CHAPTER 3:   MATERIALS AND METHODS

3.1   Plant tissue culture

3.1.1   Plant material and its tissue culture system

A total of eighty *Musa acuminata* cv. Berangan (AAA) tissue culture plantlets were purchased from the Malaysian Agriculture Research Institute (MARDI in Klang. The meristems were excised at a size of 1-2 cm leaving a meristematic dome with either one or two leaf initials. Meristems are usually excised at a length of 0.5-1 mm. However, according to Strosse *et al.,* (2001), for rapid multiplication, a relatively larger explant is advised despite its higher susceptibility to blackening and contamination. To further curb the problem of blackening, the media used for the growth of explants were supplemented with 10 mg/L of ascorbic acid which is an antioxidant as recommended by the authors.

In this study, the meristems were cultured in MS media supplemented with 5 mg/L 6-benzylaminopurine (BAP), 10 mg/L ascorbic acid, 30 g/L sucrose and Phytagel at 2 g/L. The pH of the media was previously adjusted to 5.8 and autoclaved at 120°C for 20 min at 15 psi.

The excised meristems were cultured on MS media and incubated in the dark for 2-3 days as a step to reduce oxidation that causes the blackening of the explants. The cultures were maintained in the incubation room with light period of 16 hours per day. Explants were left to adapt in tissue culture conditions for approximately 45 days. The established cultures were subcultured every 2 weeks to new media by dividing shoot clusters into individual shoots. Contaminated explants were rescued by submerging the explants in 70% ethanol for 2-3 min and later rinsed in sterile distilled water before subculturing into fresh media.

3.1.2 Explant preparation for particle bombardment

Fourty meristems were used for the genetic transformation process via particle bombardment. Achieving high rates of gene expression can be affected by the size and thickness of the target tissue as thinner tissues allow better penetration of the particles. Therefore, the meristems used were further reduced to approximately 0.5-1 cm in length. The meristems were also excised into half from the shoot clusters obtained to expose the inner cells of the meristem for direct contact with the tungsten particles during bombardment. Both halves of the meristem were then placed in petri dishes with MS media supplemented with BAP (5 mg/L) and ascorbic acid (10 mg/L). The agar acts as a support to help the explants absorb shock from the bombardment and also keep the tissues moist during incubation (Heiser, 1995). Particle bombardment may damage cells in the target tissue and has a very high mortality rate in the bombarded explants. To reduce this effect, subsequent to bombardment, explants were placed in the dark overnight before transferring to new media after 1 to 2 days.

3.2 Plasmid material

In this research, the plasmid pCAMBIA1304 containing the *EPO* gene was used with and without the KDEL sequence (pCEPO and pCEPOKDEL). The plasmids were developed in an earlier study conducted by Prof. Rofina Yasmin Othman's laboratory, University Malaya. The plasmids were stored at -20°C and were in the concentration range of 0.5 – 1.2 μg/ μL

3.2.1 Preparation of pCEPOKDEL and pCEPO

3.2.1.1   Transformation into *Escherichia coli* cells

Frozen competent DH5α *E.coli* cells were obtained from previous studies and were chosen for the transformation of the plasmids in this study. The competent cells were thawed in ice and 50 μL were aliquoted into 1.5 mL tubes and kept on ice. 50 ng of plasmid DNA were added to the *E.coli* cells. The contents were mixed by swirling gently. The tubes were then incubated on ice for 10 minutes. The tubes were then heat shocked in a water bath at 42°C for 45 secs. The tubes were then rapidly transferred to an ice bath for 5 mins. Then, 900 μL of LB broth were added to the tubes and incubated for 1 hr at 37°C. Subsequently, 100 μL from each culture was then spread on LB plates supplemented with 50 μg/mL kanamycin and incubated overnight at 37°C.

3.2.1.2   Colony selection

Colony selection for the transformed bacteria was carried out after 12 to 16 hrs incubation. This procedure was carried out under sterile conditions. The single bacteria colonies formed in the plates of the transformed cultures were selected using a sterile toothpick. The single colonies were cultured onto a gridded DNA library master plate containing LB agar supplemented with 50 mg/L kanamycin (Figure 1). The same colonies were also resuspended in 30 μL of distilled water in individual 0.5 mL tubes. The DNA library master plate was then incubated at 37°C overnight. The colony suspensions in the tubes of distilled water was boiled at 99°C for 10 mins before carrying out PCR screening using the *EPO* primers (refer to 3.3). The PCR screening was carried out to determine if the selected bacteria colony contained the recombinant plasmid.
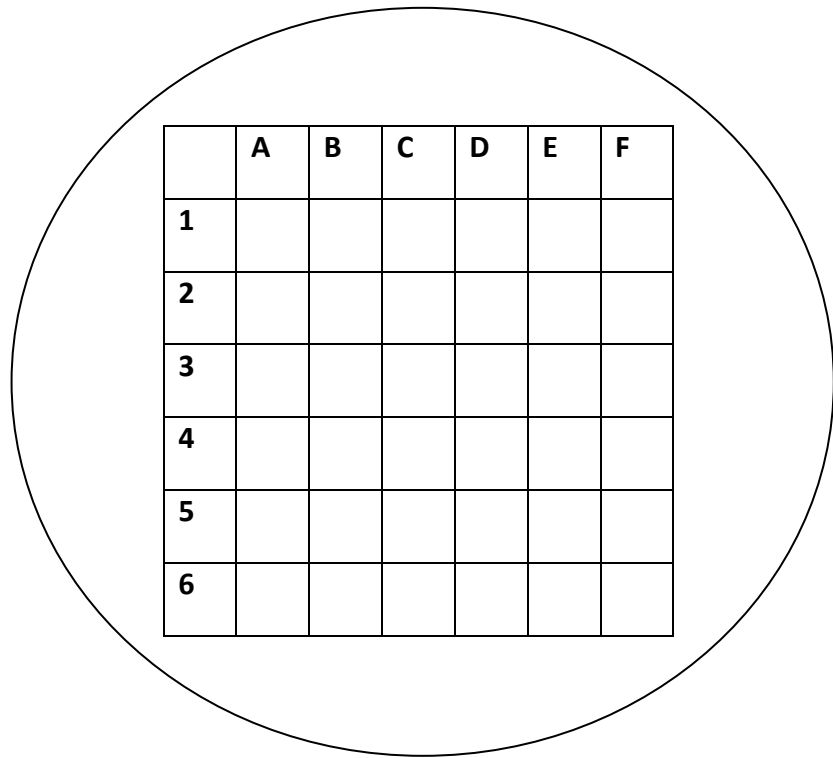
Figure 3.1: Schematic diagram of DNA library plate

3.2.1.3   Plasmid minipreparation

The colonies that showed the presence of the recombinant plasmid were chosen for plasmid extraction. The alkaline denaturation methodology was employed for plasmid extraction. This technique is based on the concept that there is a narrow pH range at which non-supercoiled DNA is denatured whereas supercoiled plasmids are not (Brown, 2001).

The selected colonies were picked from the DNA library plate prepared earlier. Using a sterile toothpick, the bacterial colony was inoculated into a Universal bottle containing 10 mL of LB media supplemented with 50 μg/mL kanamycin and left to incubate at 37°C shaken at 250 rpm overnight.

Then, 10 mL of the bacterial culture was transferred to a 15 mL Falcon tube and the cells were pelleted by centrifugation at 2,656 x g for 15 mins. The supernatant was discarded and the pellet was air dried. The bacterial pellet was resuspended in 200 μL of Solution 1 (50 mM Tris.Cl, 10 mM EDTA, 50 mM glucose buffer). The suspension was then transferred to a new 1.5 mL eppendorf tube and 200 μL of freshly prepared Solution 2 (10% SDS, 10M NaOH) was added and left at room temperature for 4 mins. Then 200 μL of Solution 3 (5M potassium acetate and glacial acetic acid) was added and the mixture left on ice for 15 mins and after which the suspension was centrifuged at 17,382 x g for 10 mins.

The supernatant containing the supercoiled plasmids were transferred to a new 1.5 mL tube and 5 μL of RNase A (10 mg/mL) was added to remove all the RNA molecules in the supernatant. The supernatant was left to incubate in a water bath at 37°C for 3 hrs. After 3 hrs, 600 μL of phenol was added, vortexed and then centrifuged for 3 mins at 17,382 x g for 3 mins. The aqueous layer formed was transferred into new 1.5 mL tubes. Then 600μL chloroform was added, vortexed and centrifuged at 17,382 x g for 3 mins.

The aqueous phase was transferred into new tubes and 0.1 Vol of 5M NaCl and 2.5 Vol of isopropanol was added and the tubes were left on ice for 20 mins and centrifuged for 15 mins at 17,382 x g. The supernatant was discarded and the pellet was washed with 1 mL of 70% ethanol and centrifuged again at 17,382 x g for 5 mins. The supernatant was discarded and the pellet was vacuum dried for approximately 10 mins. Finally, the pellet was dissolved in 50 μL of sterile distilled water and the DNA was stored at -20°C until required.

