Chapter 4

Results and Discussion

The results of the experiment entitled "Genome Editing through CRISPR Cas9 for Improvement of Beta Carotene in Groundnut [*Arachis hypogaea* (L.)]" are presented in this chapter. Experiments were conducted to β-carotene increase of different Indian cultivar of Groundnut and optimize protocol for regeneration and transformation of Groundnut via direct organogenesis from healthy explants. Transformed plants were characterized at molecular level to detect the targeted mutation in (lycopene-ε-cyclase (*LCYε*) gene.

Desired traits for plants have been attained by selective breeding through the ages; however, it is not an efficient way for a single gene modification since traditional breeding depends on random recombination and integration. Recent advances in genetics have contributed to the precise plant genome editing so that researchers can pinpoint specific gene(s) for specific purposes. The CRISPR and its associated protein (CRISPR/Cas9) system is one of the latest Genome editing technique that is more precise and effective than ZFNs and TALENs (Gaj *et al.*, 2013).

The integrated approaches of genetic engineering of soybean have great value because of its commercial importance. Groundnut is the subject of new areas of research including functional genomics, proteomics, and metabolomics, it is a crop of choice among legume or oilseeds. Groundnut is well known for many traits of interest, such as mutants in male sterility, improvement for agronomic traits and disease resistances. It is susceptible to *Agrobacterium* infection, and is therefore amenable for current plant transformation techniques.

Several techniques to introduce foreign genes into plants have been developed. However, *Agrobacterium* mediated gene transfer is the primary means of transformation in most plants (Pino *et al.*, 2010). Several genera and species including Groundnut are now being genetically transformed through this technique.

However, effective, efficient, and reproducible in vitro regeneration protocol is a prerequisite for making in vitro genetic manipulations of any crop in general and

tomato in particular. In vitro regeneration and transformation are genotype specific and hence efforts are needed to optimize the methodology for each genotype before venturing into any possible practical applications of genetic engineering. Standardization of in vitro regeneration and transformation protocols are the two important aspects of genetic transformation of crop plants including Groundnut (Rathod *et al.*, 2017).

4.1 *In vitro* plant regeneration studies in Groundnut [*Arachis hypogaea* (L.)]

Development of a suitable in vitro regeneration protocol of any plant species is paramount pre-requisite for genetic transformation studies. The tremendous improvement in the fields of plant genetic engineering and plant molecular biology led to the incorporation of variety of traits in plant species which include improvement in agronomic traits crops *i.e.,* herbicide tolerance, biotic and abiotic stress resistance, improvement in food quality, value addition, enhancing of shelf life and synthesis of various pharmaceuticals (molecular pharming) etc.

Direct and Indirect method of genetic transformation mediated by Agrobacterium tumefaciens provides an opportunity to improve the quality of all major crop species (Pattanayak and Kumar, 2000; Char *et al.*, 2017; Lee *et al.,* 2019; and Zhang *et al.*, 2019).

Success of tissue culture in Groundnut markedly influence by age of the explant, plant growth regulators, explants orientation, pH of the medium and physiological status of ex-plant materials (Radhakrishnan *et al.*,)

4.1.1 Optimization of protocol for regeneration

The seeds extracted from ripe, dried pods were rinsed for five minutes under running water. The seeds were moved to a sterile flask in the laminar airflow cabinet and surface sterilized by adding 70% ethanol to it. For 30 seconds, the seeds were washed. After the ethanol was removed, 0.1% HgCl2 was added, and the mixture was gently stirred for three minutes. The HgCl2 solution was disposed of after three minutes, and the seeds were washed three to four times with sterile distilled water (Radhakrishnan *et al.,* 2000). In the laminar hood, the testa of the sterilized seeds were

aseptically extracted using forceps and a sterile scalpel. After dividing the seeds into two halves, or cotyledons, the embryo that was joined to the cotyledons was removed, a process known as de-embryonation. Direct use of the de-embryonated cotyledons as explants for culture was made. A sharp surgical blade was used to gently remove each leaf from the bunch that was present at the embryonic axis. De embryonated cotyledonary were cut in half with a scalpel blade and carefully placed on a 110 x 40 cm petri plate that contained full strength MS basal media supplemented with different amounts and combinations of plant development regulators (Table 4.1 to 4.3).

4.1.2 Effect of BAP on shoot regeneration from de-embryonated cotyledons explant

Cotyledonary nodal explants were inoculated in shoot initiation medium supplemented with BAP (2,5,10,15 and 25 mg/l^{-1}) for shoot induction Seven different concentrations of BAP (Table 4.1) were tested for growth and differentiation. Within one week of culturing, color of the cultured explants changed from yellow to light green and expansion of explants were reported, but there was no change in media color. In second week of sub-culturing explant start to become green. On De embryonated cotyledonary, BAP showed considerable interaction and a variety of responses. In comparison to the other combinations, the medium containing 15 mg l^{-1} BAP produced the maximum shoot regeneration (84.33%). The media containing 2 mg l^{-1} BAP had the lowest shoot regeneration (42.66%). The lowest level was found in the control group, which was devoid of any growth regulator.

4.1.3 Effect of BAP and GA on Shoot multiplication and elongation

Seven different media compositions of BAP and GA were tested for in vitro shoot regeneration studies in cotyledon explants (Table 4.2) During third week of culturing, explant showed shoot multiplication and elongation of explant was observed. The combined effect of BAP and GA had noteworthy interaction and exhibited varied response on the cotyledon ex-plants of soyabean. MS media supplemented with 3.0 mg l^{-1} of BAP and 0.1 mg l^{-1} of GA (SB6) showed highest Shoot multiplication and elongation (87.67%) (Table 4.2). The lowest shoot regeneration (45.33%) was reported on media containing 0.5 mg l^{-1} of BAP and 0.1 mg l^{-1} of GA (SB1). (Fig. 4.5 A-H)

4.1.4 Effect of different concentrations of NAA on root initiation from *in vitro* developed shoots

Multiple shoots regenerated from de embryo cotyledon explants were excised with sharp scalpel blade and cultured on half strength of MS media supplemented with different concentrations of NAA (Table 4.3) Regeneration of roots started within two weeks of inoculation. Very profuse and cottony type of rooting was observed. Basal portion of shoot showed little callogenesis.

MS media supplemented with 2.5 mg l^{-1} of NAA (SI 6) showed highest root multiplication and elongation (78.33%) (Table 4.3) The lowest root regeneration (49.67%) was reported on media containing 0.1 mg l^{-1} of NAA (SI^{-1}). Within two weeks of culture, root initiation began. all regenerated shoots showed root initiation. After a month well-developed multiple fibrous roots emerged. (Fig.4.6 A-E)

4.1.5 Hardening of regenerated plantlets of Groundnut

Without causing any harm to their fragile root system, well-developed plantlets that had recovered from an in vitro groundnut tissue culture were taken out of the culture bottle. Since any traces of agar might promote fungal infections, loosely adherent calli were carefully removed and cleaned with water to get rid of adhering MS media. Roots were cleaned and then immersed in Hoagland solution for some time. In the meantime, plastic containers with a hole in the bottom are filled with a 50:50 potting mixture of cocopeat and sandy soil. The regenerated plantlets' root sections were carefully positioned over the mixture and then covered with it again. To preserve relative humidity, the plantlets were covered with perforated plastic bags and irrigated with 0.1% carbendazim. To maintain the ideal relative humidity during the first several days of establishment, plants were watered once daily and sprayed with Hoagland solution once a week. The number of pores in the enclosed plastic bag rapidly grew after a week. After a month, the plants were moved into larger pots with potting mix and maintained in a net house environment to continue growing and developing. In accordance with the soil moisture state, plants were watered (Fig.4.6). Cotyledon explants respond differently to various combinations and concentrations of regeneration ability, according to this study's optimization of the groundnut cotyledon regeneration procedure.

