SUMMARY

Pigeonpea (*Cajanus cajan* [L.] Millsp) is an excellent grain legume of the dry areas widely cultivated and necessary for the protein requirement in the Indian subcontinent. It has multiple uses as a food source, cattle feed and firewood. It is a short-living perennial shrub and has a sophisticated deep root system which makes it drought resistant. It also helps in nitrogen fixation in soil.

The major problems that decrease the potential yield of pigeonpea include the reduced availability of highly eminent seeds of enriched genotypes in appropriate quantity, privileged crop management, biotic factors such as diseases caused by bacteria, fungi and abiotic stresses such as salinity, drought etc. Almost 71% of the earth surface is occupied by saline water (Shweta Mittal *et al.,* 2011) and high salinity is the common phenomenon for the dry and semi-arid regions. The addition of salt to water decreases both osmotic potential and availability of water to roots which ultimately leads to osmotic stress.

The synthesis and accumulation of osmo-protectants such as proline is the most common strategy of that plants use to tolerate osmotic stress conditions (Xiong and Zhu, 2002). It scavenges ROS, adjusts cellular osmolarity (Yancey, 2005), and protects macromolecules by stabilizing the protein structure. Pigeonpea genotypes with increased tolerance to various pests, diseases and abiotic stresses, low allergic proteins are in great demand. The use of transformation techniques for the production of new breeding materials are much desirable to attain maximum crop yield. Osmo-protectants protect plant cells from dehydration and are accumulated to certain levels without disrupting plant metabolism (Yamaguchi-Shinozaki and Shinozaki, 2005).

The accumulation of high amounts of proline mainly depends on salt tolerance and on the nature of plant species. With this background the present study was undertaken. The title indicates three main aims, the first one was to regenerate potential plants from different explants of pigeonpea genotypes and the second one was to transform pigeonpea genotypes with *Vigna*

aconitifolia P5CSF129A gene (mutated version) and the third was to assess transgenic plants showing stable incorporation and expression of the target gene both at biochemical and molecular levels.

Regeneration of whole plant is important for successful propagation of plants through tissue culture by using a number of explants. The standard *in vitro* regeneration techniques are necessary for producing transgenic plants. Three different explants viz., whole seeds, embryonic structures and cotyledons from three different genotypes of pigeonpea viz., LRG 41, ICPL 85063 and LRG 30 were utilized for regeneration experiments. The 15-17h soaked seeds, embryonic structures and cotyledons excised from 15-17h soaked seeds were cultured on MS medium complemented with various concentrations of BAP (8.8, 13.3 and 17.7mM).

All the explants showed bulging from the nodal region. BAP being a cytokinin, induces multiple shoot buds from the axils on either side of primary shoot when the top growth is suppressed. On the other side, cotyledons initiated callus and shoots from the end where embryo was attached. The explants, when grown on MS medium supplemented with 8.8 and 13.3mM BAP, produced shoots within a week. When the same explants were cultured on MS medium supplemented with 17.7mM BAP, they developed a number of multiple shoot buds within 3 days. This concentration is considered to be the optimum concentration. The explants were elongated on MS elongation medium supplemented with 0.57mM NAA and 0.86mM $GA_{3.}$ Plants rooted on MS rooting medium with 2.46mM IBA and finally they were transferred to disinfected soil and further to polyhouse.

Among the various explants, embryonic structures produced the largest number of plantlets than the other explants. Regeneration through multiple shoot induction was more efficient from ICPL 85063 (10.0 ± 0.3) than from LRG 41(5.0 ± 1.6) and LRG 30 (4.8 ± 0.9). Based on these results, embryonic structures from three different genotypes of pigeonpea were used for further transformation experiments.

Genetic transformation is the mode of delivery and controlled expression of genes into a recipient genome which can be achieved via techniques such as physical methods

(electroporation) and biological method (*Agrobacterium* mediated gene transfer). *Agrobacterium*-mediated gene transfer method was used in transformation studies with embryonic structure as explants from three different genotypes of pigeonpea. Two constructs *Agrobacterium tumefaciens* strain LBA4404 harboring binary plasmid pCAMBIA1301and pCAMBIA2300 containing *uid*-A (i.e., β-glucuronidase) as reporter gene linked to the CaMV35S promoter and *hpt*II gene for hygromycin resistance under the control of a nopaline synthase promoter and two stress-genes, were used in this study. These genes increase the tolerance of plants against toxic effects of soil salinity.

The embryonic structure were co-cultivated with *Agrobacterium* for 3days and subcultured on to antibiotics containing MS media supplemented with 250mg/l cefotaxime and 25mg/l hygromycin. After a week, the explants were tested for *gus* activity. The differential response of pigeonpea genotypes for transformation was attributed to the inherent capacity of the genotype for the number of sites available for incorporation and the position of such sites for expression, so that they can readily recombine with Ti plasmid and express the incorporated genes.