3.3   Polymerase Chain Reaction

3.3.1   PCR with *EPO* primers

Recombinant plasmid verification was carried out by amplifying the *EPO* gene using the Polymerase Chain Reaction (PCR) under these conditions:

1. Initial denaturation   1 min 95°C

2. Denaturation   1 min 95°C

3. Annealing   30 secs 55°C

4. Elongation   1 min 72°C

5. Repeat step 2   Additional 34 cycles

6. Final elongation   5 mins 72°C

7. Cooling   10 mins 25°C

The PCR reaction was performed using the Peltier Thermal Cycler PCR (PTC-100 Programmable Thermal Controller, MJ Research, Inc.). The primers that were used for this reaction were the *EPO* Forward primer 5'- CAT GCC ATG GCC CCA CCA CGC CTC ATC TGT-3' and the *EPO* Reverse primer 5'- CGA CTA GTC AAT TCA TCC TTA GAT CTG TGA TGG TGA TGG TGA TGC CAT CTG TCC CC-3'

The constituents of the PCR mix were prepared as follows:

1. Distilled water / DNA (1μg)   14.0 μl

2. Buffer 10X   2.5 μl

3. $MgCl_2$ (50mM)   3.0 μl

4. dNTP (10mM)   1.0 μl

5. Forward primer (10μM)   1.0 μl

6. Reverse primer (10μM)   1.0 μl

7. Taq polymerase (2.5 u/μl)   0.5 μl

    Total volume   25 μl

The PCR miniprep screening was carried out under the same conditions. However in the final mix, plasmid DNA was used instead of distilled water. PCR cDNA verification was also carried out under the same condition. In this case, cDNA was used instead of distilled water.

3.4 Preparation of pCEPO plasmid

3.4.1 Gel extraction

PCR with the *EPO* primers was carried out on pCEPO to identify the presence of the *EPO* gene. Since only the presence of the *EPO* is vital in the verification of this plasmid, thus verifying the presence of this gene in the plasmid would be sufficient. The

verification of the presence of the *EPO* gene in the plasmid was carried out firstly by isolating the fragment of gel showing the presence of the DNA band of interest, purifying the DNA and sequencing it.

Gel extraction was carried out to extract and purify the DNA fragments from normal or low-melt agarose gels in either Trisacetate (TAE) or Trisborate (TBE), or to purify PCR products directly from PCR amplification. In this study, the DNA was purified using the agarose purification protocol provided by MEGAquick-spin™ PCR and Agarose Gel DNA Extraction System.

The PCR product was electrophoresed with 1% Agarose gel. The gel was then stained using ethidium bromide and viewed under the UV lamp. To reduce nicking, the gel was irradiated for the absolute minimum time possible.

A 1.5 mL microtube was weighed and the weight was recorded. The DNA fragment of interest which has an expected size of approximately 500bp was excised with a minimal volume of agarose using a clean scalpel. The gel slice was transferred to the earlier weighed microcentrifuge tube and the weight was recorded. The weight of the empty tube was subtracted from the total weight to obtain the weight of the gel slice. BNL Buffer was added at a ratio of 300μL of solution per 100 mg of agarose gel slice. (3 volumes of BNL Buffer to 1 volume of gel). The mixture was vortexed and incubated at 55°C for 10 mins or until the gel slice is completely dissolved. One gel volume of isopropanol was then added to the dissolved gel solution and was mixed well by pippetting. One spin column (blue color) was placed in a collection tube for the dissolved gel slice. The dissolved gel mixture was loaded to the spin column and centrifuged at 13000 rpm for 1 min. The flow-through was discarded after centrifugation and the spin column was placed back into the same collection tube.

Then, 700 µL of Washing Buffer was added to the column and centrifuged at 13000 rpm for 1 min. The flow-through was discarded after centrifuging and the spin column was placed back into the same place collection tube. To dry the membrane, the spin column and collection tube assembly was centrifuged for 1 min at 13000 rpm. This step is crucial as it is important to dry the spin membrane since residual ethanol may interfere with other reactions. The spin column was then placed to a clean 1.5 mL microcentrifuge tube and 30-100 µL of the Elution Buffer was added directly to the centre of the column without touching the membrane with the pipette tip. This was incubated at room temperature for 1 min and subsequently centrifuged for 1 min at 13000 rpm. The spin column was discarded and the microcentrifuge tube containing the eluted DNA was stored at -20°C

3.4.2 Sequencing

The purified DNA containing only the gene of interest was sent for cycle sequencing at First Base Laboratories Sdn Bhd. Sequencing is a fundamental requirement for modern genetic manipulation as it provides the whole sequence of the gene of interest thus allowing accurate analysis and easier manipulation of the product sequence (Primrose *et al.,* 2001).

3.4.3 Computer analysis

Multiple alignment using CLUSTALW and Basic Local Alignment Search Tool (BLAST) was carried out on the product sequence. BLAST was conducted by comparing the product sequence with the human *EPO* gene sequence. The human *EPO*

gene sequence in the GeneBank database at the National Centre for Biotechnology (NCBI) (GENE ID: 2056) was compared against the product sequence. Both sequences were compared for similarities to enable verification of the presence of the *EPO* gene in the product sequence.

Carrying out multiple alignment using CLUSTALW on the sequence data of the product and the *EPO* gene sequence would indicate accurately the presence of any base similarities or differences in both the sequences. Similarities would be clearly indicated with an asterisk (*) symbol.

3.5   Preparation of pCEPOKDEL plasmid

Due to the reduced volume of pCEPOKDEL plasmid obtained, the three tubes of plasmids obtained were pooled together into one tube for the rest of the verification and transformation processes. Verification of this plasmid was carried out by PCR using *EPO* primers and this was further verified by the sequencing of the whole plasmid. This is because, sequencing the gene of interest would only verify the existence of the *EPO* gene and not the KDEL sequence, therefore, sequencing the whole plasmid will be able to show the presence of not only the *EPO* gene but also the presence of the KDEL sequence and its position relative to each other.

3.5.1   Sequencing

The plasmid was sent for cycle sequencing at First Base Laboratories Sdn Bhd.

3.5.2 Computer analysis

Multiple alignment using CLUSTALW and Basic Local Alignment Search Tool (BLAST) was also carried out on the plasmid sequence.

3.6 Biolistic-mediated transformation

The explants that were excised in Section 3.1.2 were utilised for biolistic-mediated transformation. The explants were bombarded with pCEPOKDEL and pCEPO using the Biolistic Gun PDS-1000/He™ System (Bio-Rad, USA). The explants were bombarded using 1.2 μm tungsten as microcarriers and at helium pressure of 1350 psi with a distance of 6 cm from the target tissue as previously established by Wong (2004). The biolistic transformation was carried out according to the standard protocol of the manufacturer (Bio-Rad). RNA was extracted from the bombarded explants 25 days and 50 days after transformation.

3.7 Verification of transformants

In this study, the verification of transformants was determined by assaying the expression of the green fluorescent protein (GFP) in the explants one week after bombardment and also by carrying out PCR on the cDNA obtained through Reverse Transcriptase PCR (RT-PCR) of RNA extracted from the samples.

RNA obtained from the explants was reverse-transcribed to obtain the DNA copy of the RNA template. This was carried out to determine if *EPO* was expressed in the explants. The gene coding for the *EPO* protein if expressed in the explants will initially be

transcribed into mRNA molecules. By harvesting the RNA and reverse-transcribing it to cDNA, it would be able to determine not only the presence of the *EPO* gene in the explants but also its functionality. This method may not be able to determine if the mRNA is further translated to *EPO* protein but it can establish the presence of the *EPO* gene in the explants.

3.7.1 Visualisation of green fluorescent protein

The gene coding for green fluorescent protein (GFP) was incorporated into the plasmid pCAMBIA1304 as a reporter gene. The GFP was visualised at 430 to 460 nm wavelength with the Olympus IX71 microscope (Japan).

3.7.2 RNA extraction

RNA was extracted from the explants that were bombarded with pCEPOKDEL and pCEPO 25 days and 50 days after transformation. This was carried on explants that survived the biolistic transformation and not on explants that died, blackened or were contaminated. RNA extraction was carried out using the RNeasy Plant Mini Kit from Qiagen. The procedure used was according to the manufacturer's instructions (Qiagen). This technology uses a combination of selective binding properties of a silica-based membrane with the speed of microspin technology. To eliminate DNA contamination, DNase digestion is required in this procedure. This was carried out by incorporating DNase digestion in the RNA extraction procedure. In this study, the RNase-Free DNase Set from Qiagen was used for this purpose. Using this product provides efficient on-column digestion of DNA during RNA purification.

