Chapter – 4

TRANSFORMATION OF AGROBACTERIUM

4.0 Introduction

Genetic engineering of compatible solutes into non-accumulating plants has yielded enhanced plant stress tolerance. (Tarczynski et al., 1993; Kishor et al., 1995; Jain and Selvaraj, 1997; Nuccio et al., 1999). Glycine betaine(GB) is one such osmoprotectant, which is present in animals, bacteria, cyanobacteria, algae, fungi, and many drought- and salt-tolerant angiosperms (Rhodes and Hanson, 1993).

All known pathways for the synthesis of GB start with choline and proceed through reactions that involve one or two enzymes for the oxidation of choline to GB (Hayashi and Murata, 1998). The one-enzyme reaction for this conversion is catalyzed by choline oxidase (COD), and is present in soil bacteria *Arthrobacter globiformis* and *Arthrobacter pascens*. The two-enzyme reaction conversion is catalysed by a ferredoxin dependent choline monooxygenase (CMO) and an NADH-dependent betaine aldehyde dehydrogenase (BADH) localized in the chloroplasts of higher plants. In mammalian cells and in microorganisms such as *Escherichia coli*, there exists a two-enzyme reaction which is catalyzed by a NADH-dependent choline dehydrogenase (CDH) and BADH.

The COD pathway clearly has an advantage to be used in generating genetic transformants, over the CDH/BADH and CMO/BADH pathways because a single transformation with the relevant gene should introduce the pathway for conversion of choline to GB. The other advantage is that COD does not require any cofactors for the catalysis (Sakamoto et al., 2001).

In order to study the possibility of developing saline tolerance in plants, a choline oxidase gene was requested from Dr. GOPALAN SELVARAJ, PLANT BIOTECHNOLOGY INSTITUTE, NRCC, CANADA who kindly gifted the same

in the form of Binary vector pHS724 containing the gene of interest *cox* (choline oxidase).

This was amplified in *E. coli* DH5α through $CaCl_2$ method. In order to transform this gene of interest into the *Agrobacterium tumefaciensce*, triparental mating was carried out between *E. coli* DH5α bearing gene of interest (doner strain), *E. coli* DH5α bearing plasmid pRK2013 (helper strain) and the *Agrobacterium* LBA4404.

4.1 Materials and Methods

4.1.1 Cultures 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 a 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 pHS724 recombinant plasmid were maintained on LA plates with 50 µg/ml kanamycin.

4.1.2 Media & 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

4.1.3 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 Methods:

4.2.1 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

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 12000 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 12000 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 12000 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.

4.2.2 Competent Cell Preparation:

The protocol from Sambrook *et al.* (1989) was used for preparation of competent *E. coli* with an efficiency of ~10^6 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 4500rpm 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 4500rpm 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.

4.2.3 Agarose gel electrophoresis

Requirements:

TAE full form 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

4.2.4 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 pHS724 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 (Fisher 1995)

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.

4.5.5 Ketolactose Test (Bernaerts M. & Ley J., 1963)

Reagents:

Benedict's reagent [100 ml]: 17.3g Sodium citrate, 10.0g Na_2CO_3, 1.73g $CuSO_4.5H_2O$

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*.

4.6 Results and discussion:

4.6.1 Transformation of pHS724 into *E.coli* DH5α:

$CaCl_2$ mediated transformation was carried out in order to amplify the plasmid using the vector *E.coli* DH5α. The presence of the plasmid in *E.coli* DH5α was checked by restriction digestion pattern as well as phenotypic expression (kanamycin resistance).

