CHAPTER-4

Generation of DGAT2 Transformants of *J.curcas*

4.0 Introduction

Genetic transformation for improving traits is another valuable method for the development of *J.curcas* variety. Recent advances in genetic transformation and the availability of gene databank has made it possible to transfer the gene of interest to the recipient species to produce transgenic progeny with desired characteristics. This may help surpass certain existing limitations of the breeding programmes and also may help reduce the time required to generate those improved varieties.

J.curcas is still in its infancy with respect to genetic improvement. The application of metabolic engineering and RNAi technology may show their potential in basic research and as tools of modern plant breeding. Research and development should aim at metabolic engineering of the oil bio-synthesis pathway. There is a possibility to increase the yield of oil from *Jatropha* seeds through genetic transformation (Mukherjee et al, 2011).

Jatropha seeds contain 22-44% oil in its seeds. Main constituent of oil is TAG (Triacylglycerol). DGAT (Diacyl glycerol acyltransferase) is the enzyme that catalyzes the committed step of TAG synthesis from DAG via the Kennedy pathway. A number of studies with both mammalian (Mayorek et al, 1989; Tijburg et al, 1989) and plant (Ichihara et al, 1988; Perry and Harwood, 1993a, b; Settlage et al, 1995; Perry et al, 1999) systems have suggested that DGAT may catalyze a rate limiting reaction in TAG bio-assembly. Hence increasing the amount of this enzyme may increase TAG biosynthesis thus leading to increase in oil content per seed. Similar work has been done in *Arabidopsis* where seed specific over-expression of an *Arabidopsis* cDNA encoding DGAT resulted in an enhancement of seed oil content and seed weight (Jako et al, 2001). He et al, 2004 have reported the cloning and characterization of a cDNA encoding DGAT from castor bean. Increase in levels of DGAT enzyme can be brought about by increased

expression of the DGAT gene. This can be done by cloning cDNA copy of DGAT gene or using DGAT gene whose sequence is highly similar to that of *Jatropha curcas* DGAT and cloning this gene into a binary vector and its transformation into *Jatropha* followed by its expression under a seed specific strong promoter.

Of the available transformation methods, *Agrobacterium* mediated transformation is the most frequently used method for the development of transgenic plants. In the current study an attempt to obtain genetically transformed *Jatropha curcas* is made. DGAT gene from *Vernicia fordii* is used for the purpose of genetic transformation. *V.fordii* belongs to same family (Euphorbiaceae) as *Jatropha curcas* and shows 84% identity as found using blast search. DGAT2 construct used in this study was kindly provided by Prof. Jay Shockey from Southern Regional Research Centre, USDA-ARS, New Orleans, USA.

4.1 Literature Studies

Diminishing petroleum reserves and environmental awareness has led to an increased demand for oil plants and their derivatives as substitutes for petrochemicals in industrial applications such as biofuels, biolubricants, nylon precursors etc. However, oil plants have certain limitations such as low yields, toxicity, limited geographical cultivation area and susceptibility to insect pests. Fortunately, genetic engineering offers a novel opportunity to overcome these limitations. Recent studies have envisaged the potential of TAG synthesis and accumulation in oil seed crops as the main lipids store in plants are TAG's. Three key steps are involved in the production of storage oils. First is the fatty acid synthesis in plastids, second is fatty acid modification by enzymes located in endoplasmic reticulum (ER) and thirdly packaging of the fatty acids in TAG's which subsequently accumulate in oil bodies that bud off from the ER. Formation of TAG can be achieved in several ways as shown in figure 4.1

Generation of DGAT2 Transformation of *J.curcas*

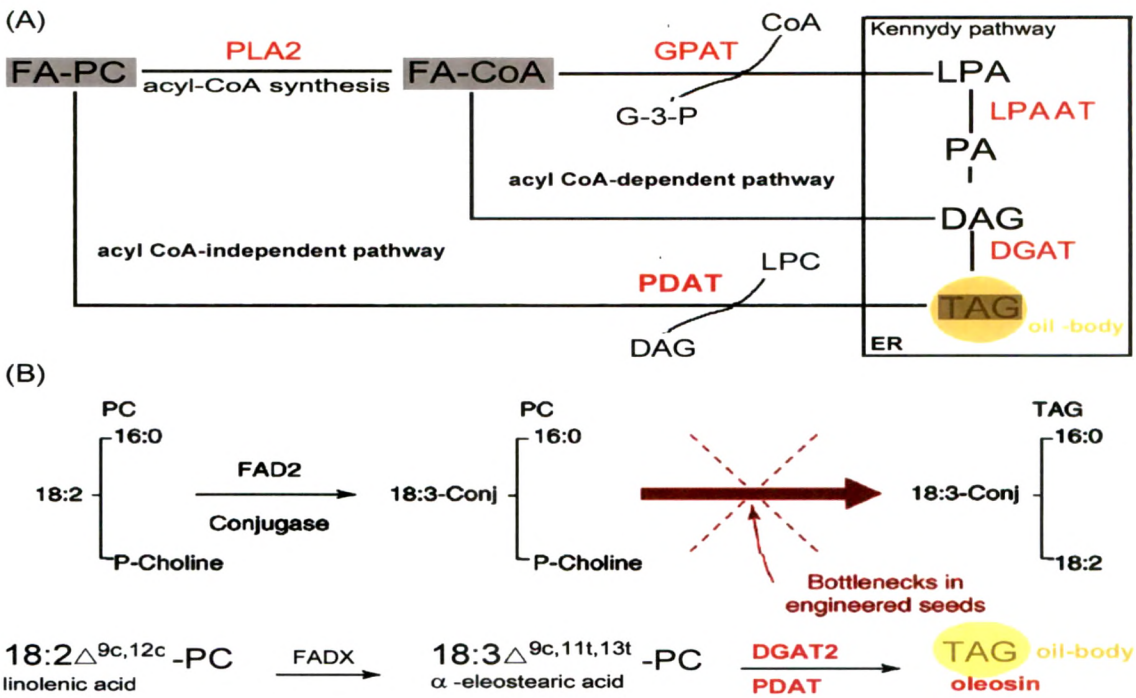


Figure 4.1 (A) The general pathway of TAG synthesis and accumulation. The first is classical Kennedy pathway catalyzed by enzyme GPAT, LPAAT and DGAT. The second is acyl-CoA independent pathway catalyzed by enzyme PDAT. The fatty acids flux between FA-PC, FA-CoA and TAG determine the final TAG level. (B) The bottlenecks in engineering plants to accumulate TAG enriched novel fatty acids are the low production of TAG but high FA-PC (Chen et al, 2008).