3.7.3   Reverse Transcriptase-PCR (RT-PCR)

Single-stranded cDNA was reverse-transcribed from total RNA of the explants using the High-Capacity cDNA Reverse Transcription Kit from Applied Biosystems. This procedure was able to quantitatively convert 2 μg of total RNA to cDNA in a 20 μL reaction. RT-PCR was carried out to confirm the functionality of the *EPO* gene in the bombarded plants. RT-PCR forms a ds-DNA copy of the RNA template. The presence of the *EPO* sequence in the cDNA would then verify the formation of the *EPO* mRNA in the bombarded explants.

According to the manufacturer's instructions, this reaction was carried out under these conditions:

1. Step 1   10 mins 25°C

2. Step 2   120 mins 37°C

3. Step 3   5 secs 85°C

4. Step 4   ∞ 4°C

The RT master mix was prepared on ice using these constituents:

1. 10X RT buffer   2.0μl

2. 25X dNTP (100mM) mix   0.8 μl

3. 10X RT random primers   2.0 μl

4. Multiscribe ™ RT (50U/μL)   1.0 μl

5. RNase inhibitor   0.5 μl

6. Nuclease free water   3.2 μl

3.7.4   PCR on cDNA obtained

The presence of the *EPO* gene in the explants was verified by amplifying the *EPO* gene using PCR on the cDNA obtained. PCR was carried out on the cDNA (as previously described in 3.3.1).

3.7.5   Purification of the PCR product

Once again the MEGAquick-spin™ PCR and Agarose Gel DNA Extraction System (Section 3.4.1) was used to purify the PCR product obtained from the PCR of the cDNA obtained from the bombarded explants. This procedure was carried out to remove excess primers and nucleotides left in the PCR mix. The product attained was used for sequencing. Sequencing of the PCR product was carried out to verify the sequence of the *EPO* gene in the transformants.

3.7.4   Sequencing

The purified PCR product obtained was sent for commercial sequencing at First Base Laboratories Sdn Bhd.

3.7.5   Computer analysis

Multiple alignment using CLUSTALW and Basic Local Alignment Search Tool (BLAST) was carried out on the sequence of the transformant.

CHAPTER 4: RESULTS

4.1 Plant material

4.1.1 Plant propagation

The excised meristems of *Musa acuminata* cv. Berangan were cultured in MS medium supplemented with 5 mg/L 6-benzylaminopurine (BAP), 30 g/L sucrose, 10 mg/L ascorbic acid and Phytagel at 2 g/L (see Figure 4.1) The meristems showed high propagation potential with 66 explants out of 80 explants produced multiple shoots. This indicated that 82.5% of meristems regenerated after culturing (Table 4.1).

However there were problems of contamination and the contaminated explants were rescued using the method described in Section 3.1.1. The meristems also suffered severely from blackening of its tissue material and this problem was curbed to reduce the rate of mortality of the explants as described in Section 3.1.1. The results summarised in Table 4.1 shows that after 50 days of culture, 11.25% of the explants suffered from contamination whilst the remaining 6.25% explants died due to blackening of the whole explants. The surviving plants were used for the transformation procedure.

4.1.2 Plant material for transformation

Propagated shoots and roots were excised as described in Section 3.1.2. The target tissue or meristems were placed on petri dishes with MS media supplemented with BAP (5 mg/L) and ascorbic acid (10mg/L) (Figure 4.2). Fourty surviving meristems were used for transformation experiments (as described in 3.6).

Condition of cultures	Number of explants	Percentage (%)
Living explants	66	82.5
Contaminated	9	11.25
Blackened	5	6.25
TOTAL	**80**	**100**
STANDARD DEVIATION	**34.12**	**42.65**
STANDARD ERROR	**19.69**	**24.62**

Table 4.1: Number and percentage of surviving and dead cultures after 50 days of culturing

Figure 4.1: Propagation of banana meristems excised from cultures obtained

from MARDI. Scale bar represents 1 cm.

Figure 4.2: Excised meristems for bombardment. Scale bar represents 1 cm.

4.2   Plasmid preparation

4.2.1   PCR analysis of plasmids for both pCEPOKDEL and pCEPO with
         ***EPO* primers**

PCR was carried out on the plasmids obtained from the transformation in *E.coli* cells. This step was carried out to verify the presence of the *EPO* gene. The PCR product was electrophoresed in a 0.7% w/v agarose gels with 1X TBE buffer and run at 120 volts for 25 mins (Figure 4.3 and 4.4). The results obtained showed the presence of the *EPO* gene as a specific band with the expected size of 550bp in the electrophoresed gel.

4.2.2   Preparation of pCEPO

One positive PCR product from pCEPO was selected and electrophoresed in agarose gel and based on the expected band size formed the fragment was extracted and purified as described in Section 3.4.1. The product obtained was electrophoresed in a 1% w/v agarose gel with 1X TBE and run at 120 volts for 25 mins (Figure 4.5). A very distinct and clear band at 550bp was obtained when the purified product was subjected to gel electrophoresis.

The purified plasmid product was also sequenced to further verify the presence of the *EPO* gene as well as to confirm the homology of the DNA fragment of interest (Appendix 8.1). The sequence data of the amplified fragment was then analysed by comparing it with the human *EPO* gene sequence using BLAST (Appendix 8.2). The results indicated more than 90% homology between the *EPO* sequence of the amplified fragment and the human *EPO* mRNA. The amplified fragment sequence was aligned with the *EPO* gene sequence using CLUSTALW and the results showed a perfect

homology of the *EPO* gene in the amplified fragment with the *EPO* gene sequence with the exception of 4 bases at the 5' end of the fragment (Appendix 8.3). Difference in the original source of the EPO gene may account for this dissimilarity.

4.2.3   Preparation of pCEPOKDEL

PCR was performed using the plasmid pCEPOKDEL. Results showed the presence of the *EPO* gene in the plasmid with the amplification of the expected band size in the electrophoresed gel 550bp (Figure 4.6). The whole pCEPOKDEL plasmid was then sequenced (Appendix 8.4). Multiple alignments of the plasmid sequence and the KDEL sequence confirmed the presence of the KDEL sequence (Appendix 8.5).
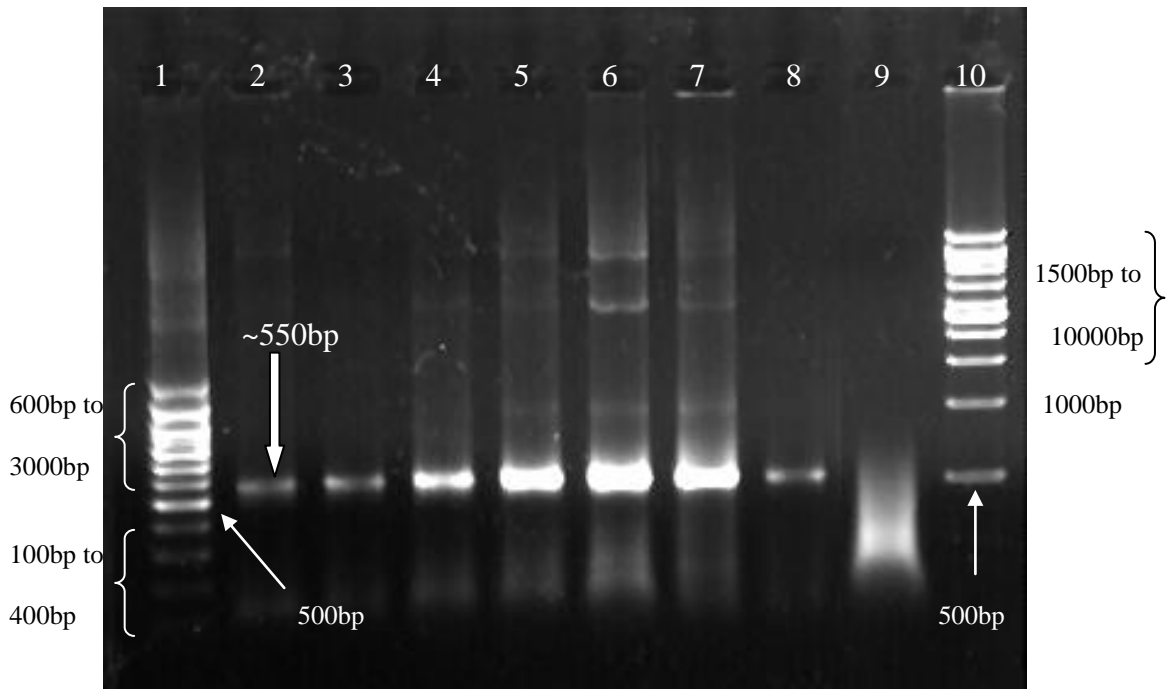
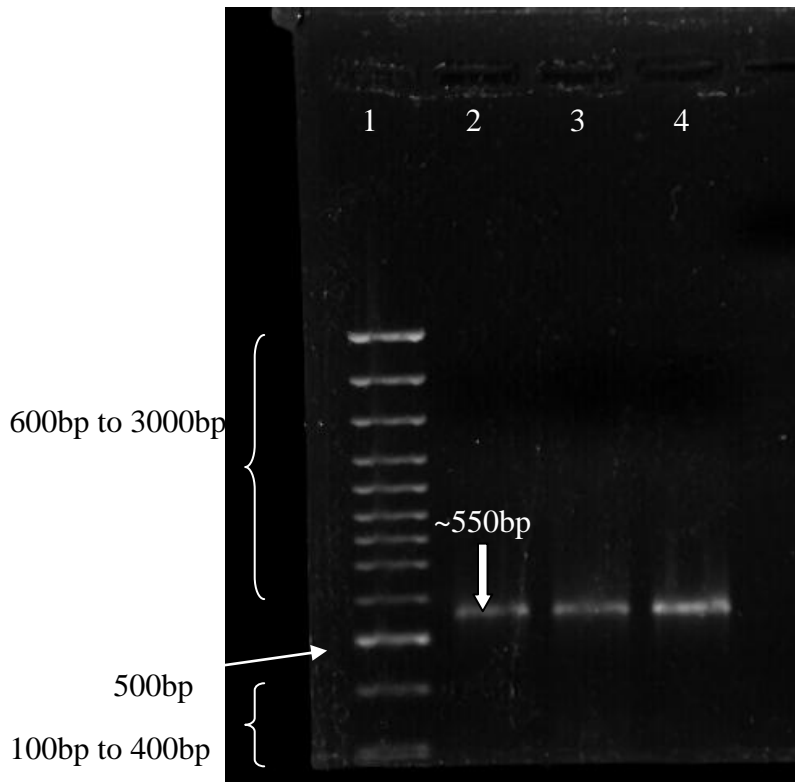**Figure 4.3:** PCR using *EPO* primers on pCEPO plasmids electrophoresed on 0.7% w/v agarose gel with 1X TBE. The expected 550bp fragment is as indicated.