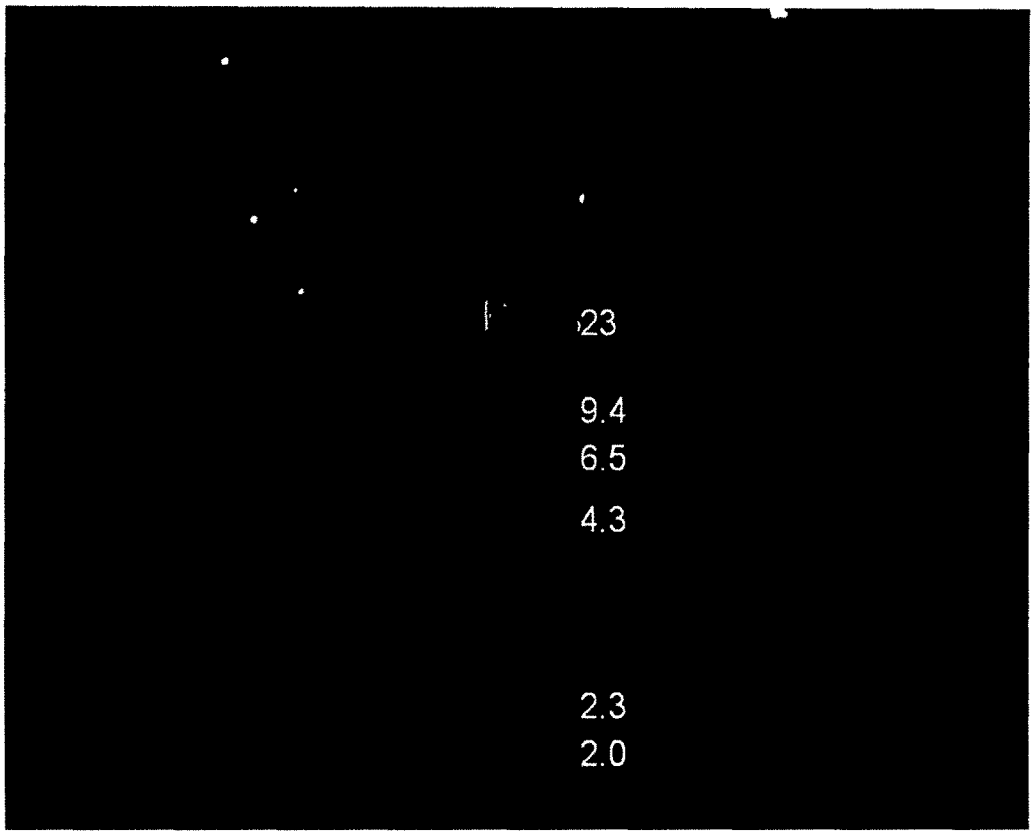

Fig.4.1 : Confirmation of the plasmid by agarose gel electrophoresis.

Lane1:0.2µl Hind III digest
Lane 2: Undigested plasmid
Lane 3:0.4µl Hind III digest
Lane 4: λ Hind III marker
Lane 5:0.2µl Eco RI digest
Lane 6: Undigested plasmid
Lane 7:0.4µl Eco RI digest

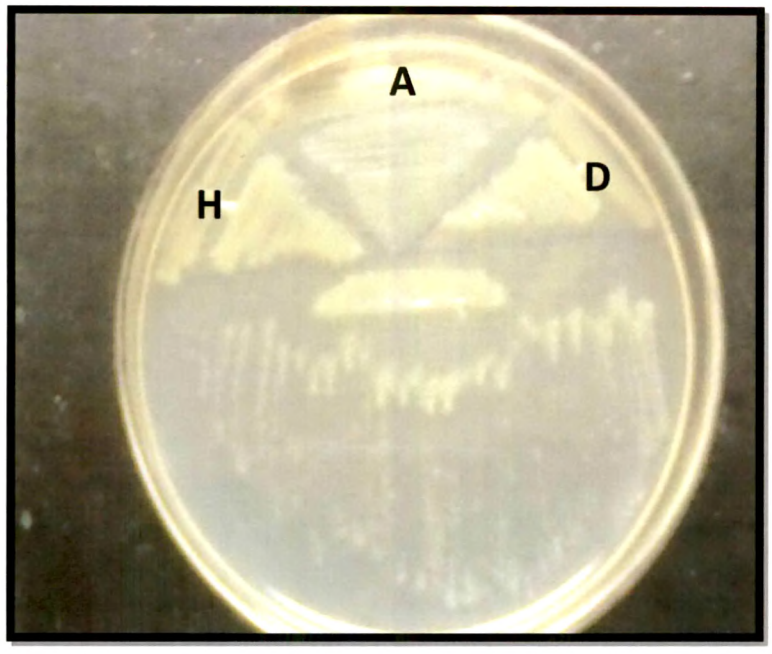

Fig. 4.2: Triparental mating (A: *Agrobacterium tumefaciens* (Rif^R Tet^R); D: Donor pHS724 (Kan^R); H: Helper strain (Kan^R)

4.6.2 Transformation of pHS724 into *Agrobacterium tumefaciens* LBA4404

Triparental mating was carried out using *E.coli* DH5α with pHS724 (kan^R) as the donor strain, *E.coli* DH5α with pRK2013 (kanR) as the helper strain and *Agrobacterium* LBA4404 A(RifR,TetR) as the recipient.

