Presentations and Publications

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Direct Organogenesis in Peanut *Arachis hypogaea* L var. GG20

Ashutosh Vasant Vadawale, Ritu Mihani, Pushpa Robin

Department of Biochemistry, Faculty of Science ,
The MS University of Baroda, Vadodara , E mail : ashutoshv@rediffmail.com

Abstract

Objective : To develop the regeneration protocol through direct organogenesis using different explants for peanut-Arachis hypogaea L var. GG20 and to study various factors affecting direct organogenesis like explant size, pre-incubation period and phytohormone concentrations.

Methods: Two different types of explants viz. mature folded leaflets and cotyledons explants prepared from dry mature seeds were used as an explants after surface sterilization and inoculated on MS medium fortified with B5 vitamins and different combinations of BAP and NAA.

Results : Immature folded leaflets from the 7 days pre-incubated seeds gave direct shoot development up to 70% within 4 weeks of incubation on MS medium with BAP 13.32 µM and NAA 2.68 µM. Cotyledon explants gave direct multiple shoot bud initiation within 25-30 days of incubation on MS medium with BAP 17.75 µM and 2.68 µM NAA. Shoots developed from both the explants rooted within 4 weeks when transferred on MS medium with IBA 3.5 µM.

Conclusion: An efficient regeneration protocol through direct organogenesis is developed for peanut-Arachis hypogaea L var. GG20 which can be effectively used for genetic transformation for desired trait.

Keywords: Organogenesis, Peanut, Phytohormone, Plant regeneration

INTRODUCTION

Groundnut or peanut (Arachis hypogaea L.) is one of the principal economic oilseed legumes and is largely cultivated in many tropical and subtropical regions of the world. The seeds are mostly used for vegetable oil, carbohydrates and proteins for human as well as animal consumption, as a legume, it also improves soil fertility by fixing nitrogen and increases productivity and thus play an important role in the agricultural economy. However, the crop is affected by many biotic and abiotic stresses which cause major losses in terms of quality and quantity.

Genetictransformationovercomestheseli mitationsandallowsintroductionofagronomically important genes across taxa [1-3]. The availability of an efficient regeneration system is however an essential prerequisite for utilization of this approach. Re-generation strategies include organogenic or embryogenic systems using a number of different explants. Advantages of leaf-derived organogenesis systems include the relative ease of obtaining material for explanting. So far, number of regeneration protocols from different explants of groundnuts have been published of which the ones that report direct organogenesis proved to be the most promising for genetic transformation[4,5]. For successful genetic modification by the production of transgenic plants, effective regeneration system is imperative. Efficient direct organogenesis enables effective regeneration and would also provide a means for applications of gene transfer. Regeneration from leaves would also provide a way for targeting gene transfer to the chloroplast DNA using biolistic method.

Although several reports on efficient regeneration from diverse explants of peanut have been published, many of them are genotype dependent. Till date, organogenesis in peanut have been reported using different explants, mainly from immature leaflets[4,6,7,8,9] and from cotyledon explants [15].

The present paper reports direct in vitro organogenesis from two different explants of Peanut var. GG 20 which is extensively cultivated and economically important peanut variety of Gujarat.

MATERIALS AND METHODS

The pods of peanut cultivar var. GG20 was obtained from the Agriculture University, Junagadh, Gujarat.

Mature dry seeds were washed under running tap water for 25 min. It was then subjected to, treatment with a solution of detergent Extran for 10 min, treatment of antifungal agent Bavistin for 10 min and finally washed thoroughly in sterile that apart from hormones concentrations, the size and physiological status of the explants also determined response of the explants.double distilled water and then surface-sterilized in 0.1% aqueous mercuric chloride for 10 min, rinsed 3-4

Table: 1. Influence of peanut leaflet developmental stage on percent organogenesis and total number of shoots per responsive explants after 30 days of incubation

Number of pre-incubation days	Leaflet characteristic	Percentage of Organogenic responsive explants	Average shoot buds after 30 days (no)
1st	white, folded	0	0.00 ± 0.00
3rd	whitish-Green folded	0	0.00 ± 0.00
5th	Green folded	40	1.25 ± 0.50
7th	Green folded	70	2.71 ± 0.65
9th	Green unfolded	0	0.00 ± 0.00