Lane 1: 100bp ladder	Lane 6: Plasmid sample 5
Lane 2: Plasmid sample 1	Lane 7: Plasmid sample 6
Lane 3: Plasmid sample 2	Lane 8: Positive control
Lane 4: Plasmid sample 3	Lane 9: Negative control
Lane 5: Plasmid sample 4	Lane 10: 1kb ladder

Figure 4.4: PCR using *EPO* primers on pCEPOKDEL plasmids electrophoresed on 0.7% w/v agarose gel with 1X TBE. The expected 550bp fragment is as indicated.

Lane 1: 100bp DNA ladder	Lane 3: Plasmid sample 9
Lane 2: Plasmid sample 8	Lane 4: Plasmid sample 10
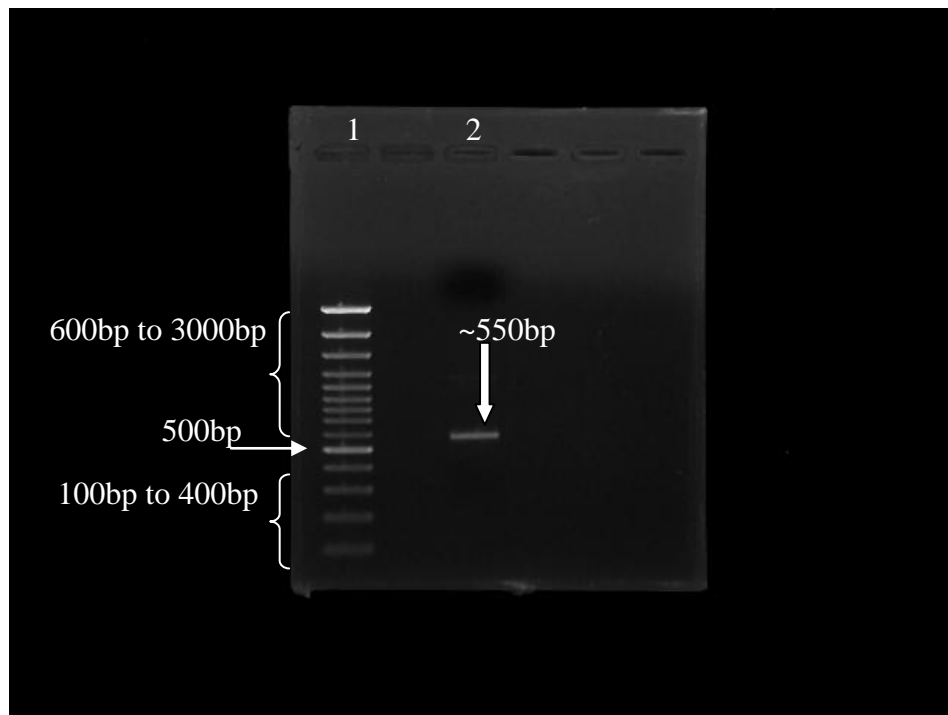
Figure 4.5: Amplified fragment of *EPO* from pCEPO plasmid sample electrophoresed on 1% w/v agarose gel with 1X TBE
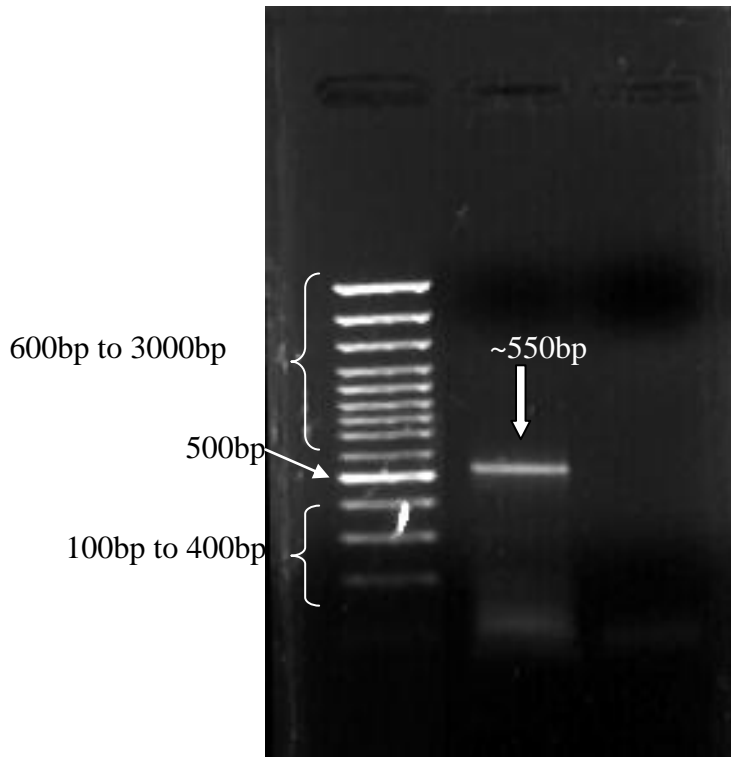
| Lane 1: 100bp DNA ladder | Lane 2: Purified PCR fragment |

Figure 4.6: Amplified fragment of *EPO* gene from pCEPOKDEL plasmid sample electrophoresed on 0.7% w/v agarose gel with 1X TBE

Lane 1: 100bp ladder	Lane 2: Plasmid sample
Lane 3: Negative control	

4.3 Transformation

4.3.1 Survival of explants

Molecular analysis was carried out on the explants that survived particle bombardment (Figures 4.7 and 4.8). The rate of survival of the meristems bombarded with pCEPO and pCEPOKDEL is as summarised in Table 4.2. The propagated bombarded plants also showed noticeable signs of growth retardation and reduced number of shoots formed as compared to the normal plants (plants which were not transformed) (Figure 4.9, 4.10 and 4.11).

No specific selection media was applied at this stage as only limited numbers of explants were used in this study. Confirmation of transformants were made *via* visual analysis of GFP expression and molecular analysis.

4.3.2 Visualisation of GFP

Successful transformation event was suggested by the visualisation of green fluorescence (Figure 4.13) while red spots show explants with no recombinant proteins expressed (Figure 4.12). Table 4.3 summarises the number of explants showing putative successful transformation occurrence via visualisation of GFP. The results indicated that 9 out of 12 explants bombarded with pCEPOKDEL showed the presence of GFP and 80% of the explants bombarded with pCEPO showed the presence of GFP.