The first is the classical Kennedy pathway where acyl-CoA acts as a donor and diacylglycerol (DAG) as an acceptor. This reaction is catalysed by acyl-CoA diacylglycerol acyltransferase (DGAT) and transfers an acyl group from acyl-coA to sn-3 of DAG and forms TAG. The second is acyl-CoA independent pathway, which uses phosphatidylcholine (PC) as acyl donor and DAG

as acceptor, catalyzed by an enzyme called phospholipids: diacylglycerol acyltransferase (PDAT), which can transfer the sn-2 acyl chain from PC (phosphotidylcholine) to DAG, forming lyso-PC and TAG. Also the acyl-CoA independent pathway can be catalyzed by DGAT, using two molecules of DAG to produce TAG and monoacylglycerol (MAG) (Chen et al, 2008). Many studies have been reported on various enzymes involved in TAG synthesis like phospholipase A2 (PLA2), G-3-P acyltransferase (GPAT), phospholopid:diacylglycerol acyltransfersae (PDAT), lyso phosphatidic acid acyltransferase (LPAAT) and diacylglycerol acyltransferase (DGAT). Beisson et al, 2007 have identified atleast eight GPAT genes in *Arabidopsis thaliana*; however none of them have been implicated to have a promising role in TAG biosynthesis. PDAT has been studied by Dahlqvist et al, 2000. It catalyses the acyl transfer from PC to sn-1, 2-DAG to yield TAG however, it is not found to have any major role in TAG synthesis.

A correlation between DGAT transcript level and oil accumulation has been reported in common oilseeds as well as in plant species that accumulate unusual fatty acids. The level of DGAT expression has been shown to be directly related to TAG accumulation in mammals. Role of DGAT in pheromone synthesis in insects is also reported. With the extensive progress in genomics, transgenics and protein purification, genes encoding two major types of DGAT were characterized in eukaryotes and designated *DGAT1* and DGAT2 (Liu et al, 2012). Shockey et al, 2006 studied *Vernicia fordii* DGAT enzymes and reported that DGAT enzyme activity is encoded by at least two classes of genes in eukaryotic cells. DGAT1 proteins are; 500 amino acids in length with 10 predicted transmembrane domains (TMDs), whereas DGAT2 proteins are; 320 amino acids long with two predicted TMDs (figure 4.2). DGAT1 plays a more constitutive role in TAG metabolism in various tung tissues, whereas DGAT2 functions more directly in the production of seed-specific TAGs. Sub-cellular localization studies indicated that both the enzymes are localized to specific sub-cellular regions of the endoplasmic reticulum and these regions are not shared by these two enzymes.

Generation of DGAT2 Transformation of *J.curcas*

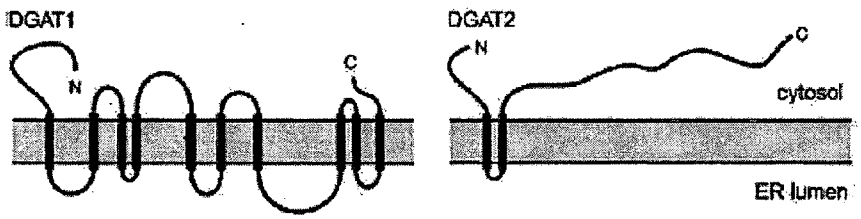


Figure 4.2: Predicted topological maps of DGAT1 and DGAT2 of *Vernicia fordii* by Shockey et al, 2002 Jain et al, 2000 have reported an increase in oil content of *Arabidopsis* seeds from 8 to 29% in selected transgenic lines by expressing a plant plastidal and a bacterial GPAT gene directly and after modification.

Genetic engineering of any crop species through genetic transformation requires a rapid and reproducible regeneration protocol. The potential of *Jatropha curcas* is well known and it is also known that it requires genetic engineering to gain commercial success and hence a few scientists have reported genetic transformation in this crop. First report on genetic transformation of *J.curcas* was given by Li et al, 2007 using *Agrobacterium tumefaciens* mediated transformation of cotyledon explants. Kumar et al, 2010 reported genetic transformation of *Jatropha* using in vitro leaf explants via *Agrobacterium tumefaciens* infection. In this study an attempt has been made at transformation of *J.curcas* via *Agrobacterium tumefaciens* carrying the gene of interest (DGAT2) via somatic embryogenesis and leaf mediated regeneration.

Generation of DGAT2 Transformation of *J.curcas*

4.2 Materials and Method

4.2.1Cultures and Maintenance:

1) *E. coli* DH5α was used for transformation of ligation mixtures and maintaining recombinant plasmids.

2) *E. coli* DH5α bearing helper plasmid pRK2013 was used as a helper strain for mobilizing the donor plasmid contained in another E. coli DH5α strain

3) *Agrobacterium tumefaciens* LBA4404 was used as a recipient in the triparental mating.

Culture Maintenance:

• *E. coli* DH5α strains were maintained on Luria agar plates.

• *E. coli* DH5α bearing helper plasmid pRK2013 was maintained on Luria agar plates containing 50 μg/ml kanamycin as the plasmid pRK2013 has a kanamycin resistance gene.

• *Agrobacterium tumefaciens* LBA4404 was maintained on AB Medium containing 5 μg/ml rifampicin & 10 μg/ml tetracycline as the strain LBA 4404 is resistant to rifampicin & tetracycline antibiotics.

• *E.coli* DH5α clones harbouring pE172 recombinant plasmid were maintained on LA plates with 50 μg/ml kanamycin. pE172 was used for transformation of somatic embryos as its under influence of seed specific promoter. pE309 was used for transformation of leaf discs as its under constitutive promoter. pE172 and pE309 were kind gifts from Prof. Jay Shockey, Southern Regional Research Centre, USDA-ARS, New Orleans, USA.

4.2.2 Media and Antibiotics:

Media:

Luria Agar (LA):

Caesin enzymic hydrolysate 10g/l

Yeast extract 5g/l

Sodium Chloride 5g/l

Agar 15g/l

Luria Broth (LB):

Caesin enzymic hydrolysate 10g/l

Yeast extract 5g/l

Sodium Chloride 5g/l

4.2.3 Antibiotic Stocks:

Kanamycin – 100 mg/ml

Tetracycline- 50 mg/ml (Dissolved in absolute ethanol)

Rifampicin - 50 mg/ml (Dissolved in 100% methanol)

Final Concentration of Antibiotics in Media:

Kanamycin - 50 µg /ml

Tetracycline- 12-15 µg /ml

Rifampicin - 5 µg /ml

4.2.4 Plasmid Extraction by Alkaline Lysis Method:

Plasmid extraction was done by Alkaline Lysis method of Sambrook et al, (1989) (Miniprep).

Reagents & Composition:

- ALS-I Tris-Cl 25mM (pH 8.0), EDTA 10mM (pH 8.0), glucose 50mM
- ALS-II 0.2N NaOH, 1% (w/v) SDS

• ALS-III 3M Potassium acetate, 5M glacial acetic acid

• Isopropylalcohol and 70% alcohol

properly allign the text

Procedure:

1) A single colony of transformed bacteria was inoculated in 5 ml of LB with proper antibiotic. The culture was incubated overnight at 37°C under vigorous shaking condition on shaker.

2) Next day the culture was centrifuged at 10,000 rpm for 10 min.

3) The supernatant was discarded & 100 µl of ice cold ALS-I was added to resuspend the pellet by vortexing.

4) 150 µl ALS-II was added & the contents of the tube were mixed by inverting 5 times, the tube was stored on ice for 10min.