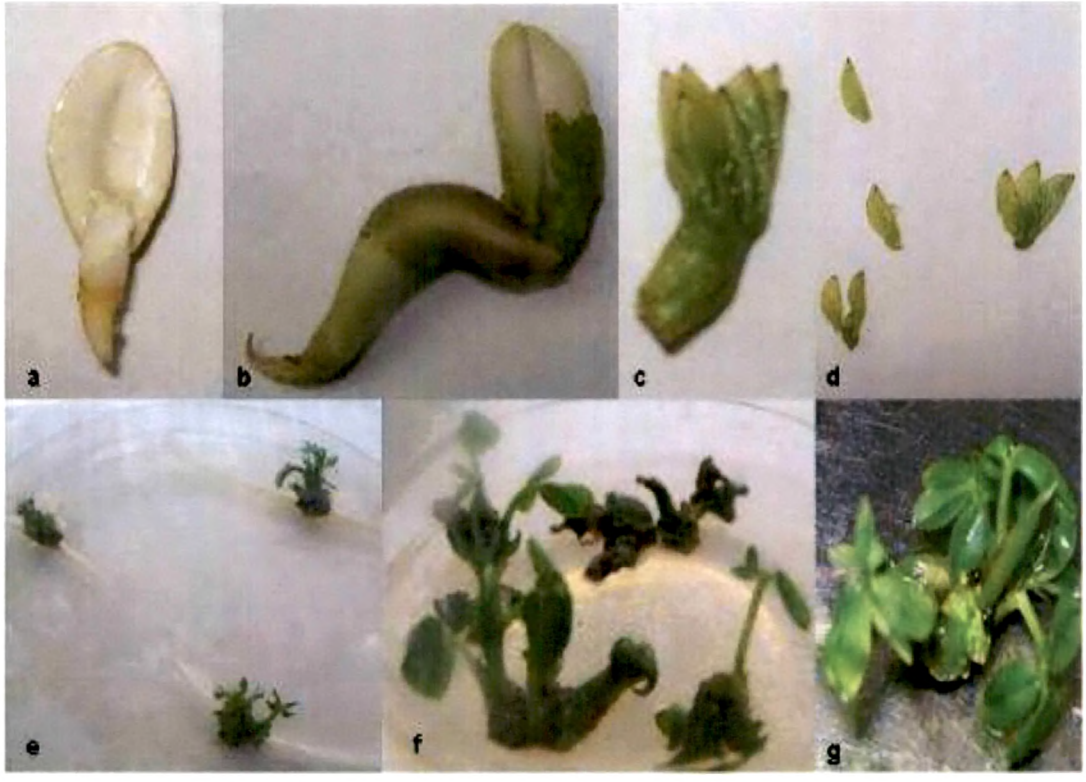

Figure1.Direct shoot organogenesis from the young green leaflets explants of peanut var.GG 20.a-c).Development of young, green, folded leaflets into the uncoated seed during 7 days incubation in MS medium BAP 2.2 µM. d) young folded leaflets separated from seeds. e) shoot bud initiation at the basal end of leaf explants after 15 days incubatiobein the uncoated on seed during 7 day incubation in MS medium BAP 13.32 µM BAP and 2.68 µM NAA.f-g)Shoot elongation and plantlet development within 30 days of incubation on the same medium.

times with sterile double distilled water. The seeds were left soaked 4-6 hours in sterile double distilled water.

In all the experiments, MS medium is used fortified with B5 vitamins[10] , 100 mg/l myo-inositol and 30 g/l sucrose and the cultures were incubated at 25 ± 28 0C under a continuous 16/8 h (light/dark) photoperiod with light supplied by cool white fluorescent lamps at an intensity of about 1600 lux. The medium was solidified with 0.65% agar (colloids, India). The pH was adjusted to 5.8 before autoclaving at 121 0C for 20 min. The experiment was performed in triplicate.

Direct Organogenesis from young leaflets.

To get the young green leaflets, seeds after surface sterilization were inoculated in MS medium with 2.22 µM BAP in petri dishes (94 mm diameter). The method of explanting has been partially adapted from Tiwari and Tuli [11].

Explants were prepared from 1-9 day-old seedlings in the initial experiments to evaluate the influence of explant age on regeneration efficiency. In the subsequent experiments, 7-day-old seedlings were used to obtain young folded leaves.

After seven days of incubation, the enlarged seeds were taken out aseptically, seed coat was removed, elongated radical was cut apart and the green young folded leaflets were separated (Fig. 1a-1d) and used as explants for direct organogenesis. These leaflets were given superficial cut on the edges and further placed on MS medium with BAP (4.44 - 17.76 µM) and NAA (2.68-5.36 µM). Within 3 weeks of incubation, direct shoot buds emerged at the basal end of the leaflets which developed and elongated (Fig. 1e-1g) in the same culture vessel in next 7-8 days. Along with the direct shoot formation at the basal end of the leaves clusters of small adventitious buds appeared which when transferred to same medium after 4 weeks of incubation, elongated into individual shoots. Fully developed and elongated plants were transferred to MS medium with IBA 3.5 µM for root induction.

Direct Organogenesis from CN explants

The method of explanting has been adapted from Sharma and Anjaiah[2]. The seed coat was removed and the embryo axis was cut surgically and removed. The seed was then cut vertically along its natural ribs to get the two individual cotyledon halves- Cotyledon node and the same were used as explants- CN explants (Fig.-2a-2c).

The explants were placed on the MS medium in such a way that the cut edge was embedded in the medium. Various combinations of BAP (4.44 -17.76 µM) and NAA (2.68-5.36 µM) for getting direct shoot bud induction were tested. 20 explants were inoculated per combination of BAP & NAA. After 4 weeks of incubation, the cultures were transferred to MS medium with reduced concentration of BAP (2.22 - 8.88) µM for elongation of the shoot buds. Fully developed and elongated plants were transferred to MS medium with IBA 3.5 µM for root induction. Well rooted plants were transferred to small portray containing mixture of soilrite:cocopeat (1:1) for acclimatization in Green House.