The E.coli β-glucuronidase (GUS) gene acts as a reporter gene system encoded by the *uid* A locus for transformation of various plants. Glucuronidase can be easily, sensitively and cheaply assayed *in vitro* and can also be assayed histochemically to localize GUS activity in cells and tissues. Twenty-five embryonic structures of each genotype were selected and analyzed for GUS expression after first cycle on selection medium (Jefferson *et al.,* 1987). The explants tested for GUS expression showed 96-100% GUS positive in the explants transformed by *Agrobacterium*. Untransformed explants did not show any staining with X-gluc even after extended assays of several days. Among the three genotypes of pigeonpea, embryonic structures of ICPL 85063 gave higher percentage of GUS expression. *Agrobacterium* strain LBA4404 harboring a binary plasmid pCAMBIA1301 vector gave better response and also more number of *gus* positives.

The over-expression of the *Vigna aconitifolia P5CSF129A* gene in transgenic pigeonpea plants enhanced the accumulation of proline and its salt tolerance. The *P5CSF129A* gene is a salt tolerance gene as it increases the level of osmolytes in pigeonpea (Chern *et al.,* 2001; Saharan

et al., 2004). It is a rate-limiting enzyme in proline biosynthetic pathway and its efficient expression in transgenic plants showed enhanced osmotic stress tolerance induced by both drought and salinity. P5CS gene is mutated at 129 position to replace Phe residue with the Ala residue using site-directed mutagenesis which is subjected to feedback inhibition (Hong *et al.*, 2000).

Genomic DNA was isolated from the leaf tissues of transformed and non-transformed T_0 generation plants. The integration of gene in the T_0 generation was assayed by performing PCR and proline content estimation. The amplification was carried out by using gene specific primers for *hpt*II and *P5CSF129A* revealed that all the hygromycin resistant plants got acclimatized showing positive amplification with both pairs of the primers. All the samples amplified 340bp fragment with *hpt*II primers and 800bp fragment with *P5CSF129A* primers, while control plants showed no amplification with either primer pair. The accumulation of osmolytes increases tolerance to osmotic stress and make plants suitable for agricultural use. The proline content was assayed on leaf tissue which were from PCR positive T_0 plants, along with non-transformed plants. Proline content was estimated from leaf dry weight of each plant independently. The proline content of transformed plants was found four times higher than the non-transformed plants.

The genomic DNA was isolated from the leaves of transformed and non-transformed T_1 generation plants. The stable establishment of genes into the genome of T_1 generation was assayed by performing PCR and Southern blotting. The amplification was carried out by using gene specific primers for *hpt*II and *P5CSF129A* genes. The PCR amplified products were separated by electrophoresis on 0.8% agarose gel. Clearly the 800bp and 340bp fragments represent the stable insertion of foreign genes in host genome. The plants exihibiting symbols of hygromycin sensitivity were not shown any kind of gene insertion. Southern analysis revealed that genomic DNA isolated from randomly selected plants of three genotypes were digested with restriction enzyme *EcoRI*, when hybridized with the PCR amplified fragment as *P5CSF129A* probe, gave the expected 3.8kb fragment in each transgenic line analyzed. There was no signal detected with wild plants.

The transgenic (T_1) plants were assayed for tolerance to salt shock (sudden exposure of salt treatment). The transgenic plants showing symbols of hygromycin resistant along with 5 non-transgenic *in vitro* germinated plants were acclimatized to soil mixture (soil and sand, 1:1) conditions with single plant in each plastic cup with equal amount of soil with 4 holes at the bottom and monitered for their reaction under exposure to 200mM NaCl. The acclimatized plants were watered with 50ml of Hoagland solution containing 200mM NaCl for 7days. The salt treated plants are used for further biochemical experiments such as proline content, lipid peroxidation, chlorophyll content and relative water content (RWC) estimation.

Pigeonpea plants expressing *P5CSF129A* gene showed an increase in plant height with leaves much greener and healthier. The proline content was also estimated from plants of all genotypes grown in tissue culture, which showed significant difference between non-transformed and transformed plants. The proline content ranging from 4,974.0 ± 3.06 to 7,987.5 ± 0.37 µg/gram dry weight was observed. The MDA content can be used as a reference for the analysis of membrane damage and free radical formation in both transformed and non-transformed plants. There is a direct correlation between the value of lipid peroxidation and membrane damage in transgenic plants. The total chlorophyll content of the transgenic leaves in acetone extract was analyzed by using spectrophotometer. The total chlorophyll concentration was higher in transgenic plants over non-transgenic ones. The leaves of transgenic plants showed higher relative water content as compared with the non-transgenic plants.

Results indicate that the embryonic structures are promising to use in transformation studies since regeneration can be readily obtained. There is genotypic variation, explant variation and *Agrobacterium* strain variation which would influence the incorporation of gene. Finally in the case of using *Agrobacterium* strain pCAMBIA1301, stable incorporation of inserts in T_0 and T_1 generation was confirmed. Significant difference was observed in transgenic ICPL 85063 which has higher proline accumulation of 7,987 ± 0.37 µg/gm when compared to the other transgenic lines.

Based on the above observations, it is concluded that the embryonic structures of ICPL 85063 are the best genotype among the *C. cajan* varieties and *Agrobacterium* strain LBA4404

harboring a binary plasmid vector pCAMBIA1301 is the most effective vector for high transformation efficiency and for enhancing salt tolerance in *C. cajan.*