5) 200 µl ALS-III was added & mixed by inverting the tubes 5 times, tube was stored on ice for 15-20 min

6) Centrifugation done at 12,000 rpm for 10 min, the supernatant was transferred to a fresh microfuge tube and equal volume of Isopropylalcohol was added. The tube was incubated at -20°C overnight.

7) Centrifugation was done at 12,000 rpm for 10min. The supernatant was carefully discarded & 200 µl 70% alcohol was added & inverted gently 2 times & centrifuged for 2 min, then the alcohol was removed carefully.

8) The microfuge tube was kept in an inverted position to completely remove last traces of alcohol. 20 µl Triple distilled water was added and kept at room temperature for 15 min for DNA to dissolve. The purity of the plasmid preparation was checked by agarose gel electrophoresis.

4.2.5 Competent Cell Preparation:

The protocol from Sambrook et al, (1989) was used for preparation of competent *E. coli* with an efficiency of ~106 transformed colonies/ µg of supercoiled plasmid DNA.

Reagents & Composition:

$MgCl_2$-$CaCl_2$ solution: 80 mM $MgCl_2$, 20 mM $CaCl_2$ ($CaCl_2$: 0.1M)

Procedure:

1) A single bacterial colony was inoculated in 5 ml of LB. The culture was incubated overnight at 37°C with vigorous shaking.

2) 1 ml of overnight grown culture was transferred in 100 ml LB and incubated at 37°C with vigorous shaking till the OD_{600} reaches 0.4. (To ensure the culture doesn't grow to a higher density measure the OD_{600} of culture every 15-20min)

3) The bacterial cells were transferred to sterile, disposable, ice-cold 50 ml polypropylene tubes. The cultures were cooled to 0°C for 10 min.

4) Cells were recovered by centrifuging at 4500 rpm for 10 min at 4°C.

5) The medium was decanted from cell pellet & tubes were placed in an inverted position for 1 min to allow last traces of the media to drain away.

6) Pellet was resuspended by swirling or gentle vortexing in 30 ml of ice-cold $MgCl_2$-$CaCl_2$ solution.

7) Cells were recovered by centrifuging at 4500 rpm for 10min at 4°C.

8) Medium was decanted from cell pellet; tubes were placed in an inverted position for 1 min to allow last traces of the media to drain away. Pellet from each tube was resuspended by swirling or gentle vortexing in 1ml of 0.1 M $CaCl_2$ and 1ml of 40% glycerol. The cells were directly used for transformation or dispensed into aliquots & frozen at -80°C.

Transformation using $CaCl_2$:

1) 100-200 µl $CaCl_2$ treated cells were transferred to a sterile chilled polypropylene tube. Upto 5 µL of DNA sample or ligation system was added to the tube and the contents mixed by swirling gently. The tube was stored on ice for 30 min.

2) The tube was transferred to a rack and kept in preheated 42°C water bath and incubated exactly for 90 sec without shaking.

3) The tube was rapidly transferred to an ice bath to chill for 2 min.

4) 400-800 µl of LB medium was added accordingly to the tube and incubated for 45 min at 37°C.

5) Appropriate volume of cells was plated onto pre-warmed LB plates with appropriate antibiotic.

6) The plates were incubated at 37 °C for 16-18 hours.

4.2.6 Agarose gel electrophoresis

Requirements:

TAE (Running buffer)

- Tris base 242g
- Glacial acetic acid 57.10ml
- 0.5M EDTA (pH 8.0) 100ml
- D/W 1000ml

50X TAE was diluted to 1X prior to use.

0.8% Agarose

4.2.7 Triparental Mating

Strains involved:

1) *Agrobacterium tumefaciens* LBA4404 (Recipient strain) - It was grown at 30°C on AB minimal medium with rifampicin & tetracycline.

2) *E. coli* DH5α harboring recombinant plasmid to be mobilized (Donor strain) - It was grown at 37°C on LB with 50µg/ml Kanamycin.

3) *E. coli* DH5α bearing helper plasmid pRK2013 (Conjugal helper strain). This plasmid when introduced into an E. coli strain harboring plasmid (Donor), mobilizes that plasmid into *Agrobacterium tumefaciens*. E. coli DH5α harboring pE172 was grown at 37°C on LB medium with 50μg/ml Kanamycin. This plasmid cannot multiply in *Agrobacterium tumefaciens* due to the lack of wide host range replicon.

Procedure

1) Four days before performing the triparental mating, *A. tumefaciens* was streaked to obtain a single colony on Luria agar plates which contained rifampicin & tetracycline and was incubated at 30 °C.

2) One day before, *E. coli* DH5α harboring pRK2013 & *E. coli* DH5α harboring the plasmid to be mobilized were streaked to obtain a single colony on LB agar with 50μg/ml of Kanamycin.

3) On the day of the triparental mating, a plate of Luria agar without any antibiotic was prepared.

4) One colony each from *E. coli* DH5α bearing pRK2013, E. coli DH5α harboring the plasmid which has to be mobilized and *A. tumefaciens* was patched separately on Luria agar plate very close to each other. With a sterile loop, all the three bacterial strains were mixed very well and the plate was left at 30 °C for 12-18 hrs.

5) On the second day from the procedure, six culture tubes which contained 0.9ml LB were autoclaved and kept ready. Six plates containing Luria agar plate with relevant antibiotics were poured and kept ready (In the medium there was an antibiotic selection for both the donor and the recipient so that the donor and the recipient that had mobilized into the *A. tumefaciens* can be maintained).

6) After mating, the bacteria on the Luria agar plate were scrapped and suspended in 1ml LB. A serial dilution was performed by transferring 0.1ml of bacterial suspension into 0.9ml of LB. Likewise 4-5 dilutions were made up to 10-4/ 10-5.

7) 100 µl of each dilution was added to Luria agar medium with appropriate antibiotics and was spread uniformly. The plates were incubated for 4-5days.

4.2.8 Ketolactose Test (Bernaerts and Ley, 1963)

Reagents:

Benedict's reagent [100 ml]: 17.3g Sodium citrate, 10.0g Na2CO3, 1.73g CuSO4.5H2O

Procedure:

1) *Agrobacterium tumefaciens* colony was patched on Luria agar plate and was allowed to grow for 12-16 hours.

2) On the next day, the plate was flooded with Benedict's reagent.

3) Within five minutes, due to the formation of ketolactose, a yellow colored zone was observed which confirmed the presence of *A. tumefaciens*.