RESULTS AND DISCUSSION

Direct Organogenesis from young leaflets.

The present report describes rapid, reproducible and efficient protocol for direct organogenesis of peanut plants from immature unfolded leaflets.

The young, green, folded leaflets from 7 days pre-incubated seeds when used as explant and inoculated on MS medium containing different combinations of BAP and NAA gave direct shoot bud initiation within 3 weeks. The shoot bud formation starts and restricted from the basal end of the leaves. MS medium with 13.32 µM BAP and 2.68 µM NAA gave 70 % direct shoot formation within 30 days of incubation. However not all leaflet gave similar response. Emerging leaflets are folded lengthwise along the middle. First three leaflet starting form the innermost pair only was able to give the direct shoot formation response. The sizes of these leaflets were 5-7 mm in length.

Thus the leaflets developmental stage showed pronounced effect on the organogenic response of the explants. The green folded and innermost first to third pair of folded leaflets developed inside seeds at the end of 5 - 7 days of pre-incubation only gave the direct shoot induction response. Neither whitish folded leaflets of 1-3 days old pre-incubated leaflets nor green unfolded leaflets of any position gave organogenic response on any of the combinations of BAP & NAA. Baker and

Wetzstein [12] also reported that 5- to 8 mm-long cut folded leaflets of peanut were superior to smaller leaflets in the embryogenesis response.

Maximum of 3 elongated shoots were observed per explants directly within a month period on MS medium with 13.32 µM BAP and 2.68 µM NAA. However, numerous shoot forming buds were found which when transferred to same medium shown further development and elongation. The results suggest that apart from hormones concentrations, the size and physiological status of the explants also determined response of the explants

Mroginski et al [9] have also reported earlier that the immature leaflet of peanut are the most responsive explants in giving organogenesis response.The results obtained correlates with Seitz [13] who reported that immature leaves induced up to 30% shoot formation on the MS medium containing NAA and BAP.Cheng et al[4] also reported that MS medium containing NAA and BAP was the most promising combination for shoot bud formation from immatureleaflet explants.

Tiwari and Tuli [11] also reported 81.5 % shoot bud formation from the young leave explants when pre-incubated for 7 days on the MS medium with combination of NAA and BAP and transferred then onwards on medium containing reduced concentrations of NAA and BAP.

However, present findings differs from these earlier reports in a way that we used green young folded leaflets and obtained direct shoot formation within one month of incubation. Direct shoot formation with least possible time is mandatory prerequisite for genetic transformation using biolistic method. The findings are also especially important for targeting chloroplast DNA transformation where chlorophyll containing tissues like leaves are required to be present in the explants.

Direct Organogenesis from CN explants

CN explants when inoculated on different combinations of BAP and NAA shown direct shoot formation response within one month of incubation. Among the various BAP and NAA combinations tested, high frequency regeneration was obtained following culture of explants on MS medium supplemented with BAP (4.44-17.76 µM) along with NAA (2.68- 5.76 µM), BAP at 17.76 µM and NAA at 2.68 was most

Figure2.Direct shoot organogenesis from the de-embryonated cotyledons of Peanut var. GG 20.a.c) Preparation of cotyledon explants. d) multiple shoot bud growth at the proximal end of the greened cotyledon explants after 20 days of incubation in MS medium with 17.76µM BAP and 2.68 µM NAA.e.f) shoot development and elongation after 30 days on the same medium. g) Root development on fully developed shoot on MS medium with 3.5 µM IBA. h) acclimatization in Green House.

effective for multiple shoot bud formation.

Problem of bacterial contamination was encountered in case of the CN explants however it could be controlled with the inclusion of treatment of antibiotic combinations (data not shown) after surface sterilization.

Explants turned green within 12-15 days of incubation on all the media combination used. After 4 weeks, shoot bud initiated at the proximal end of the CN explant (figure 2d) however not all combinations yielded shoot bud initiation. Maximum of 65 % of explants shown direct shoot bud formation from the halved CN explants. Plant as many as 6-8 shoot buds were observed which when transferred to MS medium with BAP 8.77 µM concentration developed and elongated. The findings of direct shoot regeneration from cotyledon explants is in line with the number of other reports including [14,15]. However they reported use of 2,4 D in combination with BAP. Anuradha *et al*[16] also reported 82% regeneration in the MS medium with combinations of BAP and NAA using cotyledon explants. However, they prepared the cotyledon explants keeping embryo axis intact and bisected in to two cotyledon halves from 6 day old seedlings. Therefore meristematic region of the embryo axis were present in the explants. While here we used cotyledon explants after completely removing embryo axis and then induced development of multiple adventitious buds.