4.2 Genetic transformation studies in Groundnut

4.2.1 p201B CRISPR/Cas9 vector

Addgene, a non-profit plasmid repository for gRNA/Cas9 mediated genome editing in soybeans, provided the CRISPR/Cas9 vector p201B. The pPZP backbone is the source of the binary vector p201B (Jacobs *et al.,* 2015), which is utilized to create stable groundnut transgenic plants by Agrobacterium-mediated transformation (Fig. 8). The plant selectable marker Basta, which is flanked by the left and right borders, is expressed with the CaMV 35S enhanced promoter, while the guided RNA is controlled by Mtu6 and dual 35S promoter. We choose Cas9 because of its wide spectrum of PAM recognition capabilities. For innovative genome editing applications to achieve the required alteration, gRNA selection and design are essential steps. Through Agrobacterium, this T-DNA region serves as the backbone for the transfer of guided RNA into the host system. These were important elements. Associated during our work.

4.2.2 Isolation of plasmid p201B

E. coli cultures were used to isolate Plasmid p201B using the manual alkaline lysis technique. The restriction enzyme *Swa*I was used to break down the plasmid. The Qiagen gel purification kit was used to elute the digested product. A 0.8% agarose gel was used to verify the eluted product. (Figure 4.7).

4.2.3 NEBuilder® Assembly of CRISPR vectors using ssDNA oligos

This protocol is a follow-up to the one reported in Jacobs *et al.,* 2015. It is superior in several ways: new gRNAs are created and inserted into final CRISPR vectors in a single cloning step, hands-on time is minimal, and multiple gRNAs can be created in a single reaction. Restriction enzymes are only used for vector preparation.

The primers listed are for the p201B Cas9 vectors (Addgene 59175-59178). Four DNAs are mixed, each with 20-bp overlaps; U6 promoter, gRNA ssDNA 60- mer oligo, scaffold, and p201B vector. The U6 and scaffold DNAs are made by PCR and the p201B vector is digested. Pools of oligos can be used in a single reaction, thus reducing the cost per gRNA. I have observed even distributions of inserts from a single reaction. (Fig 4.8)

Using the heat shock approach, the ligated product was converted into *E. coli* DHα competence cells. based on how many colonies developed on the plate of LA selection medium. To determine whether the dsDNA duplex is present in the vector, do the colony PCR. A 1% agarose gel was used to examine the PCR product that was produced. This serves as the primary confirmation that dsDNA has been integrated into the entrance vector p201B.

4.4 *E. coli* and *Agrobacterium* transformation and conformation by colony PCR

Groundnut genetic transformation research was conducted using *Agrobacterium tumefaciens* strain LBA4404 and *E. coli* strain DH5α. The Cas9-sgRNA-containing construct was first cloned and transformed in *E. coli*, and then in an *Agrobacterium*. Colony PCR was used to validate the presence of the desired plasmid construct (Fig.4.9).

4.5 Development of Beta Carotene edited plants from de-embryonated cotyledon explants

There are significant differences in the virulence of *Agrobacterium tumefaciens* strains among the plant hosts (Hobbs *et al.*, 1989; Davis *et al.*, 1991). Compared to normal strains, the super virulent strains have a wider host range and a greater transformation frequency (Hooykaas and Beijersbergen, 1994). For transformation to be effective, the right strain must be chosen. *Agrobacterium tumefaciens* strain LBA4404 was selected for the current investigation to infect the half-seed and cotyledonary node explants. Cotyledonary node and half-seed explants from groundnut seedlings cultivated in vitro were used in *Agrobacterium*-mediated genetic transformation investigations.

4.5.1 Using de-embryonated cotyledons as explants

A total of 239 explants were co-cultured in 5 sets of tests. Out of the 151 explants, 2,332 shoots were regenerated and placed in the Basta-containing selection medium; 552 (31%) of the shoots were able to tolerate the selection pressure. After being placed in the root-inducing media, 448 (98%) of the chosen plants produced roots,

which were then able to be hardened and moved to the PII glasshouse. Ninety-one percent of these plants were able to grow and thrive in the glasshouse. Twenty-three percent of the altered plants' shoots eventually recovered (Table 4.4 and Figure 4.1 to 4.6). In all, 239 groundnut cotyledonary explants from GJG20 were utilized for co-cultivation; table 25 summarizes and analyzes the findings at different stages. Additionally, 25 explants were kept as untransformed controls at the same time without inoculation. For co-cultivation, the cotyledonary explants were first grown on media containing 25 mg/L BAP (Figure 4.1 A-B). As previously described by Radhakrishnan et al. (2002), the de-embryonated cotyledons were positioned on their adaxial side on the media on the petri plate to guarantee that the maximal surface of the cotyledons was in contact with the medium for bacterial growth and nutrient absorption. It was found in early experiments that maintaining a high plating density was not advantageous. According to Sharma and Anjaiah (2000), the explants were plated at a density of 6-7 cotyledons per Petri plate. Within four to five days, the explants began to become green as a result of chlorophyll development. The size of the cotyledon explants increased significantly. The absorption of the hormones and nutrients provided in the medium may be the cause of the explants' growth. The explants began to develop shoot buds as a result of the increased hormone content. In the explants, many shoot buds were seen to develop (Figure 4.1D). Several shoot buds were produced by the differentiation of the organogenic tissues (Figure 4.1E) In a similar vein, Freitas et al. (1997) similarly employed a high BAP dosage of 25 mg/L. The regenerated explants showed multiple shoot bud development. By using the enhanced regeneration procedure, a very high level of regeneration may be achieved (Radhakrishnan *et al.,* 2000). The parenchymatous tissues around the proximal end of the explant are the main source of direct plantlet regeneration from de-embryonated cotyledons (Radhakrishnan *et al.,* 1999). According to earlier research by Freitas et al. (1997), only the parenchymatous cells next to the inoculation site underwent cotyledon metamorphosis. More shoots developing at the start of the culture might increase the frequency of regeneration and, consequently, the frequency of putative transgenics and changed initials. Additionally, Radhakrishnan et al. (2002) came to the conclusion that the transformation and regeneration. When cotyledons were employed as explants, frequencies were high. After being moved to a medium containing 250 mg/L cefotaxime and 15 mg/L BAP, the explants containing shoot buds were subcultured for two weeks. For selection, the

bunch of shoot buds and explants were divided into two to three pieces and placed in a medium that included three milligrams of BAP and one milligram of GA3, as well as varying doses of hygromycin (5 to 25 milligrams per liter) for two to three passages of two weeks each. Some of the shoots became brown and died as a result of the selection pressure from hygromycin. Only healthy, green shoots were advanced for more subculturing; the other shoots were thrown away. After being clipped at the base, the lengthy individual shoots were moved so they could root. The adventitious roots emerged. For primary hardening, the rooted shoots (Figure 4.5) were placed in culture tubes with Hoagland solution and cultivated for a week at 70% relative humidity in a hardening chamber. After being transferred to clay pots, the major hardened shoots were put in the PII glass house (Figure 4.6). For subsequent development, a total of 386 GJG20 plants were cultivated and kept in a PII glass house (Figure 4.6). These alleged transgenic plants had no morphological defects of any kind and seemed phenotypically normal. Generally speaking, it was found that the non-co-cultured explants reacted more quickly and produced more shoot buds than the converted explants. This suggested that agro-infection may have had an impact on the induction of shoot buds during the transformation phase.