4.2.9 Vector Map:

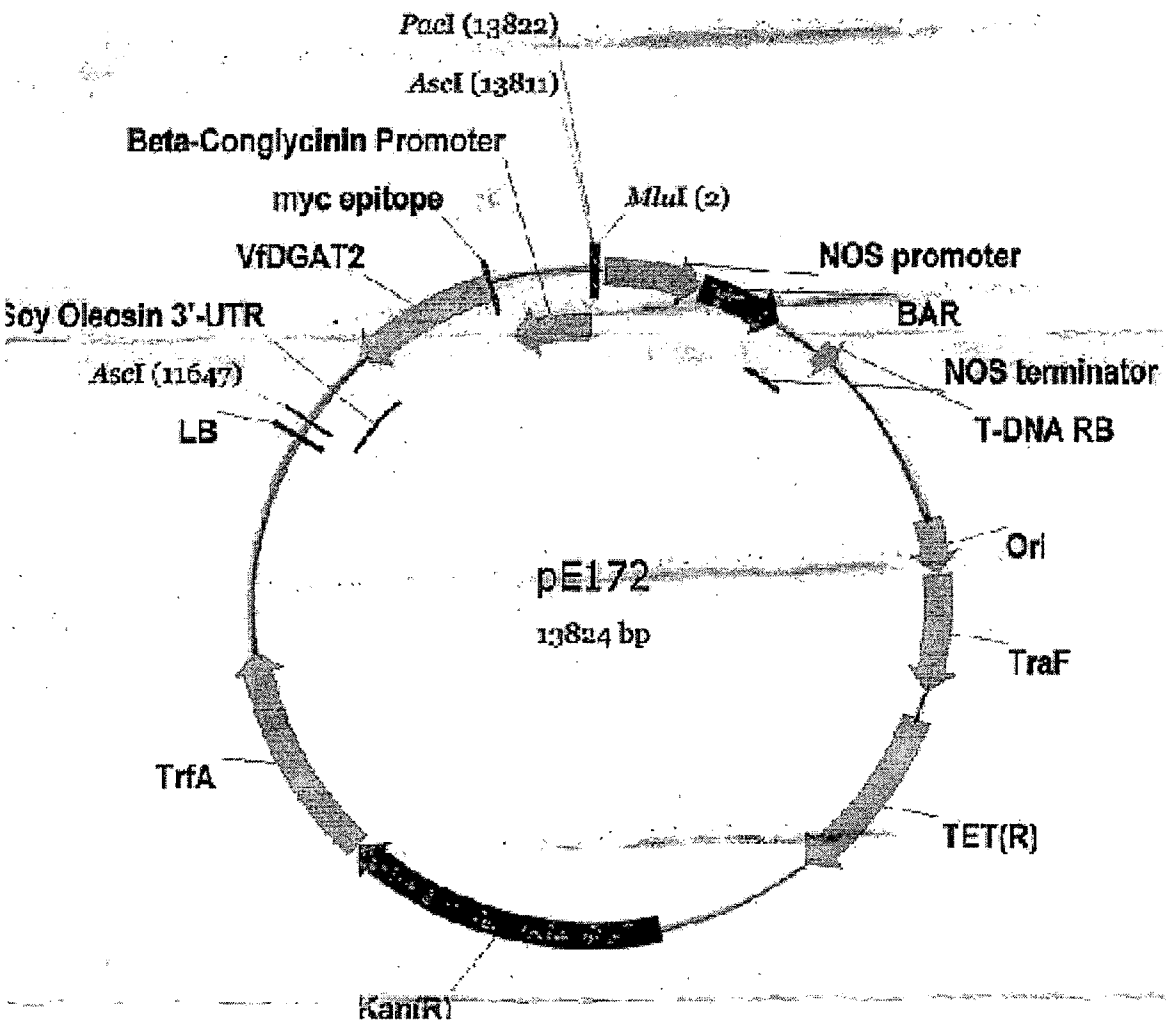


Figure 4.3: Map of pE172

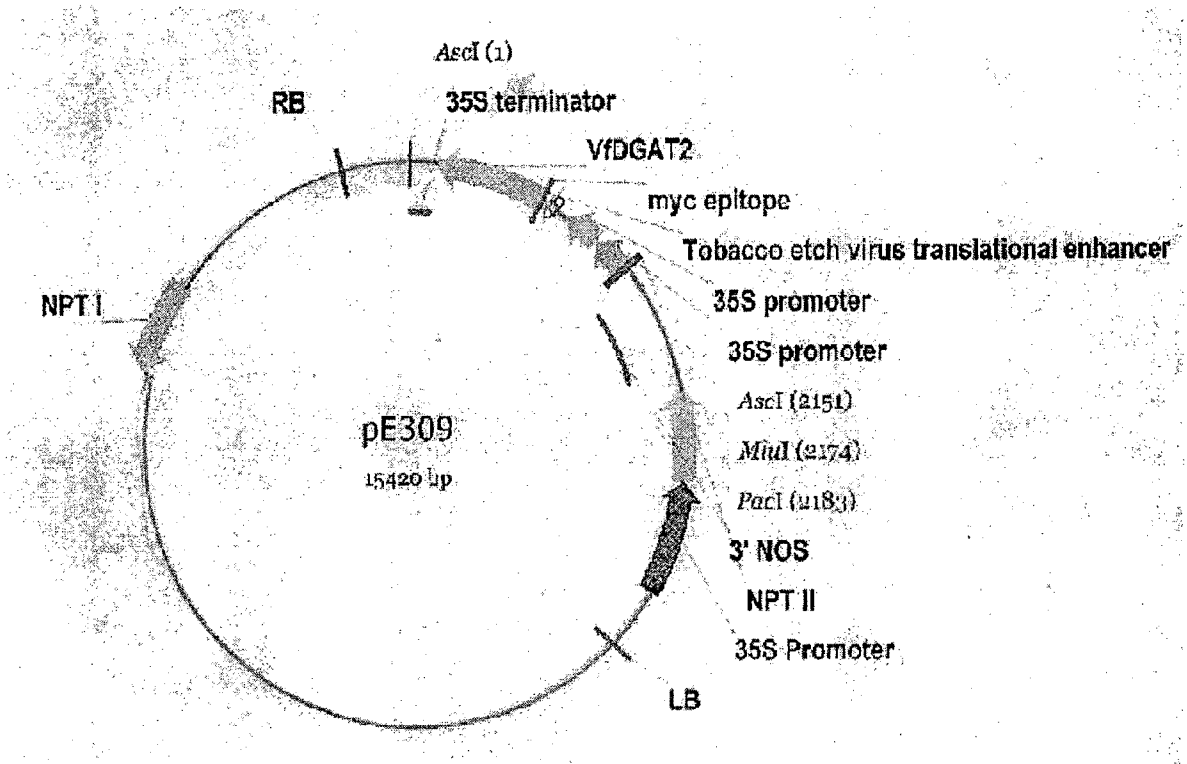


Figure 4.4: Map of pE309

4.3 Co-cultivation

4.3.1 Preparation of *Agrobacterium* suspension for co-cultivation

A single colony of *Agrobacterium* strain containing the gene of interest was incubated in 25 ml of YEP medium and grown overnight on a shaker at 200 rpm at 28°C to an OD at 600 of 1.4–1.6. The bacterial culture was centrifuged at 5,000 rpm and the pellet was resuspended in 25 ml of liquid suspension medium containing MS salts (Murashige and Skoog, 1962), 100 mg/l myo-inositol, 30 g/l glucose and 100 ml acetosyringone. The suspension was kept at 28°C for 5 hours

and used for explant infection. Different explants as explained above were infected with *A. tumefaciens* suspension culture for 20 min at room temperature under continuous shaking, blot dried on sterile filter paper and finally transferred on to regeneration medium. Regeneration medium used was with and without supplementation of acetosyringone. Explants were co-cultivated for different duration on this medium. Explants submerged in bacteria-free MS served as control.