4.3.3 Molecular analysis of transformants

RNA was extracted from surviving explants bombarded with pCEPO and pCEPOKDEL using the method mentioned in Section 3.7.1. Extraction was carried out once after 25 days bombardment and once again after 50 days bombardment on explants that survived the transformation process (Table 4.2)

Extracted RNA was then converted to cDNA and used as target material for RT-PCR. The steps taken for RT-PCR are as described in Section 3.7.2. The cDNA was then subjected to PCR with *EPO* specific primers to confirm the presence as well as the expression of the *EPO* gene in the transformants (Figures 4.14, 4.15 and 4.16). 1 out of 4 explants bombarded with pCEPO subjected to RT-PCR showed the expression of the *EPO* gene after 25 days post-bombardment and 50 days post- bombardment, 80% of the explants showed the presence of the *EPO* gene. Bombardment with pCEPOKDEL on the other hand showed no transformation event after 25 days bombardment and after 50 days bombardment only 1 out of the 6 explants subjected to RT-PCR showed the presence of expressed *EPO* (Table 4.3). The transformation efficiency was between 5 - 20% where only 1 out of 20 explants bombarded with pCEPOKDEL expressed the *EPO* gene while 4 out of 20 explants bombarded with pCEPO showed the presence of *EPO* (Table 4.4). Selected PCR products amplified from both transformation events (pCEPOKDEL and pCEPO) putatively coding the *EPO* gene were purified using the MEGAquick-spin™ PCR and Agarose Gel DNA Extraction System (Section 3.4.1) and sent for sequencing. The sequencing data obtained from both the PCR products (Appendix 8.6 and 8.7) was subjected to BLAST, both sequences indicated the presence of the human *EPO* mRNA (Appendix 8.8 and 8.9). When aligning both sequences with the *EPO* sequence, the amplified sequence showed more than 80% similarities with the original *EPO* sequence (Appendix 8.10 and 8.11).

DNA for bombardment	Days after bombardment	Total number of bombarded explants	Number of surviving explants	Percentage of surviving explants	Number of dead explants	Percentage of dead explants
pCEPO	25 days	20	4	20%	16	80%
pCEPO	50 days	20	5	25%	15	75%
pCEPOKDEL	25 days	20	12	60%	8	40%
pCEPOKDEL	50 days	20	6	30%	12	60%

Table 4.2: Rate of survival and death of bombarded explants

DNA used for bombardment	Time of post-bombardment	Number of explants subjected to RT-PCR	Number of explants with positive results (presence of *EPO* gene)	Percentage of positive cultures
pCEPO	25 days	4	1	20%
pCEPO	50 days	5	4	80%
pCEPOKDEL	25 days	12	0	0
pCEPOKDEL	50 days	6	1	16.7%

Table 4.3: Results of RT-PCR on bombarded cultures

DNA used for bombardment	Total number of explants bombarded	Total positive results proved by RT-PCR	Percentage of transformation
pCEPO	20	4	20%
pCEPOKDEL	20	1	5%

Table 4.4: Transformation efficiency (after 50 days post-bombardment)
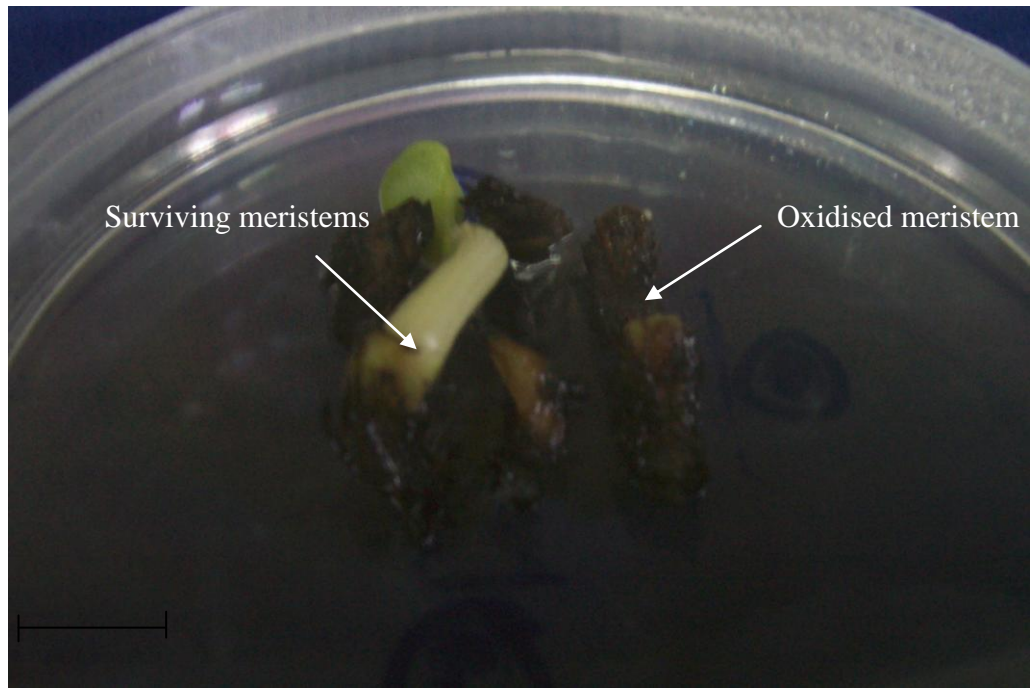
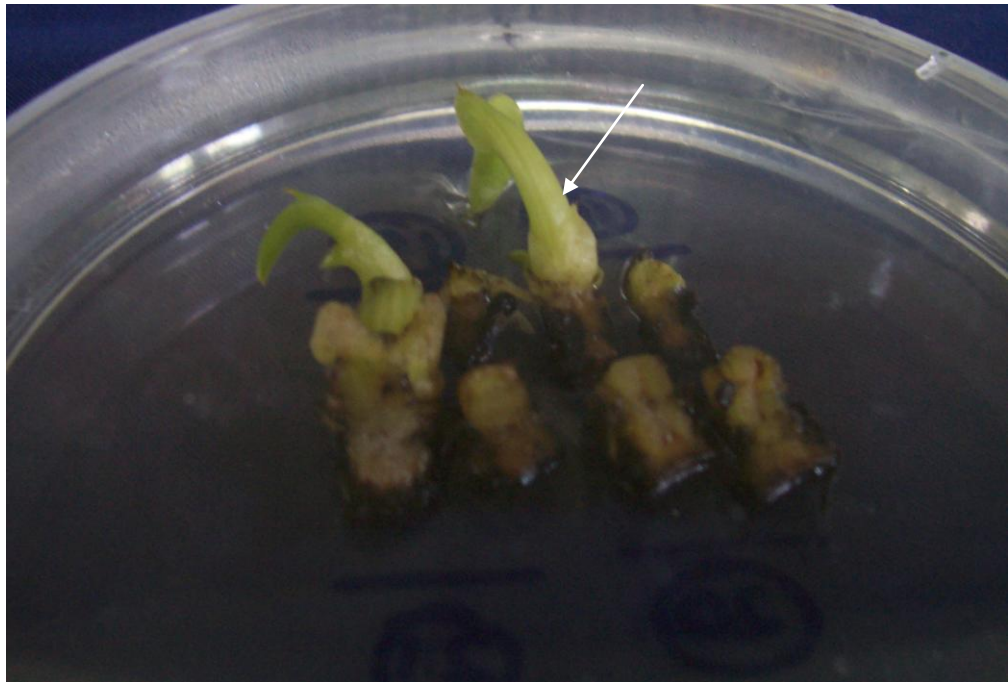**Figure 4.7:** Surviving and oxidised meristems after bombardment. Scale bar
represents 1 cm

Figure 4.8: Surviving meristems used for molecular analysis. Scale bar
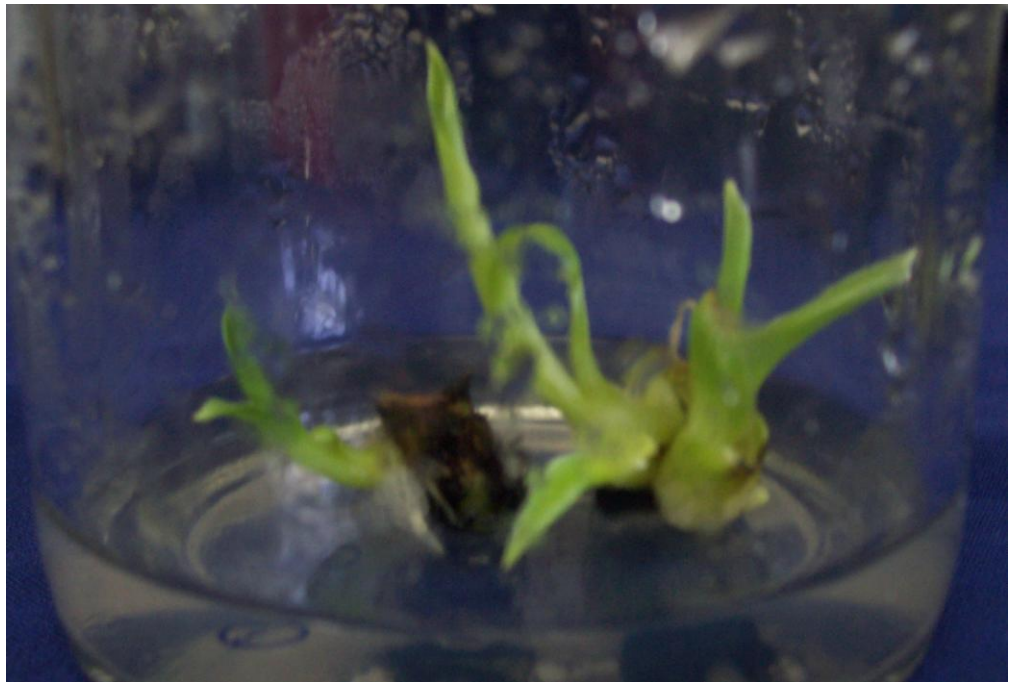represents 1 cm