4.6 Molecular analysis of putative transformed plantlets of Groundnut

4.6.1 Confirmation of Cas9-gRNA integration into the transformed plants by PCR

The confirmation of Cas9-gRNA integration into the in vitro regenerated transformed plants was done by PCR amplification of components gene of construct by using specific primers and visualizing the amplified DNA on electrophoretic gel. DNA isolated from the twenty-two transformed and control plants by the CTAB method and quality was checked by agarose gel electrophoresis on 0.8% gel. Isolated DNA was subjected for polymerase chain reaction analysis using gene specific designed primers (U6 and Cas9) for the confirm of transfer and integration of CRISPR/Cas9-gRNA gene construct from *Agrobacterium tumefaciens* to the genome of soybean plants. The annealing temperature was determined for each primer by gradient PCR. The amplified products were visualized by electrophoresis on 1.2 % agarose gel and photographed by (Syndene G: Box) imager gel documentation system. The result showed that, out of twenty-two transformed plantlets analyzed by the PCR amplification fourteen plants

were detected for the presence of U6 and Cas9 genes. The expected fragments length of U6 and Cas9 was obtained for respective genes in twelve plants (Fig.4.10 a & b), whereas no amplification was found in the control/wild plants. The PCR results demonstrated that only 57.1% of transformants contained all CRISPR/Cas9 components, indicating that Cas9 gene was not integrated in 42.8% of transformed plants during T-DNA transfer.

The most popular technique for analyzing a locus of interest is PCR-based amplicon creation. It uses complementary oligonucleotides as primers and a polymerase for cyclic DNA amplification, and it necessitates an understanding of the target DNA sequence of the altered locus. For the screening of genome-edited plants, a variety of standardized reference PCR techniques for the detection of transgenic constructs and traditional GMOs are available and used (Du *et al.,* 2017; Broccanello *et al.,* 2018; Chen *et al.,* 2018; Grohmann *et al.,* 2019 and Li *et al.,* 2020).

4.6.2 Nucleotide sequencing for gene specific PCR products of transformed and control plants

DNA was isolated from all PCR conformed, transformed and control plants. Fatty Acid Desaturase (FAD) gene amplified through PCR using gene specific primers, products were purified and qualitatively checked by electrophoresis on 1.2% gel. The purified products were sent for nucleotide sequencing by out sourcing from (Eurofins Labs Private Ltd.) to identify the mutation in the targeted genes. After the arrival of nucleotide sequencing data, it was subjected to quality check using BioEdit software. The clear peaks were observed in sequence chromatogram of all the samples sequenced indicating good quality of sequence data and very low sequencing error or back ground noise.

Many researchers have determined that conventional Sanger sequencing is appropriate for detecting targeted gene alterations of known sequences, even if the mutations are minor. According to reports, it is possible to amp up and sequence the changed locus, particularly from homogenous samples. NGS or targeted deep sequencing of a particular region proved a workable method for transgenic identification and was also modified for genome-edited plant analysis (Fraiture *et al.,* 2015; Staats *et al.,* 2016 and Li *et al.,* 2020). When compared to whole genome sequencing, focused

area sequencing by NGS greatly decreased the effort and expenses for detection and quantification; nevertheless, WGS does not require previous knowledge of a particular genetic mutation and may be used as an untargeted detection method for unknown mutational changes (Wahler *et al.*, 2013; Pauwels *et al.*, 2015 and Holst-Jensen *et al.*, 2016).

4.6.3 Bioinformatic analysis for the detection of targeted mutation induced in the lycopene-ε-cyclase gene

In the present study, BioEdit software was used to align the nucleotide sequence data to identify the mutation. The alignment results showed that, among the transformed Groundnut plants three *LCYε* transformed lines of Groundnut (GJG- 20) showed substitution type of mutation near PAM sequence and within the distal end of gRNA location indicating the specific double stranded break and insertion of mutation by CRISPR/Cas9 system (Fig.4.11 & 4.12). Mutation rate was calculated and found to be 13.63%. Moreover, the substitution of thymine residue occurred with cytosine in two lines (T3 and T4) and adenine residue occurred with cytosine in one line (T4). A variety of targeted mutation types were observed in CRISPR/Cas9 mediated genome editing tool, including deletions, insertions, and combined mutations (Cai *et al*. 2015 and Xianjun *et al.*, 2015)

Natural and exogenous mutagenesis fails in most cases because of the presence of highly sophisticated and efficient repair system. Cells respond to DNA damage by instigating robust DNA Damage Response (DDR) pathways, which allow sufficient time for specified DNA repair pathways to physically remove the damage in a substrate dependent manner (Chatterjee and Walker, 2017), this observation is consistent with what is known about the mechanisms of DNA repair (Puchta, 2005).

The present study demonstrated that the construct p201B containing Cas9 and gRNA together can generate precise mutation via NHEJ *A*grobacterium mediated delivery of Cas9 is the most efficient method available agreeing with the data presented by (Jacob *et al*,2015). The result presented here are significant with respect to both advancement of CRISPR-Cas9 based genome editing technology and plant biotechnology.

4.7 Estimation of Carotenoids in peanut seeds

Peanut mature seeds tissue (dry weight; DW) of transformed lines, as well as three non- transformed control plants were used for quantitative estimation of carotenoids (Figure: - 4.13 & 4.14). The β-carotene content was enhanced up to 1.5- 2 folds in the edited lines with respect to non-transformed control plants (0.82 µg/g). Line GJG20 showed the highest content of β-carotene (3fold; 3 µg/g). Line 559 accumulated high content of α-carotene (1fold; 1.2 µg/g) and β-carotene (3 fold; 3 µg/g) than in control α-carotene (1.5 µg/g) and β-carotene (3 µg/g). However, in lines G11, G12, G13 no significant increase in α-carotene and β-carotene content was observed compared to control. Line GJG20 showed the higher β-carotene equivalents (3 µg/g) than in control (0.82 µg/g).

Table 4.1 Different combinations and concentrations of BAP used in MS medium for shoot regeneration from De-embryonated cotyledon.

Sr. No.	**Media Code**	**BAP (mg l^{-1})**	**Percent shoot regeneration**
1	SB-0	2	**42.67**
2	SB-1	5	53.00
3	SB-2	10	57.00
4	SB-3	12	73.67
5	SB-4	15	**84.33**
6	**SB-5**	**25**	79.33
	SEM		1.35
	C.D. at 5 %		4.10

Table 4.2 Different combinations and concentrations of BAP and GA3 used in MS.

Sr No	**Media Code**	**BAP (mg l^{-1})**	**GA (mg l^{-1})**	**Percent shoot Multiplication and elongation regeneration**
1	SE-0	0.0	0.0	26.30
2	SE-1	0.5	0.1	45.33
3	SE-2	1.0	0.2	61.33
4	SE-3	1.5	0.3	67.00
5	SE-4	2.0	0.4	79.00
6	SE-5	2.5	0.5	81.67
7	SE-6	3.0	1.0	87.67

Table 4.3 Effect of various concentrations of NAA on root regeneration from multiple shoot explants in Groundnut.

Sr. No.	Media Code	NAA (mg l^{-1})	Root induction and multiplication
1	RI-0	0.0	11.05
2	RI-1	0.1	49.67
3	RI-2	0.5	50.33
4	**RI-3**	**1.0**	**86.33**
5	RI-4	1.5	69.67
6	RI-5	2.0	54.00
7	RI-6	2.5	78.33
	SEM		1.44
	C.D. at 5 %		4.37

Table 4.4 Details of the co-culture and beta Carotene editing plants developed from de-embryonated cotyledons cv. GJG20 using the p201B gene construct.