4.4 Explants and Media Composition:

4.4.1 *Agrobacterium* mediated transformation of somatic embryos of *J.curcas*

Seeds from mature fruits of *Jatropha curcas* plants (1.5-2 years old) were collected and their cotyledons used as explants. Seeds were kept under running tap water for 30 minutes followed by wash with a mild detergent. They were again kept under running tap water for 30 minutes. They were treated with 70% ethanol for 30 seconds followed by three rinses with distilled water. Surface sterilization was done with 0.1% mercuric chloride for 2 minutes. Traces of mercuric chloride were removed by giving five rinses with sterile distilled water. Surface sterilized seeds were de-coated under aseptic conditions and inoculated on MS medium supplemented with 4.0 mg/l 2,4-D. The medium was fortified with 3% sucrose and agar (0.8%) was used as a solidifying agent. The pH of the media was adjusted to 5.8 before addition of agar and was autoclaved at 121°C for 20 min. Cultures were maintained at 24±2°C for 14 hour photoperiod.

4.4.2 *Agrobacterium* mediated transformation of leaf discs of *J.curcas*

Young leaves at 3rd and 4th node from the apex were collected from 1.5-2 year old plants grown on campus. The explants were thoroughly washed with tap water for nearly half an hour followed by washing with soap solution. The explants were surface sterilized with 0.1% mercuric chloride for 2 to 3 minutes followed by five rinses with sterile distilled water. Leaves

were then excised into small pieces of 1x1cm and inoculated on Murashige & Skoog (MS) basal medium. A week after inoculation on MS basal medium, the explants were cultured on (4.5 µM-27.0 µM) BAP and (3.5 µM-7.5 µM) IBA.

4.5 Determinaiton of phytotoxic level of selective and bactericidal antibiotics:

Leaf explants and somatic embryos were inoculated on MS medium containing different levels of kanamycin (30, 40, 60, 80 and 100mg/L) respectively for selection of transformants. In a separate study different levels of cefotaxime (50mg/L, 100mg/L,150mg/L and 200mg/L) were tested for controlling *Agrobacterium* infection after transformation.

4.6 Evaluation of factors influencing transformation:

A range of parameters were evaluated and each experiment included three replicates of ten explants each. These parameters included length of pre-culture period (1, 4, 5, 6, 7 days) of explants in regeneration medium prior to *Agrobacterium* infection, effect of acetosyringone, effect of wounding and length of co-cultivation medium (3, 4,5, 6 days).

4.7 Results and Discussion Gene name is always written in italics

Binary vector pE172 contains DGAT2 gene under a seed specific promoter and binary vector pE309 contains DGAT2 gene under a constitutive promoter were used in this study. In present investigation an attempt has been made to study the over-expression of DGAT2 in oil seed crop *J.curcas*. For this two different regeneration systems have been taken into consideration. Over-expression studies of DGAT2 under beta congylcinin promoter (seed specific) were done with somatic embryos (detailed investigation reported in chapter three of this thesis). Over-expression studies of DGAT2 under a constitutive promoter (35 S) were attempted with leaf discs as explants (chapter two covers regeneration studies from leaf discs).

4.7.1 Tranformation of *Agrobacterium* with DGAT2 by Triparental Mating:

Triparental mating was carried out using *E coli* DH5α with pE172 (Kan^R) as the donor strain, *E coli* DH5α with pRK2013 (Kan^R) as the helper strain and *Agrobacterium-LBA4404* (Rif^R, Tet^R) as the recipient. Similar experiments were carried out with pE309 (Kan^R) as the donor strain. Figure 4.3 shows the growth of transformants as observed on Luria Agar plates with Kanamycin, Rifampicin and Tetracycline.

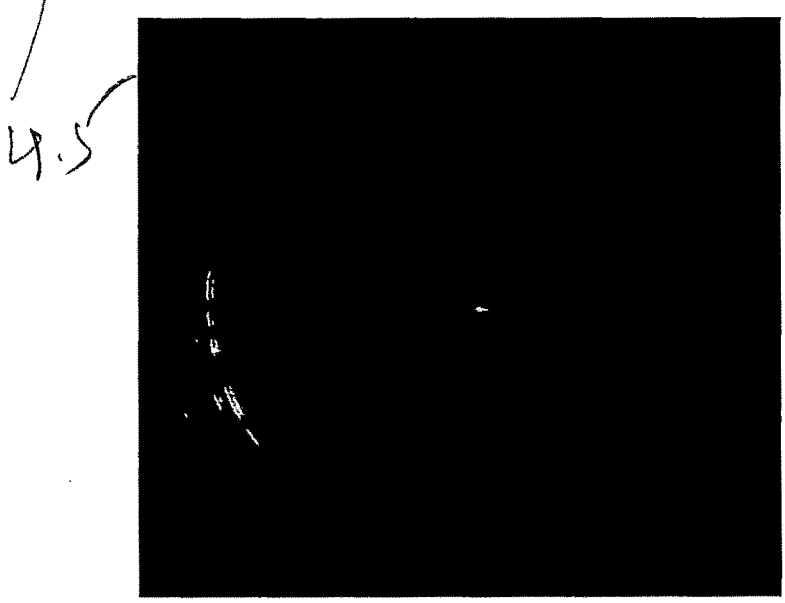
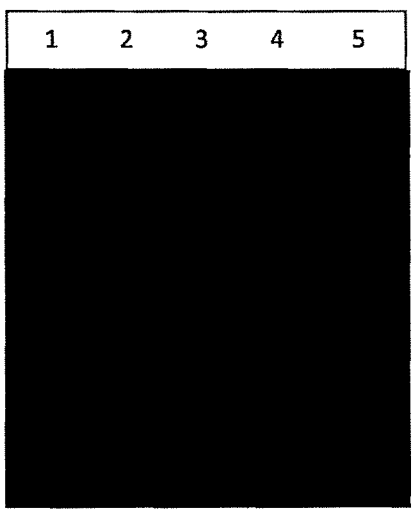


Figure 4.5: Growth of transformants was observed after plating on Luria Agar plates containing tetracyclin(10 µg/ml),rifampicin(5 µg/ml) and kanamycin(50 µg/ml).

4.7.2 Confirmation of *Agrobacterium* transformants using restriction digestion

Agrobacterium containing pE172 was digested with single cutter Bam HI (figure 4.4) and *Agrobacterium* containing pE309 was digested with single cutter Eco RI (figure 4.5).



Lane1: Lamda *Hin*dIII Marker

Lane 2: Digested DGAT2 in Donor (pE172)

Lane 3: Undigested DGAT2

Lane 4: Lamda *Hin*dIII Marker

Lane 5: Digested DGAT2 in *Agrobacterium* transformants.