	Kan	Rif-Tet	Rif-Tet-Kan
pHS724	√	-	-
pRK2013	√	-	-
Agrobacterium	-	√	√
Agrobacterium transformants	√	√	√

Table 4.1: Resistance of plasmids to antibiotics

After two days of incubation, sufficient growth was obtained. All the three cultures (also streaked individually) showed adequate growth. Growth obtained was subjected to selection pressure by spreading different dilutions of antibiotics rifampicin, tetracycline and kanamycin on Luria agar. *Agrobacterium tumefaciens* transformants would survive because kanamycin present in the medium would not allow the growth of non-conjugated *Agrobacterium tumefaciens* cells. *Agrobacterium tumefaciens* LBA4404 is sensitive to kanamycin but resistant to rifampicin and tetracycline. Rifampicin and tetracyclin would not allow the growth of both *E.coli* DH5α pHS724 transformants & *E.coli* helper strain as they are sensitive to rifampicin and tetracyclin though resistant to kanamycin.

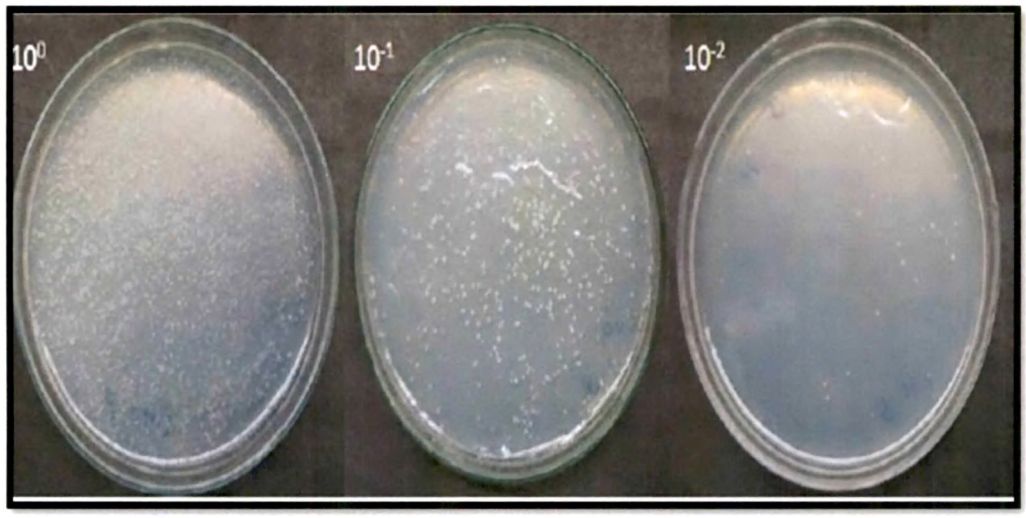

Fig. 4.3: *Agrobacterium tumefaciens* on LA plate

DILUTION	NO. OF COLONIES	cfu/ml
Undiluted	750	7.5×10^2
10^{-1}	250	2.5×10^2
10^{-2}	20	2×10^2

Table 4.2: Growth of transformants on Luria Agar plate

Fig 4.4: *Agrobacterium* and *Agrobacterium* transformants on LA plate

Agrobacterium and *Agrobacterium* transformants were streaked on the same LA plate containing antibiotics rifampicin, tetracycline and kanamycin. *Agrobacterium* was unable to grow as it was sensitive to kanamycin while sufficient growth of *Agrobacterium* transformants was obtained.

Growth obtained could be that of *A. tumefaciens* cells which had received the binary vector construct. These could be putative pHS724 *A.tumefaciens* transformants.

4.6.3 Confirmation of *A.tumefaciens* transformants by Ketolactose test

In order to test whether the colony was of *A. tumefaciens*, ketolactose test (also known as Benedict's test) was performed. This test is a confirmatory test given positive only by *A. tumefaciens* but negative by *E.coli*. Therefore, a positive Benedict's test will confirm the culture as *A. tumefaciens*.

A culture giving positive ketolactose test shows a yellow ring around its colony when flooded with Benedict's reagent due to the formation of ketolactose which reacts with Benedict's reagent to form yellow colour.

As can be seen in the figure, a yellow colour was observed around the colony which confirmed that the culture was *A.tumefaciens*.