Details of culture	SET I	SET II	SET III	SET IV	SET V	Total
Number of explants in each set	76	96	25	16	36	239
No of explants regenerated	31	47	23	29	21	151
Number of shoots in selection medium	632	593	412	339	346	2322
Number of shoots passed selection	312	98	45	39	58	552
Number of shoots rooted	234	90	37	32	55	448
Number of shoots grown in glass house	234	72	32	32	53	423
Number of shoots survived	203	70	28	32	53	386

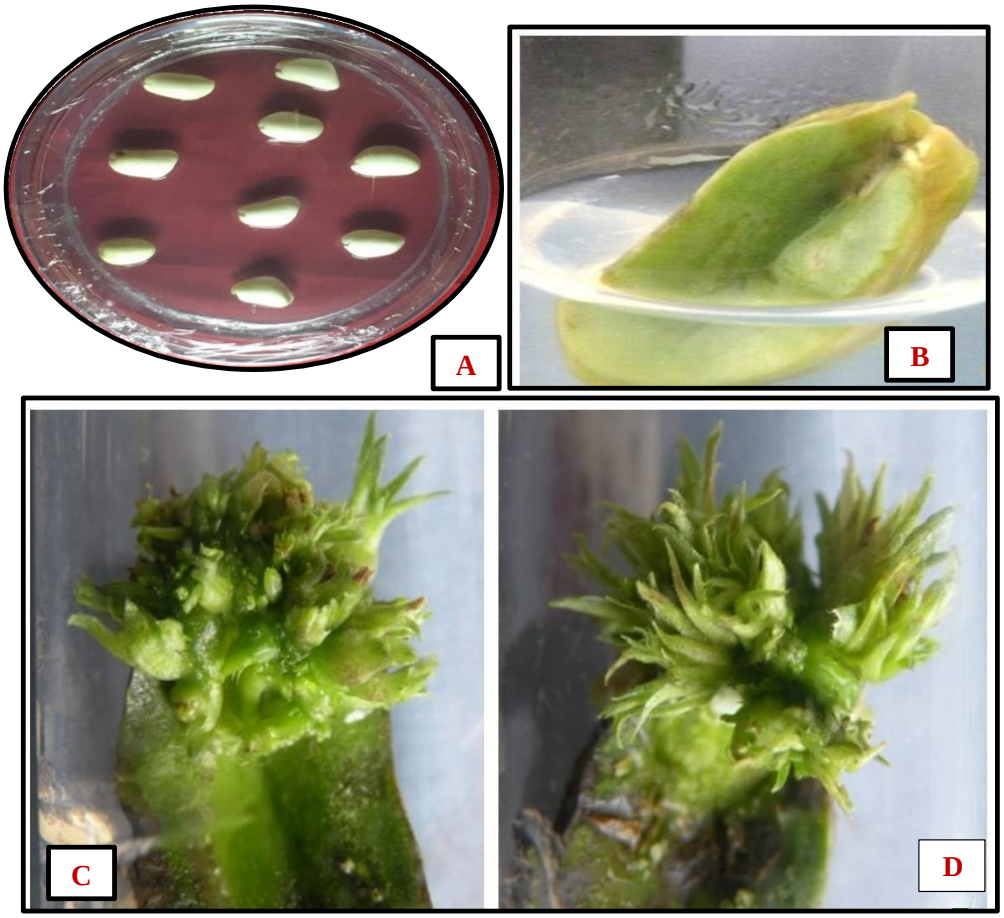

Figure 4.1 Co-culture of de-embryonated cotyledons and regeneration (A) De-embryonated cotyledons in co-culture medium containing 15 mg/L BAP, (**B**) Sub culture of green and enlarged de-embryonated cotyledons in culture medium containing 15 mg/L BAP, (**C**) Regeneration of multiple shoot buds from de-embryonated cotyledon explants, (**D**) Bunch of large number of multiple shoots with small quadrifoliate leaves

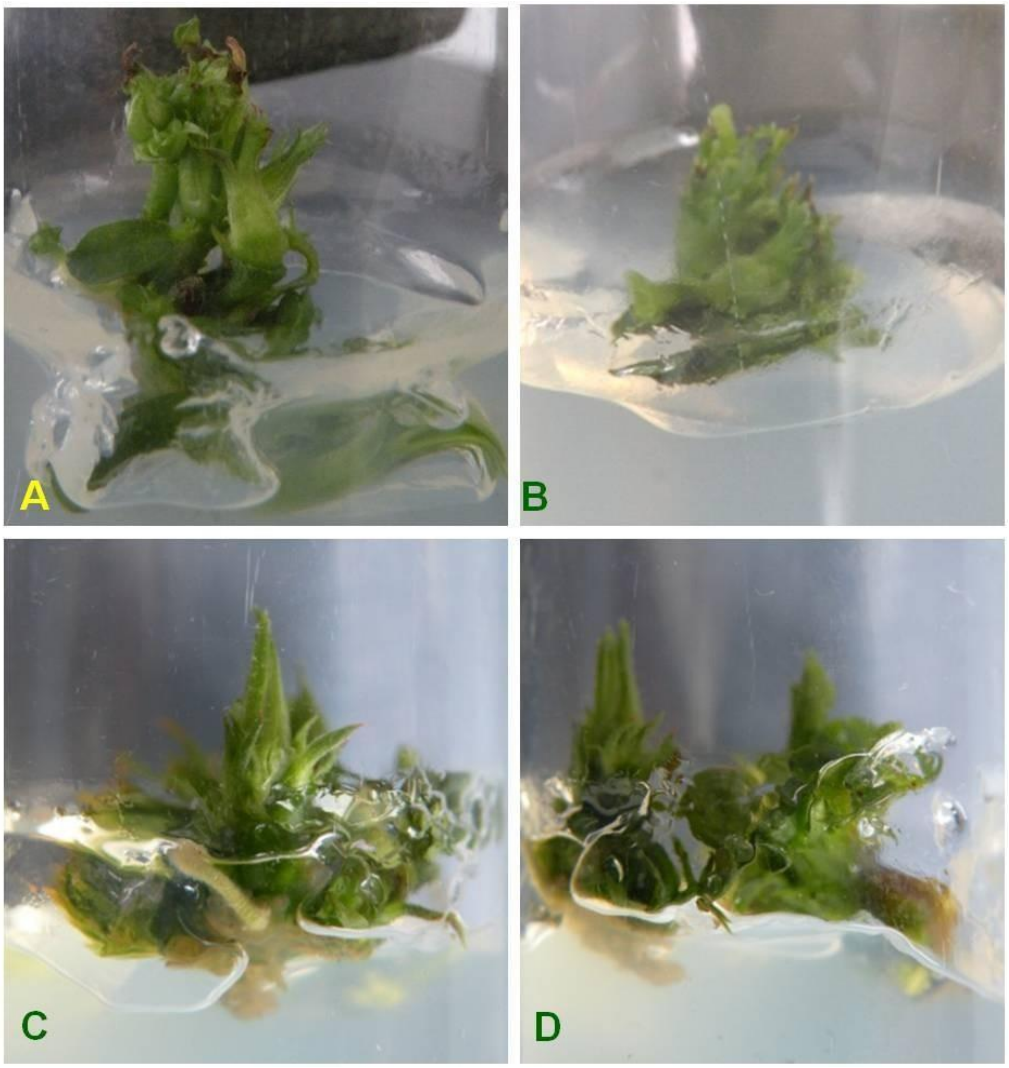

Figure 4.2: Shoot buds (developed from de-embryonated cotyledons) in hygromycin selection media A-B: Shoot buds in selection media containing 5 mg/L hygromycin, **C**: Shoot buds in selection media containing 10 mg/L hygromycin, **D**: Shoot buds in selection media containing 15 mg/L hygromycin