Figure 4.6 A: Conformation of transformation (pE172)

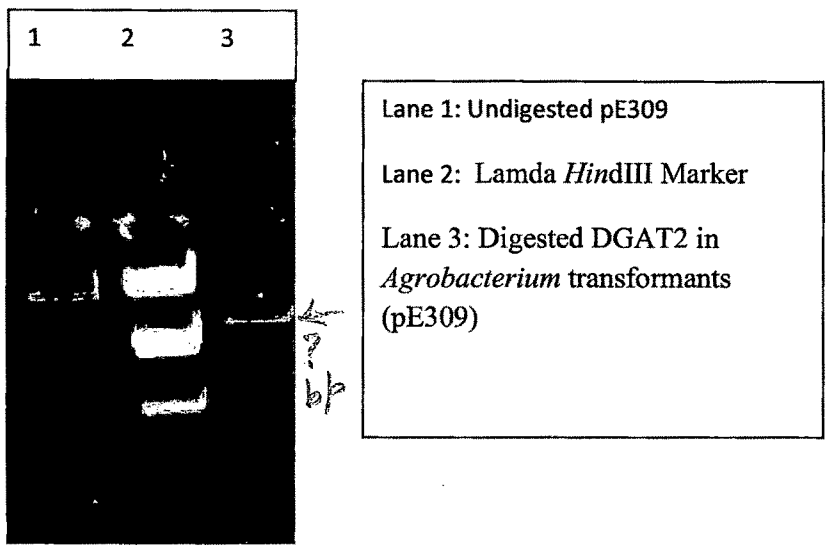


Figure 4.6 B: Conformation of transformation (pE309)

In species where low transformation efficiencies are expected, the study of the effect of several factors by comparing the percentage of recovered transformed plants may prove unsuccessful because limited numbers of transformants are produced. Therefore, it becomes important to evaluate the influence of diverse factors on the efficiency of T-DNA transfer. However, these studies can be used as a guide, and only major differences between tested parameters should be considered (Suma et al, 2008). Some others have also reported that these parameters are known to influence the transformation efficiency and the optimized conditions are host species dependent (Xing et al, 2007).

4.7.3 *Agrobacterium* infection of leaf explants

Axenic leaf discs and somatic embryos were immersed in Murashige and Skoog broth seeded with transformed *Agrobacterium.* Fifteen and twenty minutes of immersion time of explants in

Agrobacterium culture was studied and it was found that twenty minutes is more effective period for immersion as compared to fifteen minutes. Figure 4.7 shows transformed leaf discs with *Agrobacterium* containing pE309. Figure 4.8 shows transformed somatic embryos. As seen in figure 4.7 *Agrobacterium* infection is obsserved on the edges of leaf discs. These leaf discs are then transferred to primary selection medium which is MS+ Kan for both the explant types. The surviving explants are then treated as putative transformants. Figure 4.8 C shows embryos after primary selection on MS + kanamycin. These putative transformants would now be subjected to PCR analysis using DGAT2 specific primers. Overall transformation efficiency with leaf discs was found to be 3.3% and further survival of explants was also hampered. While somatic embryos showed a little higher transformation efficiency of 10.08%, the problem encountered here too was further survival of putative transformants and regeneration of survived putative transformants. Kumar et al, 2010 have reported 18.3% of transformation efficiency of leaf explants of *J.curcas* as measured by the surviving shoots on selective medium. Li et al, 2007 have reported 13% transformation efficiency of *J.curcas* cotyledon discs as explants. However, this is the first report of *Agrobacterium* mediated transformation in somatic embryos of *J.curcas*. Xing et al, 2007 have reported to have achieved more than 25% of transformation frequency of embryogenic calli of sweet potato in suspension cultures. It should be noted that solid media cultures and liquid suspension media are both different systems for achieving regeneration and hence cannot be compared.

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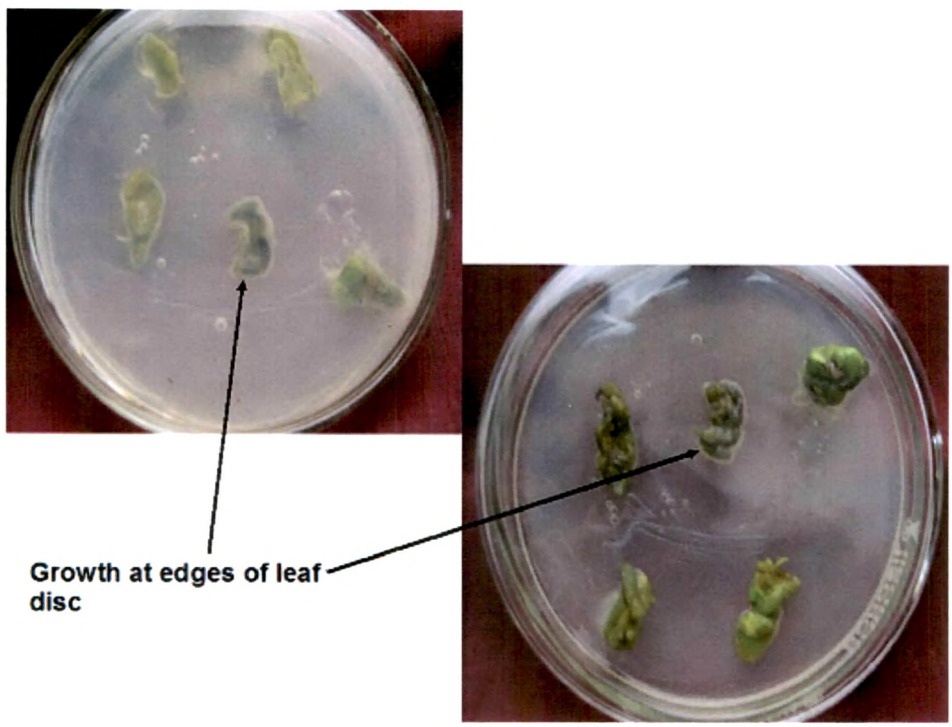

Figure 4.7: Co-cultivation of transformed *Agrobacterium* and *Jatropha* leaf discs

Figure 4.8 A: Somatic embryos on the edges of cotyledonary leaf discs

(A)

Figure 4.8 B: Co-cultivation of somatic embryos on MS medium

(B)

Figure 4.8 C: Transformed embryos on MS+ Kan

(C)

4.7.4 Evaluation of selective and bactericidal antibiotics on leaf discs and somatic embryos

These antibiotics are detrimental to tissue proliferation and growth at higher concentrations and fail to suppress bacterial growth at low concentrations (Sathyanarayana et al, 2012). The effect of increasing levels of kanamycin (20, 40, 60, 80 and 100 mg/L) was checked on leaf explants and somatic embryos. At higher concentration of kanamycin (80 and 100 mg/L), 100% bleaching of explants was observed in a week's duration. The phytotoxic effect of kanamycin was seen at 40 and 60mg/L as explants started bleaching after 10 to 12 days in culture resulting in total loss of chlorophyll pigmentation. Hence, in the subsequent experiments 20mg/L of kanamycin was used. However, Vadawale et al, 2012 reported 60mg/L as a suitable concentration of kanamycin for selection of putative transformants in *Arachis hypogea*. In a separate study, increasing levels of cefotaxime (50mg/L, 100mg/L, 150 mg/L and 200mg/L) were used, 200mg/L was found to be effective ~~concentration~~.