Confirmation of the transformation of plasmid pHS724 into *Agrobacterium* was done by checking the phenotypic expression of *cox* gene in presence of NaCl.

4.6.4 Expression of Choline Oxidase (COX) in *Agrobacterium*

Expression of *cox* gene in *Agrobacterium* was checked by streaking the culture of *Agrobacterium* transformants containing pHS724 on a medium containing NaCl along with the antibiotics rifampicin, tetracycline and kanamycin. Also, the growth of *Agrobacterium* on a medium containing NaCl and antibiotics rifampicin and tetracycline was checked.

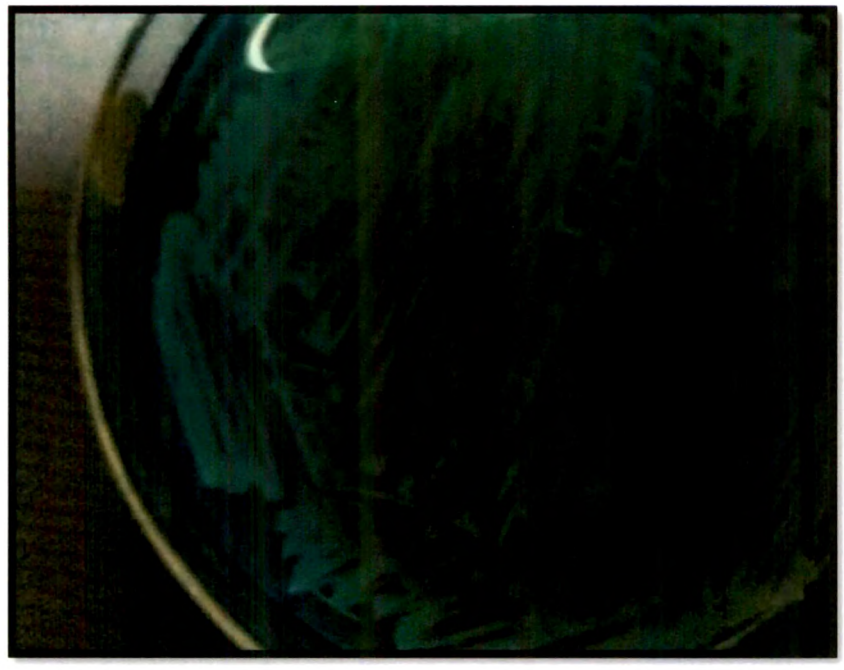

Fig 4.5: Ketolactose test

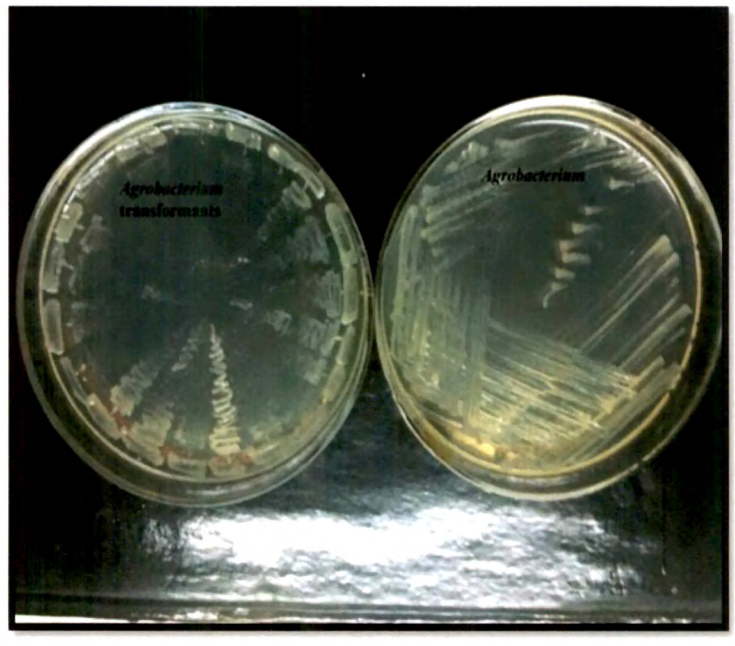

Fig 4.6: *Agrobacterium* and *Agrobacterium* transformants on a medium containing 0.1M NaCl