Table 2. Response of CN explant on MS medium supplemented with different combinations of NAA & BAP for direct shoot organogenesis

NAA (µM)	BAP (µM)	Response Greening (%)	Average shoot buds after 30 days (no)
2.68	4.44	43.33	0.64 ± 0.81
	8.88	50.00	2.00 ± 1.00
	13.32	56.67	3.09 ± 1.51
	17.76	66.67	6.09 ± 1.30
5.36	4.44	60.00	0.45 ± 0.69
	8.88	53.33	2.91 ± 1.38
	13.32	33.33	1.45 ± 0.82
	17.76	60.00	2.27 ± 1.56

CONCLUSION

The important aspect of the results shown here in this paper is the shortest duration in which direct shoot organogenesis could be achieved using different explants and therefore it can be effectively used for genetic transformation of *Arachis hypogea* L. var. GG20 for desired trait.

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RESEARCH ARTICLE

BIOTECHNOLOGY

TRANSFORMATION OF GROUNDNUT - *ARACHIS HYPOGEA L. VAR. GG20* WITH THE *COX* GENE-AN ATTEMPT TO DEVELOP SALINITY TOLERANCE

ASHUTOSH VADAWALE*, RITU MIHANI, ASHA MATHEW AND PUSHPA ROBIN

Department of Biochemistry, Faculty of Science,The Maharaja Sayajirao University of Baroda, Vadodara - 390 002. India.

ASHUTOSH VADAWALE
Department of Biochemistry, Faculty of Science,The Maharaja Sayajirao University of Baroda, Vadodara - 390 002. India.

*Corresponding author

ABSTRACT

Salinity, is one of the most serious environmental factors limiting the productivity of crop plants and agricultural production world-wide. An attempt was made to develop salinity tolerance in Groundnut-*Arachis hypogea* l. var GG20 through *Agrobacterium* mediated transformation with the *cox* gene for synthesis of Glycine betaine. *Agrobacterium tumefaciens* LBA 4404 with binary vector pHS724 containing the cox gene was co-cultivated with different groundnut explants for infection and transformation. Various factors like explant types and co-cultivation period affecting transformation and concentration of kanamycine in selection medium were studied. The successful transformants were confirmed through phenotypic expression as well as through the expression of GUS gene *(uidA* gene) in the regenerated plants.

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KEY WORDS

Salinity, Glycine betaine, Groundnut, Transformation

INTRODUCTION

Several environmental factors adversely affect plant growth and development and final yield performance of a crop. Drought, salinity, nutrient imbalances (including mineral toxicities and deficiencies) and extremes of temperature are among the major environmental constraints to crop productivity worldwide (Hamdia et al. 2010). Salinity, among all these is the most serious environmental factors limiting the productivity of crop plants and agricultural production world-wide (Ashraf, 1999). 40% of world's surface has salinity problems (Jadhav et al., 2010). However, despite advances in the increase of plant productivity and resistance to a number of pests and diseases, improvement in salt tolerance of crop plants remains elusive. In spite of considerable efforts through breeding programmes, progress to enhance salt tolerance has been very slow. Classical genetic studies have demonstrated that the ability of plants to tolerate salt stress is a quantitative trait involving the action of many genes. As a result, it has been difficult to obtain salt tolerance crop plants by traditional methods (Foolad and Lin, 1997).

Among various alternatives for development of saline tolerance in plants through genetic engineering, transfer of gene for synthesis of Osmolytes is most sought after by researchers worldwide. Osmolytes are certain organic metabolites of low molecular weight which are known collectively as compatible solutes (Bohnert et al., 1995). Metabolites that serve as compatible solutes differ among plant species and include polyhydroxylated sugar alcohols, amino acids and their derivatives, tertiary sulphonium compounds and quaternary ammonium compounds (Bohnert and Jensen, 1996). Glycine betaine (GB) among all compatible solutes is one of the most potent compatible solutes which protect the cell machinery in plants against various kinds of stresses like salinity, drought, cold etc. (Hayashi et al 1998). Among the plants which can naturally produce GB, three pathways exist for its synthesis (Hayashi et al 1998). It starts with choline and proceeds through a reaction that involves one or two enzymes for the oxidation of choline to GB. Different enzymes catalyze the reaction in different organisms. The overall reaction is

Choline ——————□ Betaine aldehyde ——————□ GB
CMO / COX /CDH *COX / BADH*

CMO : Choline monooxygenase, COX :Choline oxidase, CDH Choline dehydrogenase, BADH : Betaine aldehyde dehydrogenase.

COX pathway has the advantage of being a single enzyme which can catalyze both steps and also it does not require any cofactor for catalysis (Sakamoto et al, 2001).

Ground nut – *Arachis hypogea* L. is a major cash crop of Gujarat. It is rich source of protein, oil, and fodder and plays an important role in the agricultural economy of the state. An attempt was therefore made to develop salinity tolerance in Ground nut – *Arachis hypogea* L. var GG20.

MATERIALS AND METHODS

Plant Material, culture initiation and maintenance

The pods of peanut cultivar var. GG20 was obtained from the Agriculture University, Junagadh, Gujarat.

Mature dry seeds were washed under running tap water for 25 min. It was then subjected to, treatment with a solution of detergent Extran for 10 min, treatment of antifungal agent Bavistin for 10 min and finally washed thoroughly in sterile double distilled water and

then surface-sterilized in 0.1% aqueous mercuric chloride for 10 min, rinsed 3–4 times with sterile double distilled water. The seeds were left soaked 4-6 hours in sterile double distilled water.