4.8 Optimization of factors influencing transformation

4.8.1 Explant Pre-culture Period

Preculturing explants prior to inoculation and co-cultivation with *Agrobacterium* has been shown to improve genetic transformation frequencies in many plants like *Zingiber officinale* Rosc (Suma et al, 2008), *Lathyrus sativus* (Barik et al, 2005), *Ipomoea batatas* (L) Lam (Xing et al, 2007) and microalgae like *Chlorella* sp. and *Nannochloropsis* sp. (San et al, 2011). It was observed in the present study that one day pre-cultured explants gave similar transformation efficiency as seven days pre-cultured explants. Pre-cultivation allowed proliferation of the plant cells to provide a large population of competent cells as potential targets for transformation (Xing et al, 2007). Hence, for both leaf explants and somatic embryos seven days of pre-culture was preferred. More so in case of somatic embryos as seven days pre-culture would enable to

generate a few somatic embryos which would then be susceptible for *Agrobacterium* infection during co-cultivation.

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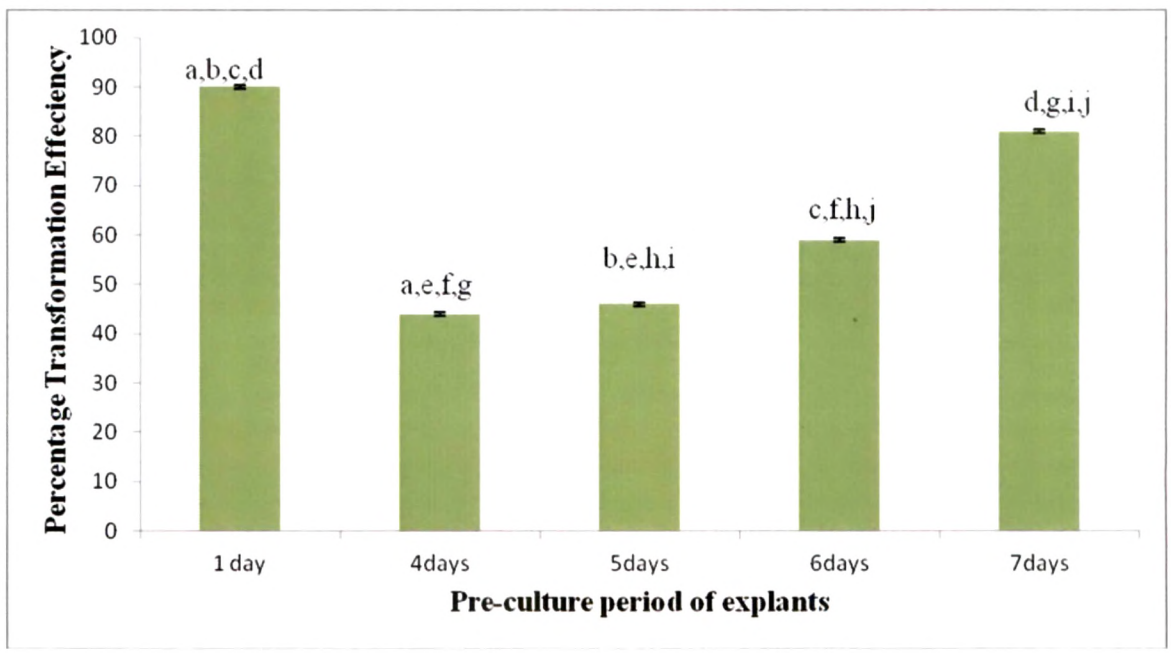

Figure 4.9: Effect of pre-culture period variation on transformation efficiency, N=30

a,b,c,d,e,f,h,i,j at p<0.0001 and g at p<0.005

4.8.2 Wounding Effect:

Wounding helps in the secretion of various phenolic compounds from the wound sites of dicotyledonous plant explants, a process which subsequently increases the transformation efficiency (Barik et al, 2005). Wounding can be through needle pricking or glass beads. There are generally two types of responses to wounding. In some plants it is found that the phenolic

compounds secreted from the wounded sites inhibit transformation efficiency whereas in some plant species it is known to improve transformation efficiency. In the present study effect of hand pricking with the help of a needle was studied. As shown in figure 4.10 needle pricking reduced the transformation efficiency. The explants so treated also showed browning. Kumar et al, 2010 have reported similar observation in *J.curcas*. Wounding increases the levels of secondary metabolites which might be responsible for tissue browning.

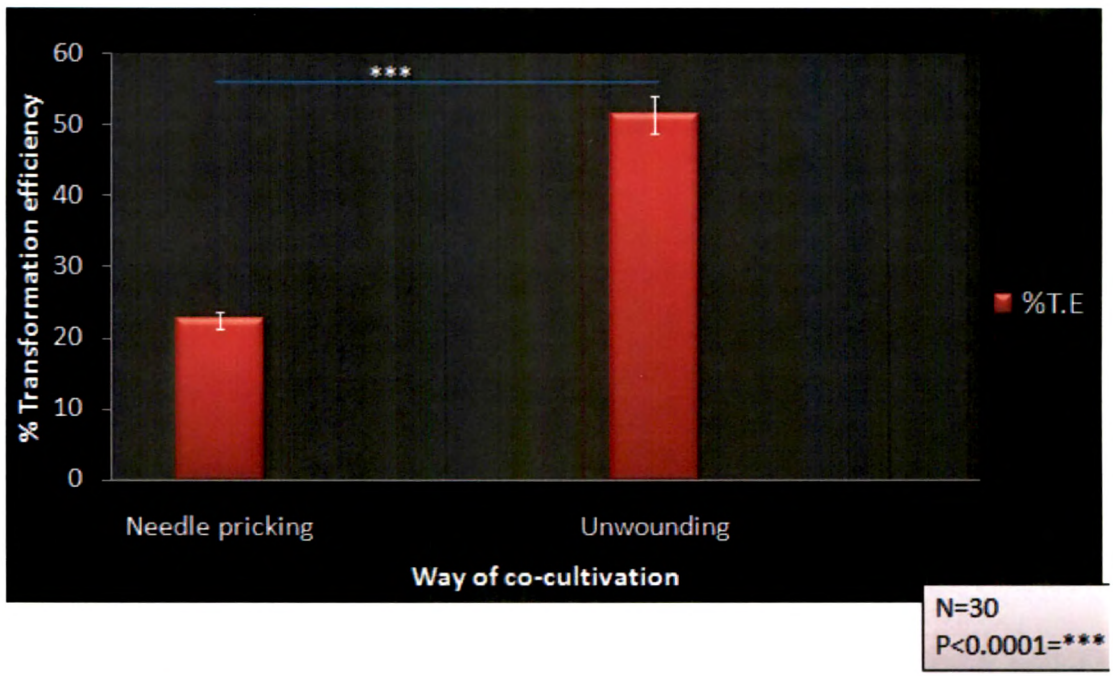

Figure 4.10: Effect of wounding on transformation efficiency

4.8.3 Effect of acetosyringone:

Phenolic compounds like acetosyringone are known to increase the transformation efficiency of *Agrobacterium* (Bolton et al, 1986). 20mg/L of acetosyringone was used in the present study (Li

et al, 2007; Kumar et al, 2010). As shown in figure 4.11 nearly 50% increase in transformation efficiency was observed with the use of acetosyringone.