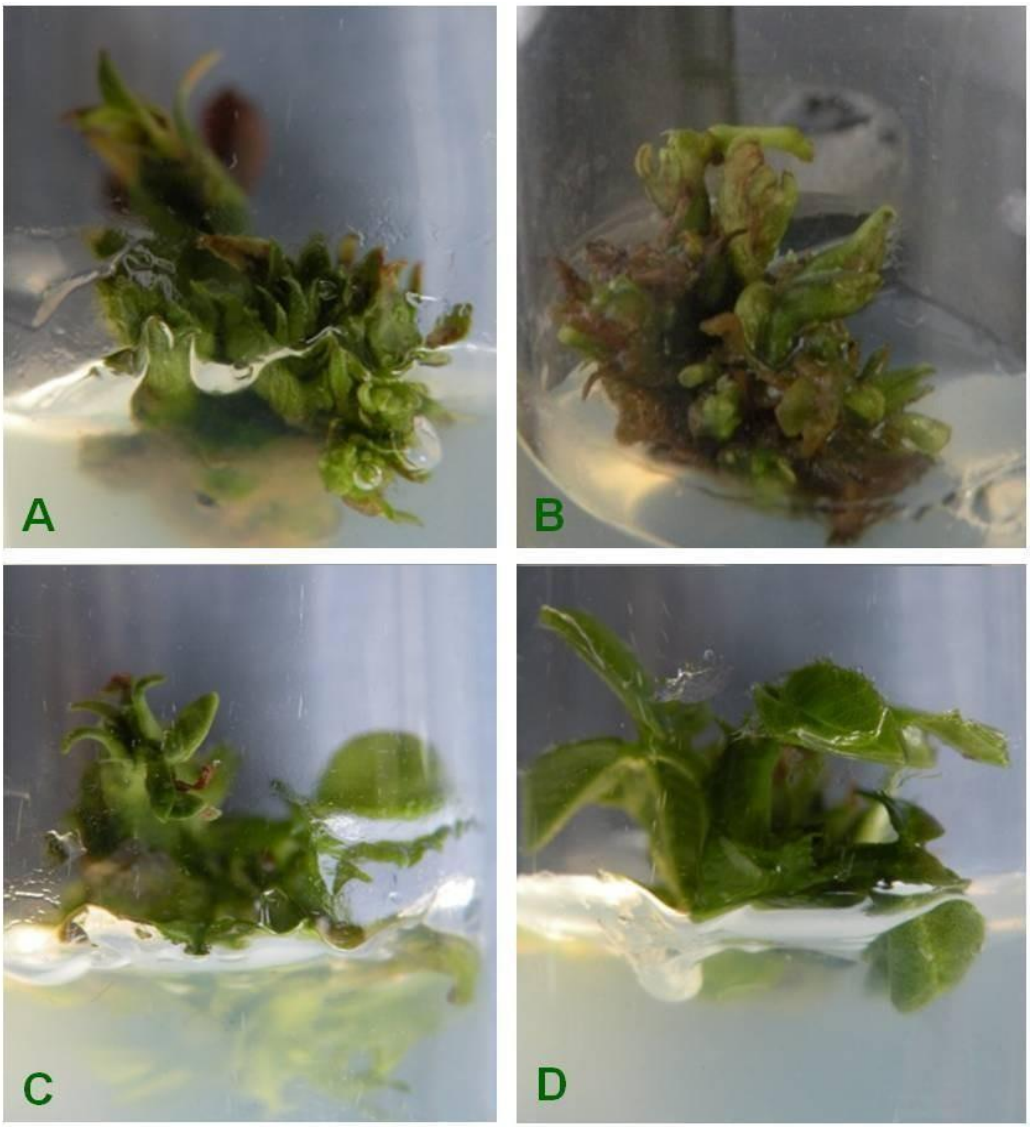

Figure 4.3 Shoot buds (developed from de-embryonated cotyledons) in hygromycin selection media (**A**) Shoot buds in selection media containing 20 mg/L hygromycin, (**B**) Shoot buds in selection media containing 25 mg/L hygromycin, (**C-D**) Survived shoot buds in hygromycin selection media.

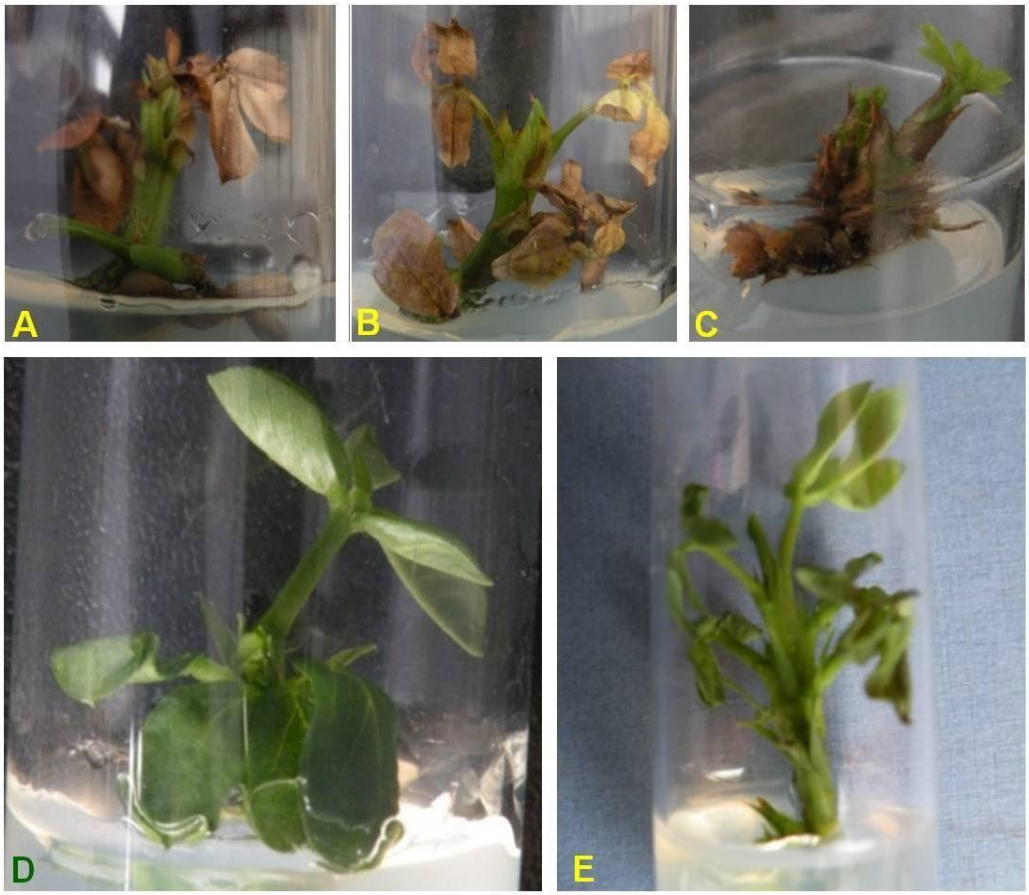

Figure 4.4 Individual shoots in hygromycin selection A-B: Individual shoots in hygromycin selection media, **D-E:** Individual healthy shoots passed hygromycin selection