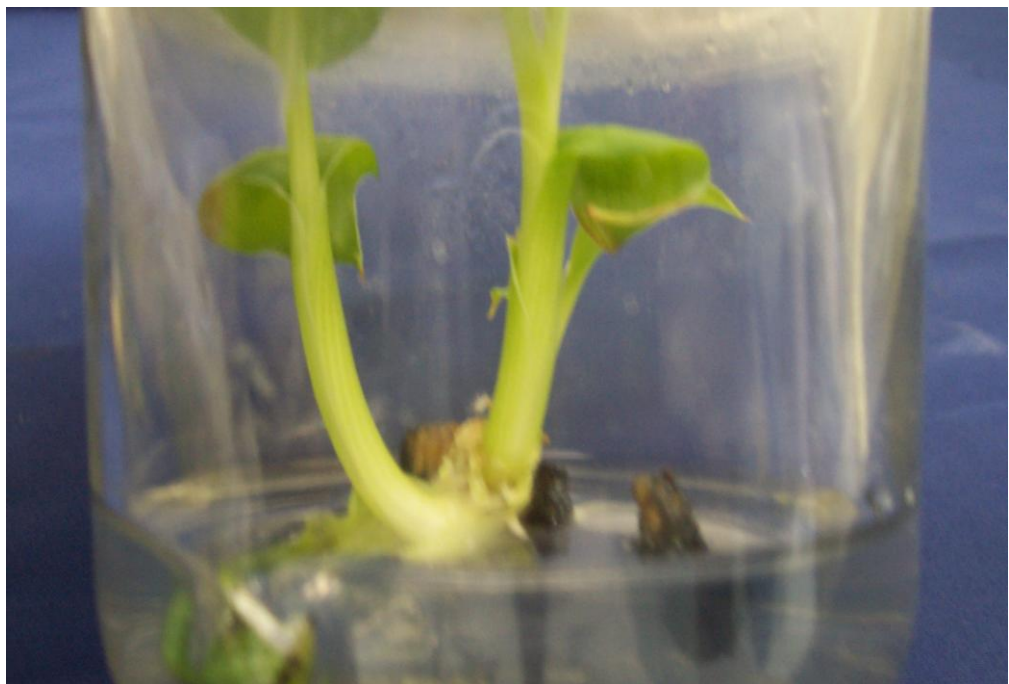**Figure 4.9:** Normal meristems at 25 days. Scale bar represents 1 cm

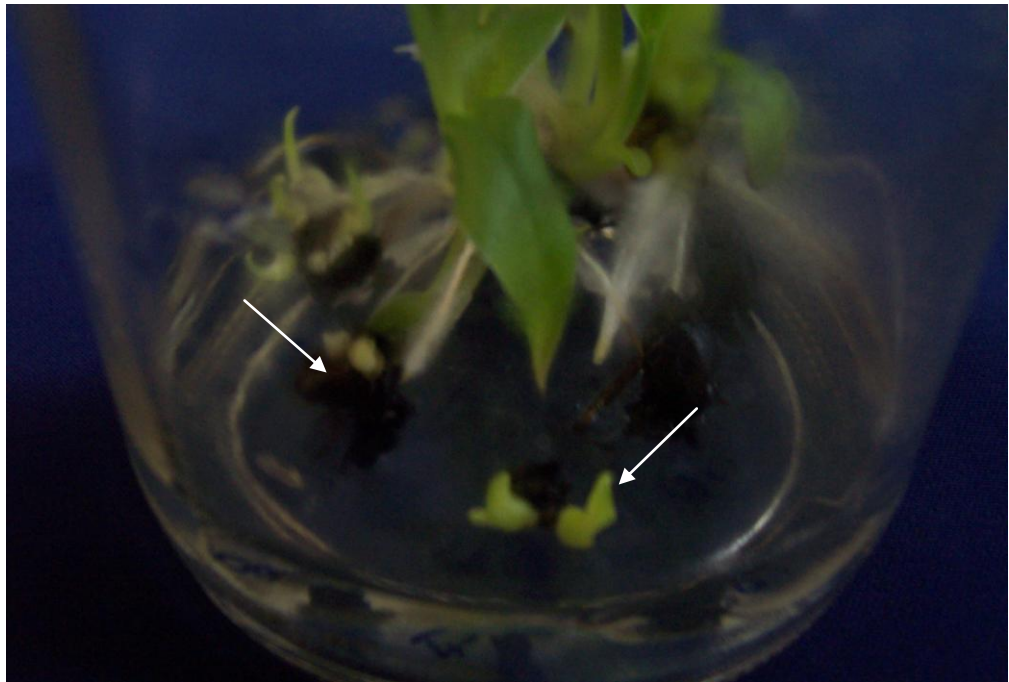**Figure 4.10:** Normal meristem at 50 days. Scale bar represents 1 cm.

Figure 4.11: Retarded growth of meristems. Scale bar represents 1 cm.

Figure 4.12: Control meristem viewed at 400X magnification. Red colour

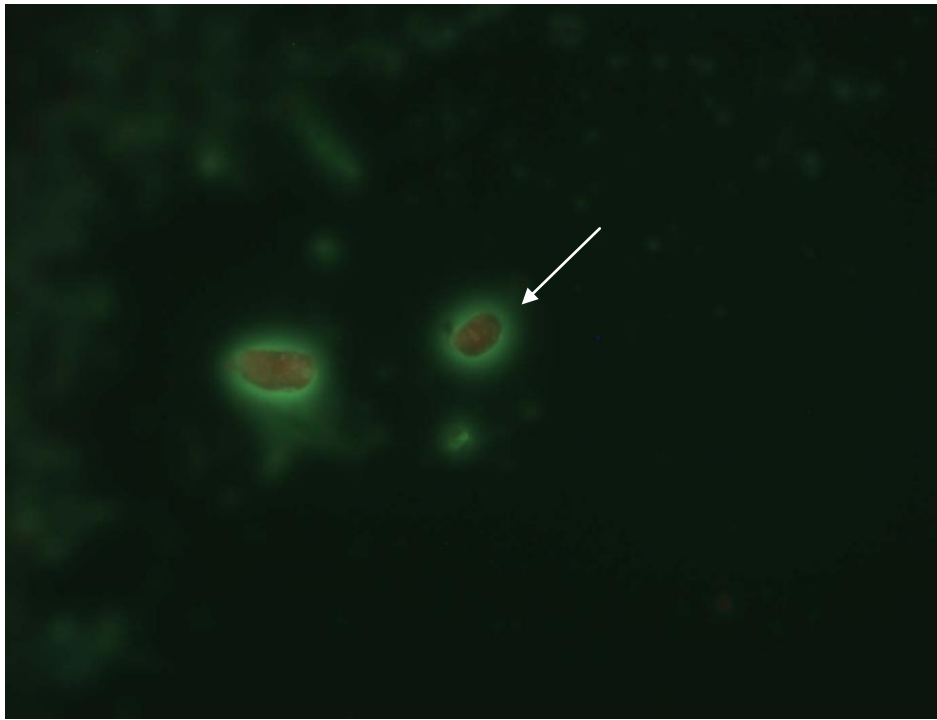indicates no expression of the recombinant protein

Figure 4.13: Transformed meristem viewed at 400X magnification. Green spots showing the presence of GFP thus confirming the presence of recombinant protein expression.