Experiments were conducted on MS (Murashige and Skoog, 1962) medium fortified with B5 vitamins (Gamborg et al., 1968), 100 mg/l myo-inositol and 30 g/l sucrose.

The media were solidified with 0.65% agar (colloids). The pH was adjusted to 5.8 before autoclaving at 121°C for 20 min. The cultures were maintained at 28 ± 1°C under a continuous 16/8 h (light/dark) photoperiod with light supplied by cool white fluorescent lamps at an intensity of about 1600 lux.

Explant preparation

Three different kinds of explants i.e. embryo axis, young immature leaves and cotyledon explants were used for transformation .

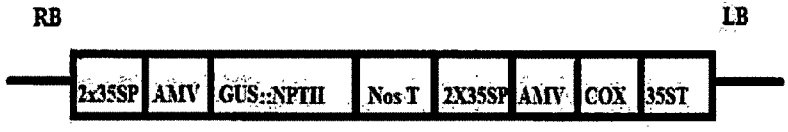
Mature dry seeds were imbibed in sterile double distilled water for 6-h after surface sterilization and kept for germination on autoclaved filter paper bridges in sterile double distilled water in test tubes. In case of embryo axis and cotyledon explants, the explants were collected from 3-day-old seedlings. While in case of young immature leaves the explants were taken from 7 day old seedlings.

In case of embryo axis, the seed coat and the radical were removed and the cotyledonary nodes were excised by cutting both epicotyls and hypocotyls approximately 2-3 mm above and below the nodal region and the embryo axis was bisected along the longitudinal plane. The meristematic region present in the nodal region was macerated by 6-8 diagonal shallow cuts by a sterile surgical blade. From each seed, two explants were obtained.

In case of cotyledon explants the embryos were surgically removed from the cotyledons and two explants were obtained.

Agrobacterium strains and plasmid vectors

The disarmed *Agrobacterium tumefaciens* strain LBA4404 (Hoekema et al. 1983) harboring the binary plasmid pHS724, kindly provided by Dr. Gopalan Selvaraj, Plant Biotech Inst., NRCC, Canada, was used as a vector system for transformation. The uidA or GUS (8 glucuronidase) gene driven by the CaMV 35S promoter and terminator sequences served as reporter gene. Plasmid pHS724 contains the gene of interest - *cox* for choline oxidase gene and kanamycin resistance gene for bacterial selection driven by a double 35S promoter, and terminated by 35S poly A signal and the *nptII* and *uidA* genes.



pHS724

The vector contains Right border and Left border sequences which can specifically recombine with the right and left border of the Ti plasmid. The *cox* gene is under the control of viral 35S promoter. GUS represents the eukaryotic reporter gene while in case of primary selection, the transformants can be screened by kanamycin resistance. The size is 15.6 Kb.

Co-cultivation and transformation

A single colony of Agrobacterium strain was incubated in 50 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 50 ml of liquid suspension medium containing MS salts (Murashige and Skoog 1962), B5 vitamins (Gamborg et al. 1968), 100 mg/l

myo-inositol, 30 g/l glucose and 100 ml acetosyringone. The suspension was kept at 28°C for 30 min 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 and transferred onto co-cultivation medium and incubated for 5 days at 21°C in the dark. Experiments were conducted on MS (Murashige and Skoog, 1962) medium fortified with B5 vitamins (Gamborg et al., 1968), 100 mg/l myo-inositol, 30 g/l sucrose, BAP (4.44-17.76 µM) and NAA (0.53-1.06 µM).

Selection of transformants, multiplication and growth conditions

After co-cultivation for 5 days, the explants were rinsed 5–6 times with sterile water separately aseptically. All explants were blotted on sterile paper to remove excess bacterial suspension and further placed on shoot induction medium which is the same as co-cultivation medium but additionally containing Cefotaxime (Alkem, India) (200 mg/l) to eliminate overgrowth of *A. tumefaciens*. All the cultures were maintained at 28±1°C under a continuous 16/8 h (light/dark) photoperiod with light supplied by cool white fluorescent lamps at an intensity of about 1600 lux.

The bud forming region of the culture were transferred to shoot multiplication and selection medium consisting of MS salts, B5 vitamins, 100 mg/l myo-inositol, 30 g/l sucrose, 6.5 g/l agar BAP (2.22-8.88µM), 200 mg/l cefotaxime and different concentrations of kanamycin (Macleods, India).

Two subcultures of 4 week intervals were done and multiple shoot buds were subcultured onto respective fresh medium. After two cycles of continuous kanamycin selection the shoots that attained > 2 cm length were excised and transferred onto MS medium supplemented with 4.30 µM α-naphthalene acetic acid to induce roots

GUS assay

The ß -glucuronidase (GUS) histochemical assay was used as a rapid way to detect the presence of the *uidA* gene (GUS) in the putative transformants as described by Jefferson *et al.* using leaf segments in regenerated shoots from explants. GUS assay was carried out by incubating the whole transformed plant tissues in GUS assay solution overnight at 37°C.