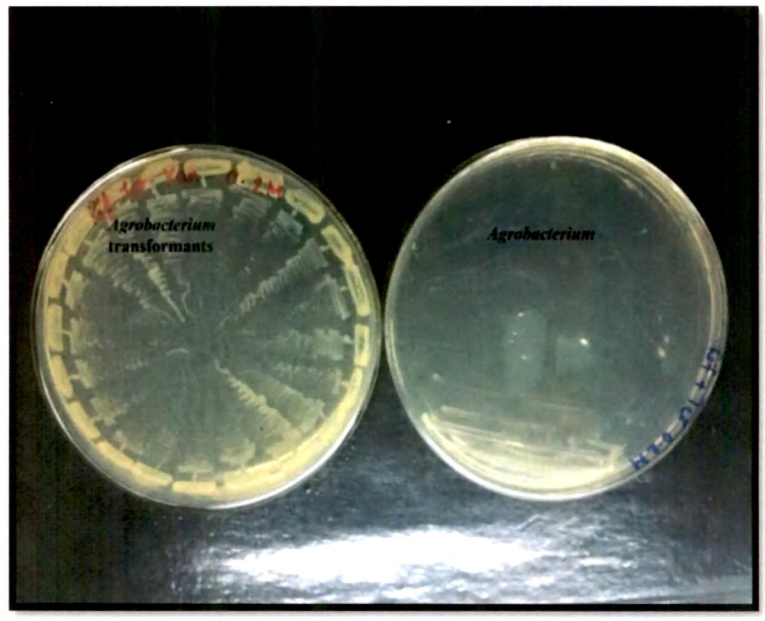

Fig 4.7: *Agrobacterium* and *Agrobacterium* transformants on a medium containing 0.2M NaCl

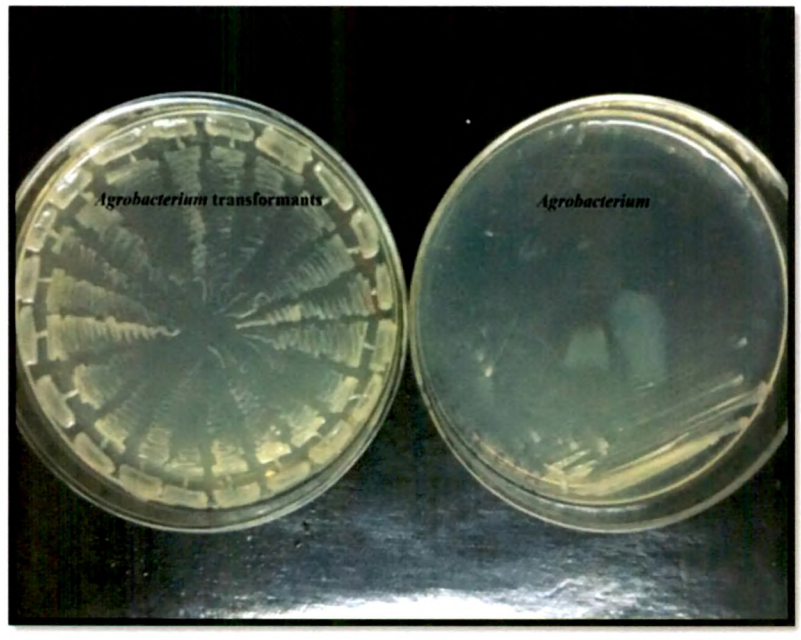

Fig 4.8: *Agrobacterium* and *Agrobacterium* transformants on a medium containing 0.3M NaCl