Figure 4.14: Transformed meristem viewed at 400X magnification. Green spots showing the presence of GFP thus confirming the presence of recombinant protein expression

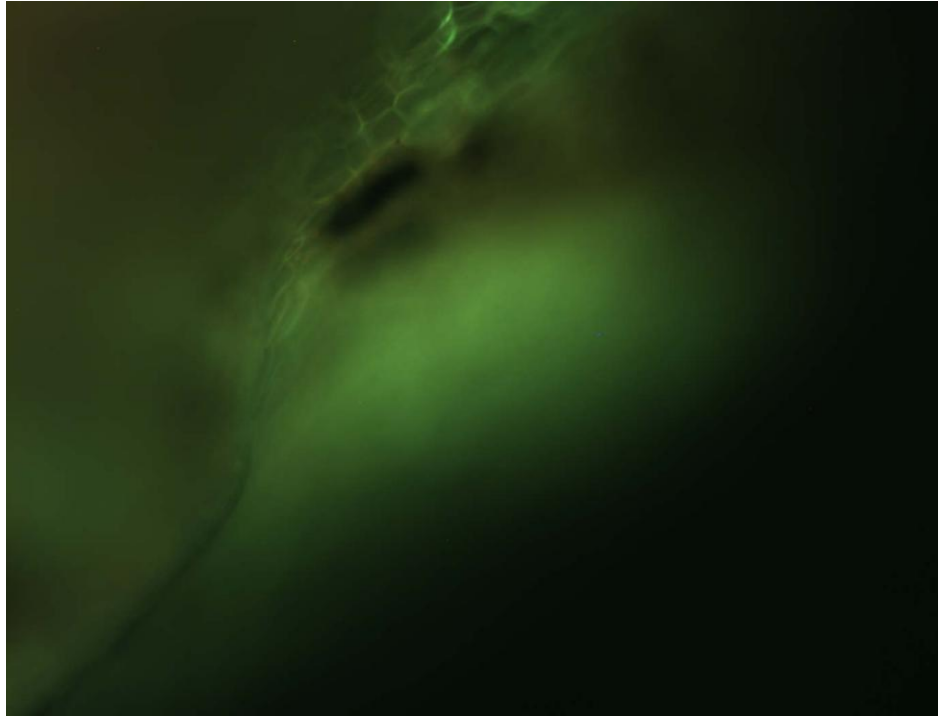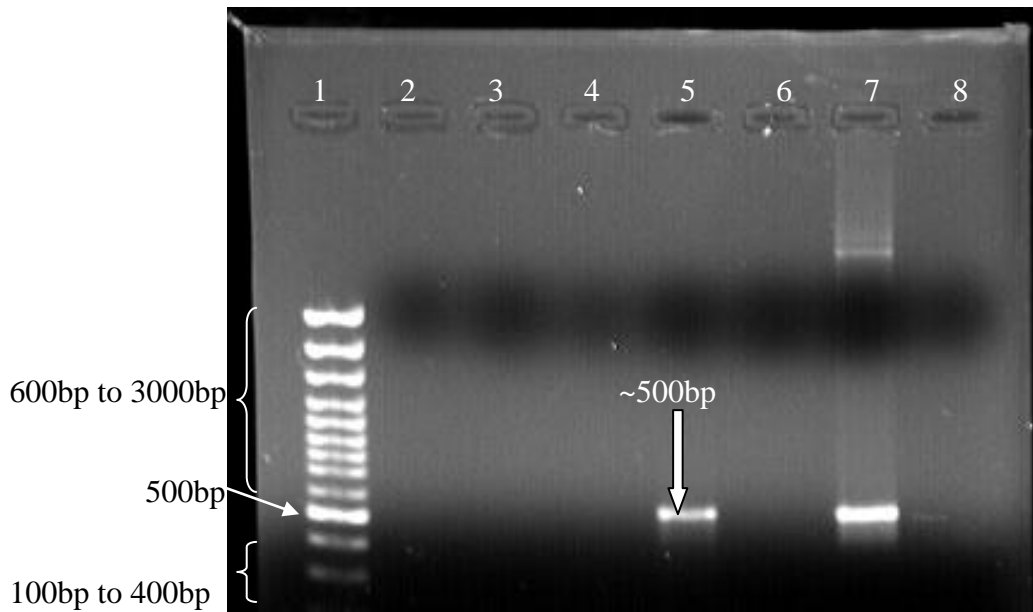**Figure 4.14:** PCR on cDNA of bombarded explants with pCEPO after 25 days

post-bombardment. Sample 19 (Lane 5) showing the presence of *EPO*.

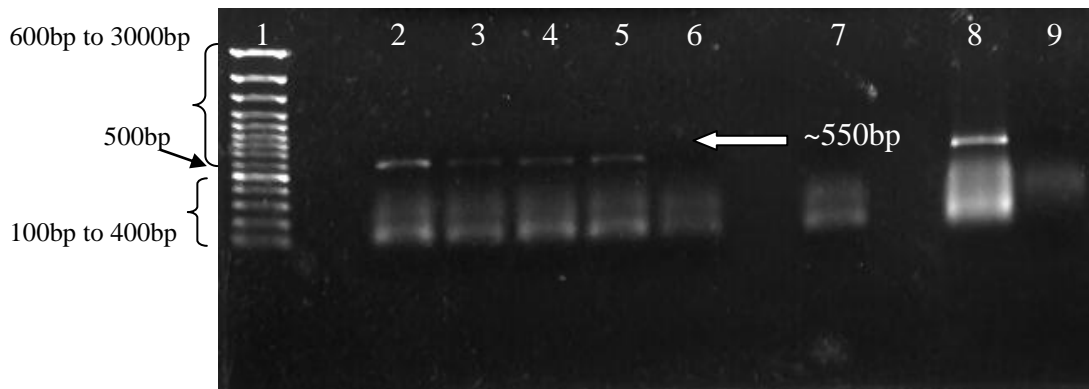| Lane 1: 100bp ladder	Lane 5: cDNA sample 19
Lane 2: cDNA sample 1	Lane 6: cDNA untransformed sample
Lane 3: cDNA sample 9	Lane 7: Positive control (pCEPO)
Lane 4: cDNA sample 10	Lane 8: Negative control

Figure 4.15: PCR on cDNA of bombarded explants with pCEPO after 50 days post-bombardment.

Samples 9 (Lane 2), 10 (Lane 3), 11 (Lane 4) and 12 (Lane 5) showing the presence of *EPO*.

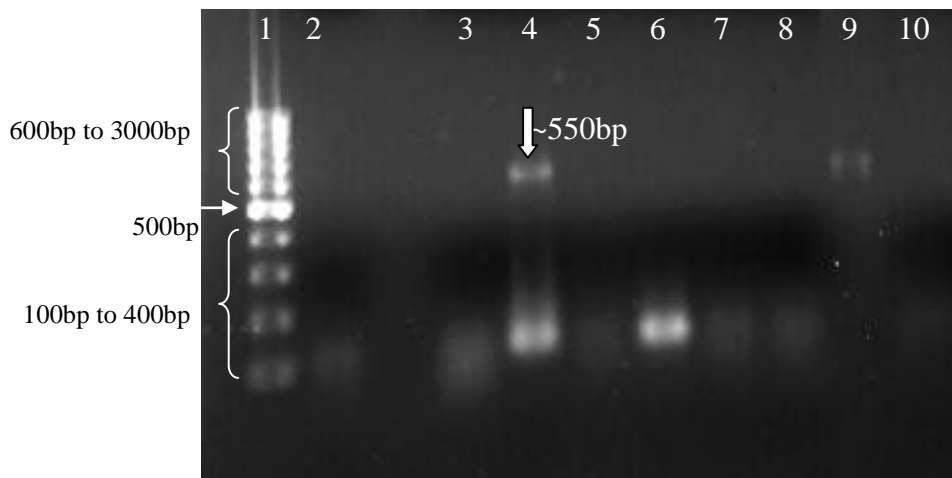| Lane 1: 100bp ladder	Lane 6: cDNA sample 19
Lane 2: cDNA sample 9	Lane 7: cDNA untransformed sample
Lane 3: cDNA sample 10	Lane 8: Positive control (pCEPO)
Lane 4: cDNA sample 11	Lane 9: Negative control
Lane 5: cDNA sample 12	

Figure 4.16: PCR on cDNA of bombarded explants with pCEPOKDEL after 50 days post-bombardment. Sample 8 (Lane 4) showing the presence of *EPO*.

Lane 1: 100bp ladder	Lane 6: cDNA sample 17
Lane 2: cDNA untransformed sample	Lane 7: cDNA sample 19
Lane 3: cDNA sample 3	Lane 8: cDNA sample 20
Lane 4: cDNA sample 8	Lane 9: Positive control
	(pCEPOKDEL)
Lane 5: cDNA sample 14	Lane 10: Negative control

CHAPTER 5: DISCUSSION

In this study, the *EPO* gene was shown to be putatively transformed and expressed in banana cells. This host system was selected as it is expected to provide an economical and efficient platform for large scale production of the protein. Recombinant proteins can also be redirected into intracellular compartments which are organelles such as the chloroplasts, endoplasmic reticulum, mitochondria, etc. The amount of recombinant protein produced can also approach industrial scale-levels and finally the health risks arising from contamination with potential human pathogens or toxins are minimised (Daniell *et al.,* 2001). Banana is an excellent host system as it can be clonally propagated, there is low risk of outcrossing and it can produce abundant fruits which is excellent for oral intake of pharmaceutical proteins (Warzecha and Mason, 2003). *EPO* is a glycoprotein involved in the regulation and maintenance of a physiological level of circulating erythrocytes (Matsumoto *et al.,* 1995). Inadequate production of erythropoietin in humans, causes anaemia. Anaemia is also found to be associated with many different diseases. The use of recombinant erythropoietin can be very beneficial to patients with anaemia. This experiment represents a first proof of concept on the potential of expressing the valuable pharmaceutical protein *EPO* in banana as an expression host. The possibility of developing constructs with high expression through targeted expression was done in this study.