RESULTS AND DISCUSSION

Explants were co-cultured with Agrobacterium strain LBA4404 carrying the binary vector pHS724 and then transferred to respective shoot induction medium to select for transformed shoots. After 5 days of incubation the Agrobacterium infection was conspicuously seen in ring fashion on cotyledons (Fig-1).

Co-cultivated explants swelled and developed shoot buds with little callus after 3 weeks on selective shoot regeneration medium. Shoot clumps which survived this selection step were sub-cultured to shoot multiplication medium. The green shoot buds along with yellowish-white shoots were transferred to fresh medium with 60 mg/L kanamycin sulfate along with respective controls. The yellowish white coloration of shoot and leaves was observed which might be due to extra genetic load of the gene of interest along with *uidA* gene and *nptII* gene present and indicate the positively transformed shoots.

There was significant reduction in qualitative growth performance of control plants compared to transgenic plants on Kanamycin rich MS medium. The kanamycin resistant transformed elongated shoots were rooted in respective medium. Root induction was observed within 3 weeks of culture. The transformation frequency in terms of number of explants producing kanamycin resistant shoots was better using co-cultivated embryo axis explants (32%) than for cotyledons explants (21%). Different factors which

affected the transformation frequency were studied.

Effect of Explant type on transformation

Co-cultivated explants showed different responses upon inoculation with Agrobacterium. The efficiency of shoot bud formation from embryo axis or cotyledon explants was not affected by Agrobacterium infection when compared with uninfected explants. On respective shoot induction medium, 50% embryo axis explants showed multiple shoot bud induction while 30% explants showed shoot bud formation in case of cotyledon explants at the proximal end. In case of young leaves explants shoot bud formation could not be achieved even after four weeks of incubation on the medium. Subsequent transfer of leaves explants also did not yield any bud formation. This might be because the leaves were too tender and young when co-cultivated with agrobacterium.

Effect of co-cultivation periods on transformation

The transformation frequency of different explants was highly influenced by the co-cultivation period. Transformation frequencies of different explants co-cultivated on respective shoot regeneration medium for different periods are shown in Table 1. After two cycles of sub culture, the maximum transformation frequency occurred in embryo axis explants (32.20%) followed by cotyledon explants (21.10%) after 5 days of co-cultivation than after 1, 2, and 3 days of co-cultivation. Thus five days of co-cultivation resulted in the highest percentage of shoot regeneration on selection medium. These differences might be because of the fact that the concentration of *Agrobacterium* was higher after 5 days of cultivation which increases the possibility of gene transfer in groundnut. Similar observations were made in alfalfa by Chabaud *et al* (1988). Similar results were also observed in cowpea (Muthukumar *et al.* 1996) and in pea (DeKathen and Jacobsen 1990, Lulsdorf *eta.* 1991).

Table 1.

Effect of co-cultivation period on shoot bud regeneration on three explants of groundnut on shoot induction medium with 60 mg/l kanamycin sulfate

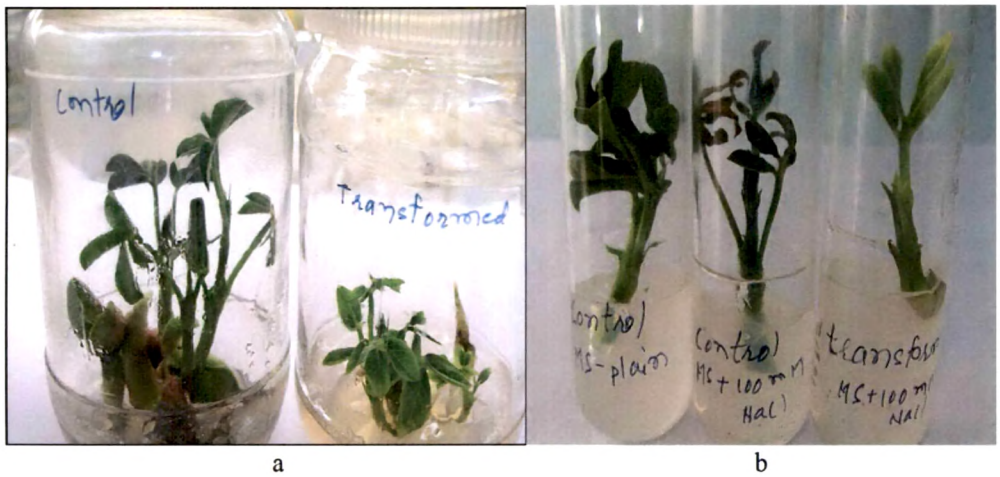
Co-cultivation period Without kanamycin (No of days)	Shoot bud regeneration frequency* (mean + SD)		
	Embryo axis	Cotyledon explants	Young leaves
1	5.50 ± 1.08	5.20 ± 1.03	3.10 ± 1.37
2	13.50 ± 2.37	10.20 ± 1.23	-
3	18.40 ± 1.07	12.60 ± 1.84	-
4	26.10 ± 3.07	18.60 ± 1.51	-
5	32.20 ± 2.94	21.10 ± 2.69	-

.* 30-35 explants were cultured per experiment and replicated three times.