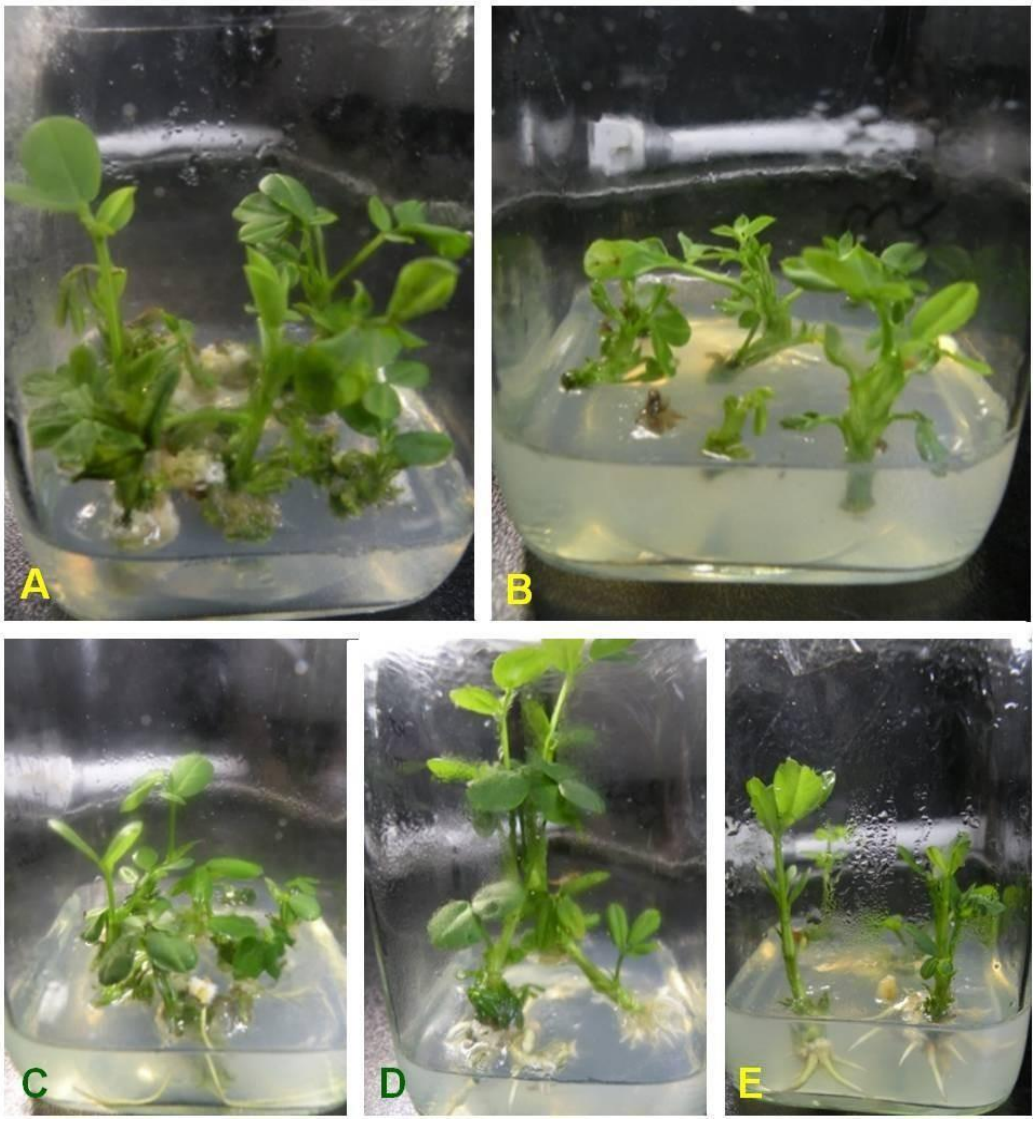

Figure 4.5 Individual shoots in rooting media A-E Individual elongated and healthy shoots passed hygromycin selection pressure and transferred in rooting media.

Figure 4.6 Hygromycin resistant individual plants (developed from de-embryonated cotyledons as explant) hardened in glass house.

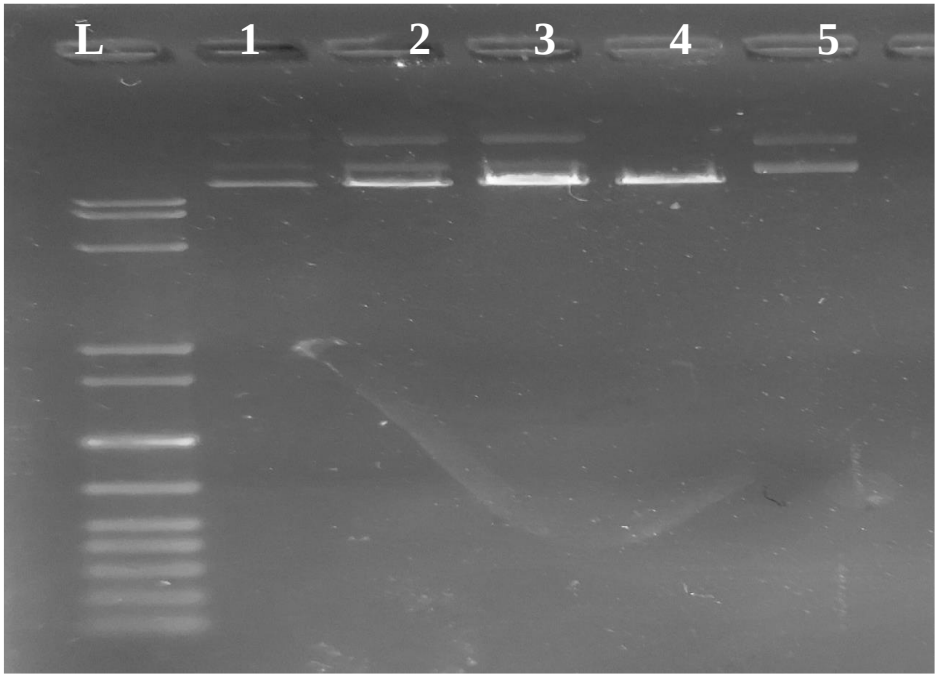

Figure 4.7 Restriction enzymes SwaI to digest p201b vector in figure L – Ladder 1kb; Lane - 1-3 Restriction digestion P201b plasmid**;** Lane 4 – Negative Control**;** Lane 5– Positive Control

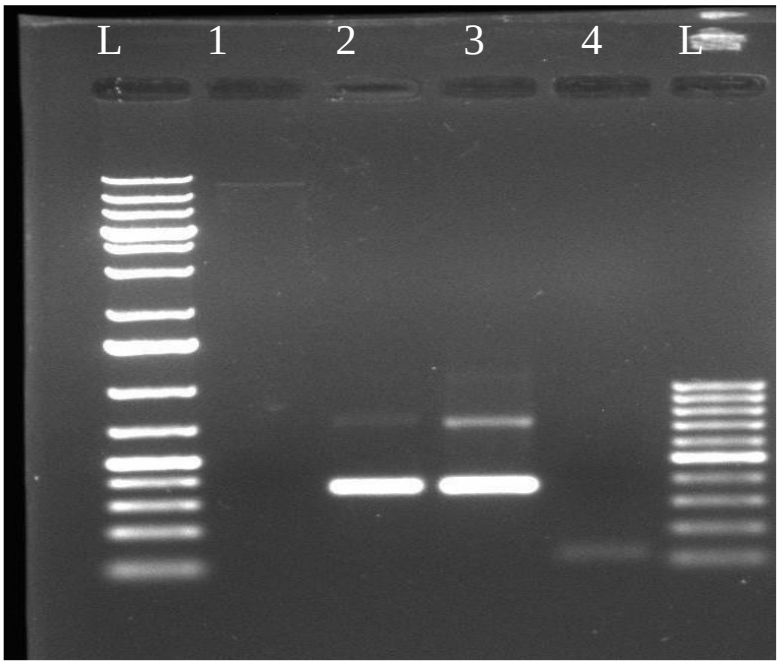

Figure 4.8 p201B vector cassette L - 1kb pulse leader**;** 1 – p201B digest product**;** 2-Spe I_MtU6 promoter**;** 3- Swal _MtU6 promoter**;** 4-SpeI_Scaffoldp; L – 100bp leader