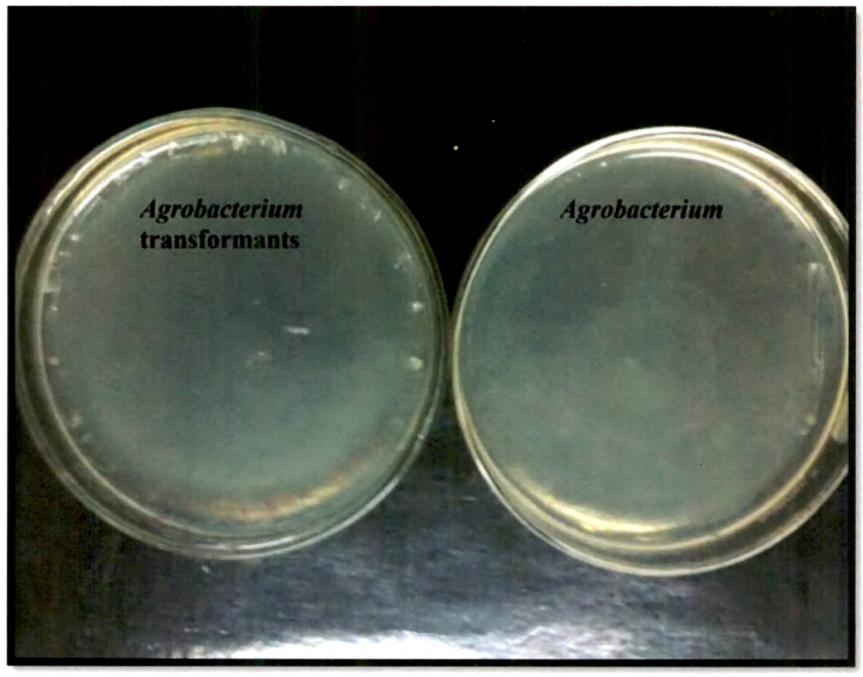

Fig 4.9: *Agrobacterium* and *Agrobacterium* transformants on a medium containing 0.4M NaCl

Luria Agar contains 0.08M NaCl. *Agrobacterium* was able to grow in 0.1M NaCl, while *Agrobacterium* transformants which contained the *cox* gene were able to survive till 0.3M NaCl. Hence the gradient could be increased from 100mM to 300mM thus increasing the salt tolerance capacity of transformants by 200mM.

4.7 DISCUSSION:

In this study, an attempt was made to incorporate the gene of interest choline oxidase (COX) responsible for production of glycine betaine, an osmoprotectant into groundnut. The plasmid containing the gene (pHS724) was amplified in the vector *E.coli* DH5α. Primary selection of the transformants was done by the presence of kanamycin resistance. The transformants were confirmed by plasmid isolation and further by restriction digestion pattern.

However, we could not see the expression of COX gene in the vector *E.coli* DH5α. This is due to the absence of inducible promoter which can be expressed in the bacteria *E.coli*. Since the gene choline oxidase is under the control of viral promoter 35S, no difference in the salt tolerance capacity was observed in *E.coli* DH5α transformants as compared to *E.coli* DH5α (control).

Further triparental mating of *E.coli* DH5α containing the plasmid pHS724 (serving as the donor strain) was performed with *Agrobacterium tumefaciens*; while *E.coli* DH5α containing the plasmid pRK2013 served as the helper strain thereby mobilising our gene of interest to *Agrobacterium tumefaciens*.

The transformants were confirmed by their ability to survive in presence of all the three antibiotics kanamycin, rifampicin and tetracycline. Phenotypic expression of the transformants was done by streaking the transformants in presence of NaCl. The transformants were able to survive in presence of 300mM NaCl while 400mM showed no growth. Hence the tolerance capacity of the transformants was increased by 200mM as compared to control. This is in full agreement with the reference (Selvaraj et al, 2000) which assert that

Glycine betaine can serve as an excellent osmoprotectant for the cell. Glycinebetaine is an important compatible solute in response to salt, drought and other osmotic stresses in many organisms. It exists in cytoplasm as a non-toxic osmolyte and its concentration increases so as to maintain the osmotic balance of the cell when plants are stressed. At the same time, it acts as a low molecular weight chaperone to stabilize the structure of the protein and enzyme so that they can keep in functional conformation. The references also quote that the salt tolerance capacity of the organism can be increased by external addition of choline in the medium. Similar attempts to generate salt tolerant plants have been made in tobacco. In this study three types of transgenic tobacco plants were acquired by separate transformation or co-transformation of a vacuolar Na(+)/H(+) antiporter gene, SeNHX1, and a betaine synthesis gene, BADH. When exposed to 200 mM NaCl, the dual gene-transformed plants displayed greater accumulation of betaine and Na(+) than their wild-type counterparts (Zhou et al.,2007). Photosynthetic rate and photosystem II activity in the transgenic plants were also shown to be less affected by salt stress than wild-type plants. Transgenic plants exhibited a greater increase in osmotic pressure than wild-type plants when exposed to NaCl. More importantly, the dual gene transformed plants accumulated higher biomass than either of the single transgenic plants under salt stress. In this study they have shown that simultaneous transformation of BADH and SeNHX1 genes into tobacco plants can enable plants to accumulate betaine and Na(+), thus conferring them more tolerance to salinity than either of the single gene transformed plants or wild-type tobacco plants (Zhou et al, 2008).