However, extended co-cultivation period for more than 2 days for young leaf led to explant abortion caused by bacterial contamination. Thus young leaf explants could not yield any transformed shoots. These results do not agree with that of Eapen et al., (1994) who obtained an average of 6.7% of shoot regeneration on selection medium containing 50 mg/l kanamycin. Cheng et al. (1996) reported that the frequency of transformed fertile plants was 0.2% to 0.3% of the leaf explants inoculated. The difference in results could be explained by the fact that they used mature leaf segments while here we used tender young immature leaves which are fragile comparatively.

Effect of kanamycin concentration in selection:

To find out optimum concentration of Kanamycin and its effect on different explants, various concentration of kanamycin were added to respective medium as shown in table 2 and their effect were observed prior to Agrobacterium transformation. At 80 mg/L, kanamycin caused necrosis in all explants. Concentrations of 80 mg/L and 100



a b

Figure - 1.

a: Comparison of transformed plant with the control plant growing on MS medium without stress

b: Comparison of performance of transformed plants with control plants.

L: control plants in MS medium without NaCl, M: control plants in MS medium with 100 mM NaCl, R: Transformed plant in MS medium with 100 mM NaCl.

mg/L kanamycin completely inhibited shoot bud formation. Kanamycin concentration of 100 mg/L caused all explants to become necrotic within two weeks of incubation of culture and killed almost all the type of explants. At 60 mg/L, kanamycin level produced highest transformation efficiency (45% and 40 % for embryo axis and cotyledon explants respectively) with normal growth of explants without any deleterious effect on both embryo axis as well as cotyledon explants while in case of young leaves even 40-60 mg/ml kanamycin also caused chlorosis and death of the explants. Thus Transformation efficiency was decreased when there was an increase in the kanamycin sulfate level beyond 60 mg/l. Thus higher concentration of kanamycin caused necrosis of explants. Accordingly, in all the experiments with transformation kanamycin concentration of 60 mg/L was used in multiplication and selection medium for the initial selection of transformants. A concentration of 40 mg/l kanamycin sulfate was used for selecting transformants in subsequent subculture to prevent possible escapes. Similar findings were also reported by Kar et al., (1996) in chickpea.

Table 2
Transformation frequency of groundnut var. GG20 as influenced by explant type and level of kanamycin sulfate.

Explant type	Kanamycin sulfate (rng/l)	No. of explants Co-cultured	No. of explants producing shoots	Transformation frequency (%)
Embryo axis	40	35	19	54.29
	60	35	16	45.71
	80	35	14	40.00
	100	35	8	22.86
Cotyledon	40	35	18	51.43
	60	35	14	40.00
	80	35	13	37.14
	100	35	3	8.57
Young leaves	40	35	11	31.43
	60	35	6	17.14
	80	35	4	11.43
	100	35	0	0.00

Confirmation of transformants
Phenotypic Expression:

Shoot growth was determined to assess the stress tolerance of the transgenic plants. There was a significant reduction in the growth of transformed plants even under non-stress conditions compared to control plants but this might be due to kanamycin-containing agar medium to select for only transgenic plants. Transformed plants which survived the selection pressure for two subcultures were inoculated in MS medium containing 100mM NaCl along with the control plants and incubated under standard growth condition. All transgenics and their corresponding untransformed controls suffered a growth reduction under conditions of salinity (100mM NaCl). This correlates with the results obtained in case of *Brassica napus* and *Arabidopsis* by Huang et al., (2000). Growth reduction due to salinity was less severe in the transformed plants than in the untransformed control (Fig-1) in presence of 100 mM NaCl in medium.

After 12 days of incubation it was observed that the control plants which were healthy and were green in color started showing sign of leaf burning and curling from the leaf margins while the transformed plants did not show such sign of burning of leaf edges

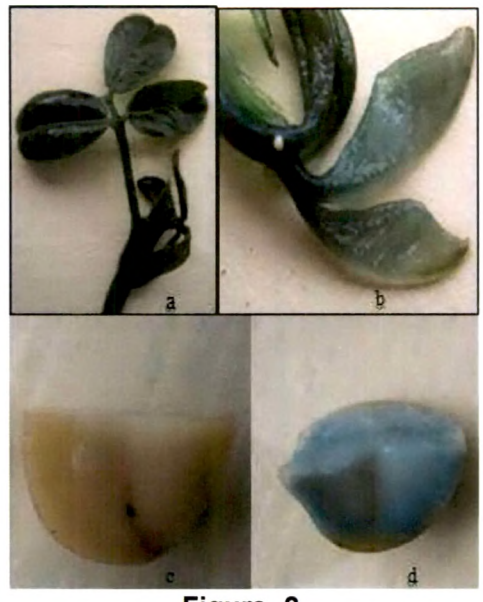


Figure -2.
X – gluc staining in the control and transformed tissues
a: control leaves with no blue staining, b: Transformed leaves with blue staining.c: control cotyledon with no blue staining, d: Transformed cotyledon with blue staining.

and remained healthy and continued to grow. This indicates the accumulation of glycine betain as a consequence of expression of *cox* gene in the transformed plants increased saline tolerance up to 100 mM level. Hayashi et al., 1998 also reported development of different level of saline tolerance in transgenic *Arabidopsis* transformed with the cholin oxidase gene from Arthrobacter globiformis (*cod* A).