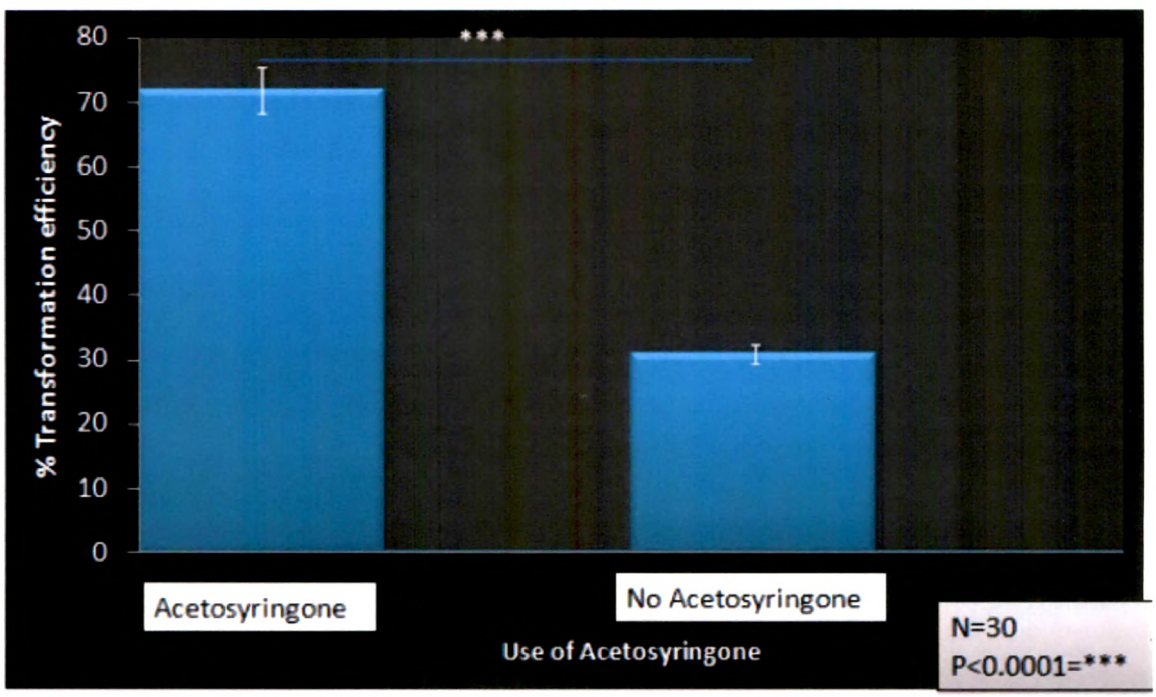

Figure 4.11: Effect of acetosyringone on transformation efficiency

4.8.4 Effect of co-cultivation:

Co-cultivation leads to induction of virulence and gene transfer. Co-cultivation period has great influence on transformation. Too short co-cultivation period is not favorable for transformation. However, too long co-cultivation period results in overgrowing *Agrobacterium* and therefore is harmful to plant cells as longer period may cause necrosis and cell death (Xing et al, 2007). Co-cultivation for 3-6 days is generally suitable for *Agrobacterium* mediated transformation (McHughn et al, 1993). In the present study, co-cultivation for 4 days yielded maximum

transformation efficiency (figure 4.12). At this time point higher number of callus forming discs and lower number of necrotic discs were observed. Co-cultivation for more than 5 days encouraged an overgrowth of bacteria with a concomitant decrease in transformation efficiency and difficulty in removal of *Agrobacterium*. Similar results have been reported for *J.curcas* by Kumar et al, 2010 whereas Li et al, 2007 have reported 3 days of co-cultivation in *Jatropha curcas*.

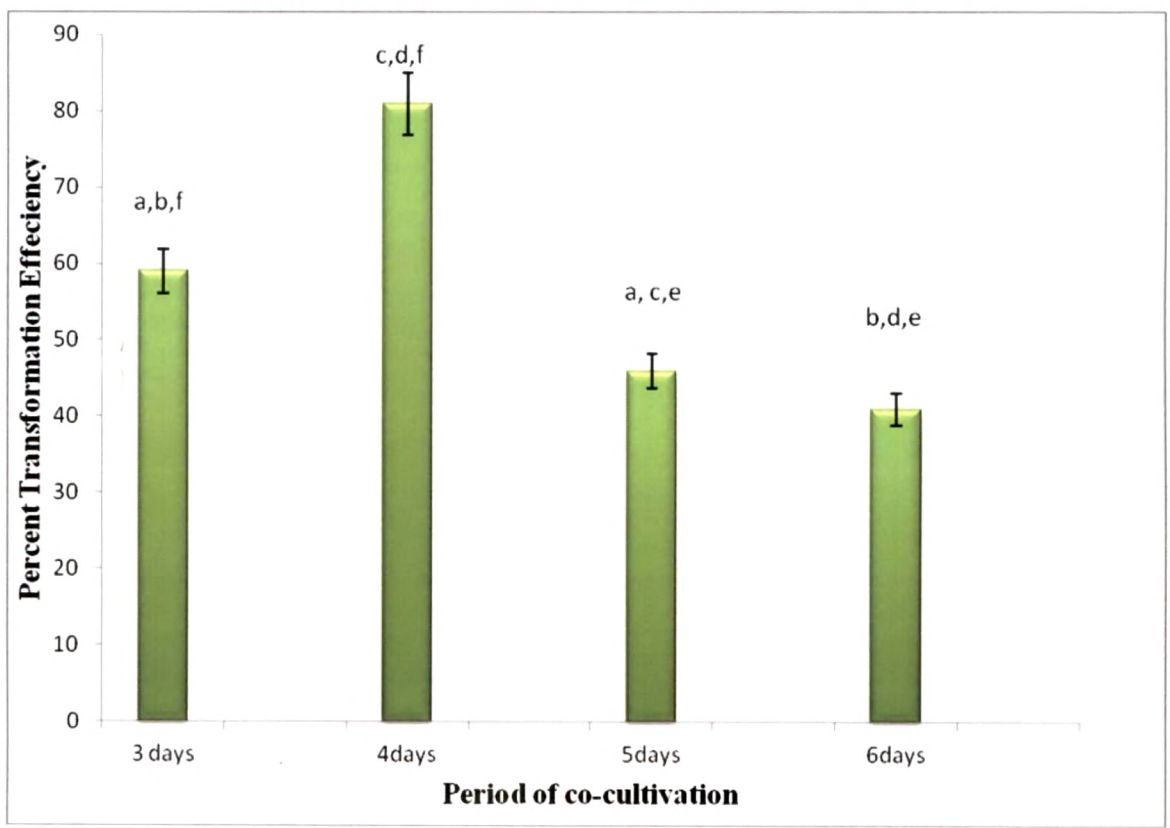

Figure 4.12: Effect of period of co-cultivation on transformation efficiency N=30, p<0.0001

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