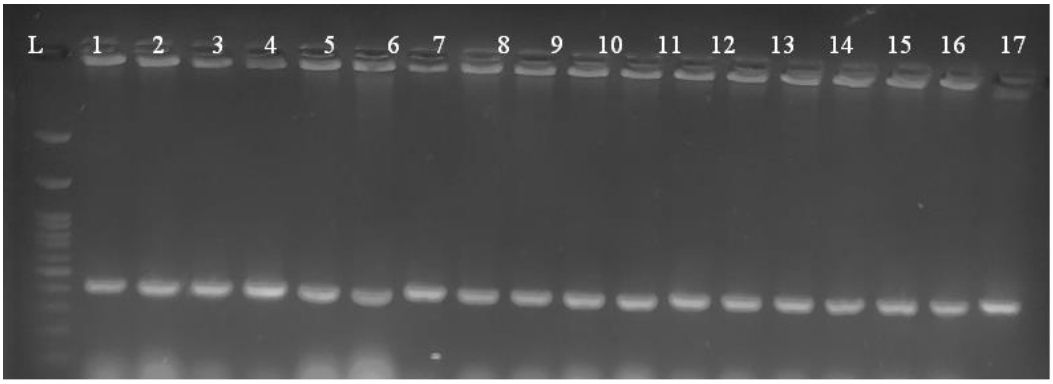

Figure A

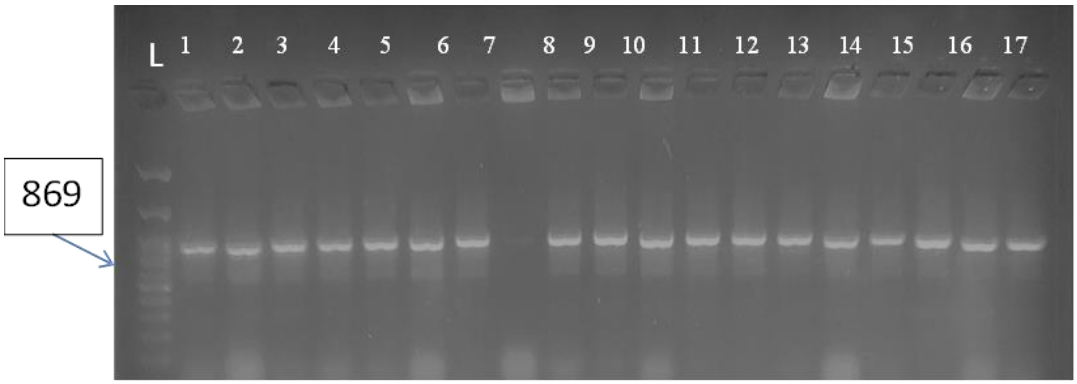

Figure B

Figure 4.9 Colony PCR analysis for presence of Cas 9

Fig A Colony PCR analysis for presence of Cas9 (439bp) in *E. coli* Strain DH5α

Fig B Colony PCR analysis for presence of Cas 9 (869bp) in *Agrobacterium* strain LBA 4404

L – Ladder 1kb,

Lane - 1-7 & 9-19 Transformed colonies Agrobacterium strain LBA 4404 Lane 19- Positive Control

Lane 8- Negative Control

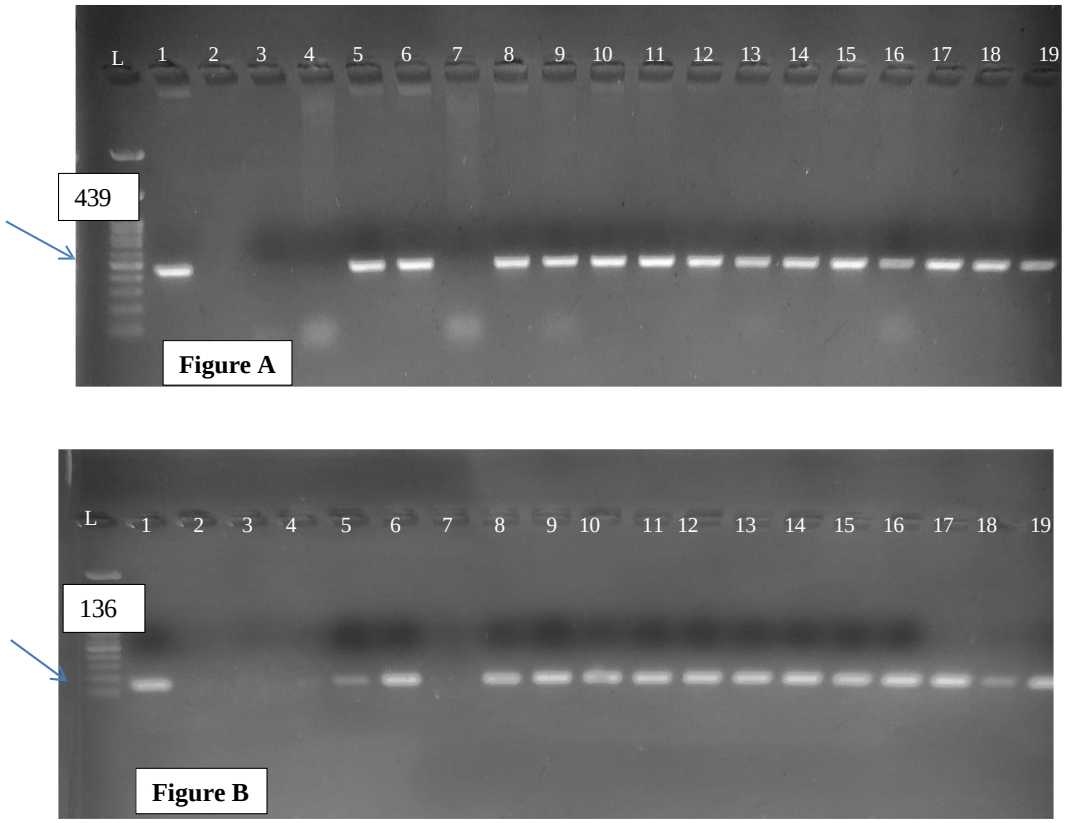

Figure 4.10 PCR analysis Beta Carotene transformed Groundnut (T0) plants

Fig A: -Amplification of U3 (136) promoter for gRNA integration and

Fig B: - Cas 9 A (439 bp)

L – Ladder 1kb,
Lane - 1 – Positive control,

Lane-2 –Negative control

Lane -5,6, 10-19 Transformed lines

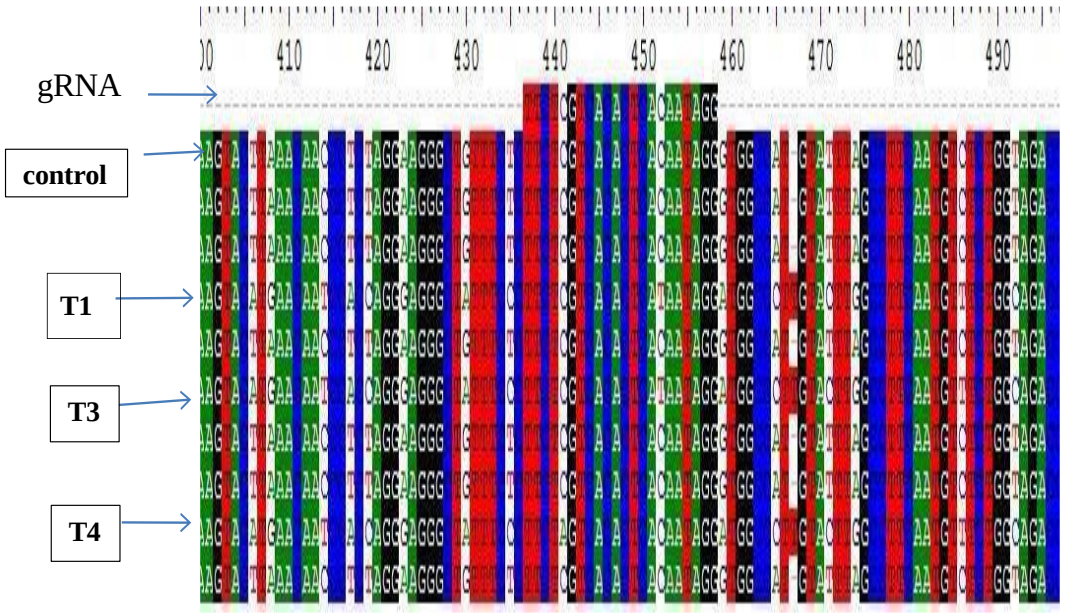

Figure 4.11 Alignment of DNA sequences obtained after sequencing result of T0 plants of Groundnut

