18 different treatment combinations, the treatment pertaining Agrobacterium suspensión of O.D 1.0, virulence inducer, (acetosyringone) at 250 μM/ml and infection time of 1.0 min was found optimum. Among all transformation parameters, bacterial growth of O.D, 1.0 and acetosyringone concentration, 250 μM/ml has shown significant impact on transformation efficiency. While there was no more impact of infection period over transformation efficiency. The treatment comprising bacterial O.D, 1.0 with 250 μM/ml acetosyringone and 1.0 infection time 1.0 min revealed 90.02% transformation efficiency. However, lowest transformation frequency i.e. 68.76% was reported in treatment of bacterial O.D. 1.5 with 150 μM/ml acetosyringone and 0.5 min. infection time.

Thus, present investigation revealed the optimization of *in planta* transformation parameters in pigeon pea and suitability of genotype BSMR853 for genetic transformation and further genetic improvement.



**KEW WORDS:** *Agrobacterium*, *in planta*, *GUS* gene Pigeon pea



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