5.1 Plasmids

The integrity of the gene construct pCEPO was verified by sequencing the purified DNA fragment of interest and in pCEPOKDEL both the EPO gene and the KDEL sequence was verified by sequencing the whole plasmid.

This is because, pCEPO does not carry the KDEL sequence therefore only the presence of the *EPO* gene needs to be verified and extracting the gel with the DNA fragment of interest would be an ideal method of verification. Whereas, pCEPOKDEL has the KDEL sequence cloned in a position adjacent to the *EPO* gene in the plasmid construct. Therefore, sequencing the whole plasmid is a better approach of verification.

The KDEL motif has been successfully used in reported studies to improve significantly the expression of foreign proteins in plant cells (Ri, *et al.,* 2006; Levitan *et al.,* 2005) although on its use in banana transformation has not been reported.

5.2   Plant propagation before and after transformation

In this research, the plant material used was *Musa acuminata* cv. Berangan and it was found to be an excellent choice as it could be propagated easily with the growth of multiple shoots. In this connection, it is shown that around 80% of the meristems used could be propagated. According to Juli (2001), the propagation of the explants is influenced by intrinsic and culture induced factors. These include genetic stability of the cultivars, the choice of banana explants, the composition of culture media used and the length of propagation time. All these factors were previously optimised in the laboratory.

In this study, the explants of choice for genetic transformation were the meristems. It has been previously shown that for the genetic transformation of banana, the choice of

explants is restricted to meristematic tissue and embryogenic cell suspensions. Based on the study conducted by Swennen *et al.,* (2002), it is said that the low transformation frequency of explants isolated from multiple meristem cultures proves that this tissue is not ideal for genetic transformation which is in contrast to using embryogenic cell suspensions for transformation which is said to be very efficient. However embroyogenic cell cultures are tedious to initiate thus were not used in this study.

In this study the meristems were found to be amenable to particle bombardment with a survival rate ranging from 20-60% after bombardment. However, these explants were found to be susceptible to bacterial and fungal contamination even though the plant material obtained was from a sterile culture.

Oxidation of the tissue material of the explants was the major problem encountered in the propagation of explants after bombardment. Oxidation is caused by the polyphenolic compounds released from wounded tissues of the explants and this undesirable exudates form a barrier around the tissue preventing nutrient uptake and hindering the growth of the explants (Strosse *et al.,* 2001). This caused the death of many explants during particle bombardment (Table 1) as the explants could no longer survive and propagate because particle bombardment injured the tissue of the explants causing oxidation and eventually death of the explants. As reported by Southgate *et al.,* (1995), the use of tungsten as the microprojectile in the biolistic gun may be inexpensive but it is toxic to certain cell types which may cause surface oxidation thus affecting DNA binding and degradation of adhered DNA. In this study, tungsten particle was used instead of gold which did not appear to cause any obvious toxic effect to any cells. No comparison study was carried out to prove this statement. However, this problem may be overcome by increasing large numbers of explants for transformation to maximise the chances of

obtaining the desired outcome, using gold particle instead of tungsten and changing the method transformation to *Agrobacterium*-mediated transformation.

The propagated bombarded banana plants also showed severe signs of retardation. This observation was also reported in a study conducted by Ban *et al.,* 2004 where the *EPO* gene was expressed in tobacco plants and signs of retardation were observed in the plants. Similarly in this study, the same attributes were observed as the transformed banana plants showed slower growth as compared to the plant that was not bombarded. The transformed plants also grew relatively short reaching less than half the height of the normal plant. According to Ban *et al.,* (2004), positive correlation between the severity of the phenotype and *EPO* mRNA levels was observed causing severely stunted phenotype in transgenic tobacco that expressed higher levels of *EPO* mRNA thus suggesting that the presence of the *EPO* mRNA was the cause of stunted growth of the bombarded banana plants. However, it is not unusual in many transformation experiments that the introduction of foreign genes may result in a developmental penalty. This may not be due to the inserted gene sequence per se but may just as well be due to potential effects i.e. the location of insertion of the transgene.

Explants that survive particle bombardment on the other hand, may or may not carry the gene of interest. This is due to the fact that particle bombardment segregates the T-DNA of the plasmid breaking that strand of DNA into many smaller fragments. On the other hand, high levels of transient gene expression of the protein in bombarded cells may not correlate to subsequent high levels of stable gene expression. This may be due to the fact that the foreign gene may not be incorporated into the genome of the host cell. Natural DNA repair mechanisms may ensure that the foreign DNA is not replicated in the next generation. In addition, the stably transformed cells may only express the foreign gene for a limited amount of time due to cell death resulting from the physical

effects of the bombardment procedure (Southgate *et al.,* 1995). Hence for analysis of stable integration, the transformed plantlets in this study would have to be studied till maturity and its stability in its progeny plants assessed.

5.3   Visualisation of GFP and molecular analysis of transformants

GFP was visualised as green luminescent spots indicating positive transformation event and red spots for no transformation event in the bombarded explants. Since the GFP protein was designed as a fusion construct with the gene of interest in the plasmid construct therefore, observing the presence of GFP can imply the possible successful expression of the *EPO* gene in the transformed explants. This is a simple and useful screening method in the absence of a simple assay for detecting the expression of the target gene itself. According to Jeoung *et al.,* (2002), early detection of plant transformation events is necessary for the rapid establishment and optimisation of banana transformation protocols. Reporter genes like GFP encode for messages which would clearly indicate the transient or stable expression of the transferred genes in the transgenic cells.

RNA extraction was carried out on the explants that showed growth after 25 days and 50 days of bombardment. This was to confirm the presence of *EPO* in the explants after a subsequent period of time after bombardment thus suggesting the stability hence the integrity of the gene into the plant genome. RT-PCR of the bombarded explants to confirm the presence of *EPO* indicated a success of rate of 17-80% showing that the presence of the *EPO* gene is stable in the banana plant for at least the period of time studied. This proves that the *EPO* gene could be successfully expressed in banana cells. However, this result serves as only a preliminary result as further studies need to be

carried out to establish the functionality of the *EPO* protein and the integration of the *EPO* gene in the banana genome.

In this study, the presence of the *EPO* gene cannot be confirmed in all the cells of the meristem as particle bombardment would disperse the recombinant DNA (rDNA) in a random fashion and the chances for all the cells in the meristem to stably carry the *EPO* gene may be difficult. Thus the positive transformation events which have been obtained in this study are not a conclusion that the *EPO* gene is present in all the cells of the meristem i.e. there remains the possibility that the putative transformed plant is chimeric. According to May *et al.,* (1995), transformation using meristematic tissue may be appealing because of its short regeneration time but may be of limited value because of the potential generation of chimeric plants. In this connection, the ability of transformed cells containing the foreign gene to regenerate into complete plantlets would be advantages.

The targeting of the *EPO* gene into the endoplamic reticulum via the KDEL sequence showed lower success in transformation as compared to the expression of the EPO gene without the KDEL sequence. It could be speculated that the higher expression of *EPO* using this construct was detrimental to the transformed cells. However the expression level of the *EPO* gene was also not assessed to show the difference between bombardment with pCEPOKDEL and pCEPO was not possible. This study only showed the presence of the *EPO* mRNA via RT-PCR of transformants. This observation supports the observation by Ban *et al.,* (2004) in tobacco cells which suggest a correlation between *EPO* expression levels and increased mortality of the cells.

Studies were also not conducted on the functionality of the protein produced in the transformants. Further analysis need to be carried out for the confirmation of the ability

of the transformed banana plant to produce functional *EPO*. The sequencing results of the mRNA obtained from the transformant showed around 80% similarities with the human *EPO* mRNA and may indicate that mutations could have occurred during or after transformation which may have an impact on the protein formed.

This study, have shown the insertion and expression of the *EPO* gene transformed explants of banana. However, further studies need to be taken to increase efficiency of transformation for subsequent analysis.

CHAPTER 6: CONCLUSION

1. In this study the *EPO* gene was putatively transformed into *Musa acuminata* cv. Berangan using pCEPO and pCEPOKDEL. The success of this transformation event was verified by visualising the GFP protein and by carrying out RT-PCR on the cDNA obtained from the bombarded explants.

2. The expression level of the *EPO* gene was not verified in this study therefore, further analysis need to be carried out to determine the expression level of the recombinant protein using the two different constructs. The functionality of the *EPO* protein was also not studied thus indicating the need for further research in this area.

3. The choice tissue material for particle bombardment in this study was not ideal as the use of meristemic tissue for bombardment would more than likely result in chimeric plants. For stable genetic transformation the use of cell suspension of somatic embryos would be a better choice for higher frequency and stable transformation of banana cells.

4. However as a proof of concept, the study was successful in suggesting the potential of banana as a biofactory for expressing valuable pharmaceutical proteins.