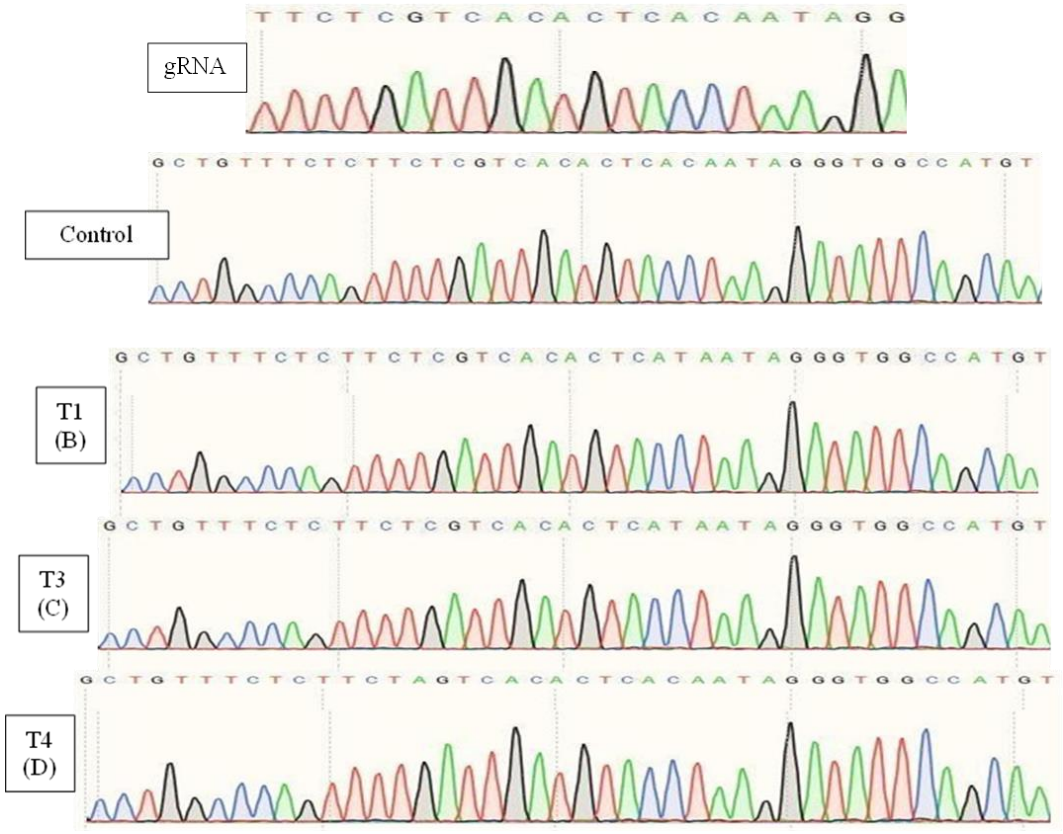

Figure 4.12 The sequencing chromatograms of LCYε gene in T0 generation
(A) Wild type non- transformed plants (B, C, D) Transformed lines of T0 generation

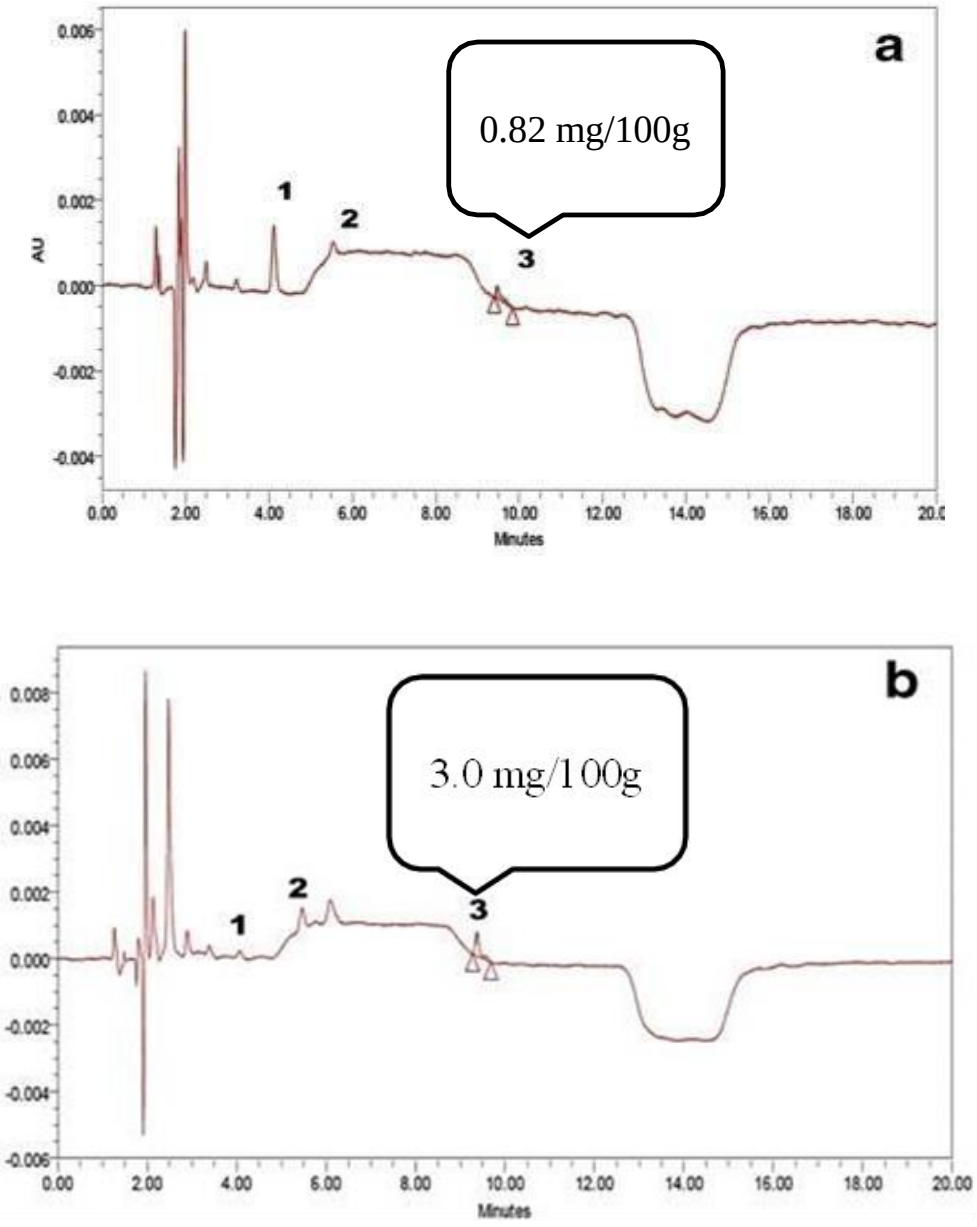

Figure 4.13: HPLC quantification of b-carotene; **A**- without genomic edited seed, b- after genomic editing concentration of **B**-carotene. Indicative peak numbers of the potential markers 1: lutein and zeaxanthin, 2: β-cryptoxanthin, 3: β-carotene

Figure 4.14: Visual observation of the altered amount of b-carotene in the groundnut cotyledon