GUS Expression

The whole regenerated plants along with cotyledons were subjected to in situ GUS assay. The expression of uidA gene was verified by histochemical staining of the leaf of the transgenic plants. The GUS positive regenerants showed the typical indigo blue colouration of X-Gluc treatment, while the control did not. Young leaves were more densely stained than other tissue of the plant and showed various GUS spots and GUS positive sectors on different leaf, shoot and cotyledon parts (Fig. 2). Both, leaves and the cotyledon parts of the transformants showed dense Gus positive sectors and not only GUS spots which indicate uniform transgene integration.

In conclusion, transgenic groundnut plants expressing cox gene were successfully generated through *Agrobacterium tumefaciens* mediated transformation conferring salinity tolerance.

ACKNOWLEDGEMENTS

Authors are thankful to Dr. Gopalan Selvraj, Plant Biotechnology institute, NRCC, Canada for providing the cDNA clone of *cox* (choline oxidase) gene and to the GSFC Science Foundation for funding the project.

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Transformation of *Agrobacterium Tumifascience* LBA 4404 with a Cholin Oxidase-Cox Gene Conferring Salinity Tolerance

Ashutosh Vadawale, Ritu Mihani, Asha Mathew and Pushpa Robin

Department of Biochemistry, Faculty of Science,
The Maharaja Sayajirao University of Baroda, Vadodara - 390 002. India

Abstract. Agrobacteium tumifascience strain LBA 4404 was transformed with the cholin oxidase gene through triparental mating. The growth of transformants was checked under appropriate antibiotic selection pressure. The successful tranformants were confirmed by the ketolactose test. The phenotypic expression of *cox* gene was checked by growing *Agrobacterium* and *Agrobacterium* transformants on medium containing different concentration of NaCl. The transformants were able to survive in presence of 300mM NaCl while non-transformants (control) could only grow and survive up to a NaCl concentration of 100 mM indicating that the tolerance capacity of the transformants was increased by 200mM as compared to control.

Keywords: Salinity, Cholin Oxidase, transformation

1. Introduction

Glycine betaine (GB) is one of the most potent compatible solutes which protect the cell machinery in plants against various kinds of stresses like salinity, drought, cold etc. (Hayashi et al 1998). Among the plants which can naturally produce GB, three pathways exist for its synthesis (Hayashi et al 1998). It starts with choline and proceeds through a reaction that involves one or two enzymes for the oxidation of choline to GB. Different enzymes catalyze the reaction in different organisms. The overall reaction is

Choline ----------→ Betaine aldehyde ----------→ GB
CMO / COX / CDH *COX / BADH*

CMO : Choline monooxygenase, COX :Choline oxidase, CDH Choline dehydrogenase, BADH : Betaine aldehyde dehydrogenase.

COX pathway has the advantage of being a single enzyme which can catalyze both steps and also it does not require any cofactor for catalysis (Sakamoto et al, 2001).

2. Materials and Methods

The gene *cox* (choline oxidase) gene was received as kind gift from Dr. Gopalan Selvraj, Plant Biotechnology institute, NRCC, Canada in the form of Binary vector pHS724 and 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.

3. Culture Maintenance

E. coli DH5α strains which was used for transformation of ligation mixtures and maintaining recombinant plasmids were maintained on Luria agar plates. *E. coli* DH5α bearing helper plasmid pRK2013 which was used as a helper strain for mobilizing a donor plasmid contained in another *E. coli* DH5α strain was maintained on Luria agar plates containing 50 μg/ml kanamycin as the plasmid pRK2013 has a kanamycin resistance gene. E.coli DH5α clones harbouring pHS724 recombinant plasmid were maintained on LA plates with 50 μg/ml kanamycin. *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.

4. Plasmid Extraction

Plasmid extraction was done by Alkaline Lysis method of Sambrook *et al.* (1989) (Miniprep). A single colony of transformed bacteria was inoculated in 5 ml of LB with antibiotic. The culture was incubated overnight at 37°C under vigorous shaking condition on shaker for a 24 hrs. Next day the culture was centrifuged at 12000 rpm for 10 min. The supernatant was discarded & 100 µl of ice cold ALS-I was added to resuspend the pellet by vortexing. 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. 200 µl ALS-III was added & mixed by inverting the tubes 5 times, tube was stored on ice for 15-20 min. 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. Centrifugation was done at maximum 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. 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

5. 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. A single bacterial colony was inoculated in 5 ml of LB and the culture was incubated overnight at 37°C with vigorous shaking. 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. The bacterial cells were transferred to sterile, disposable, ice-cold 50 ml polypropylene tubes. The cultures were cooled to 0°C for 10 min. Cells were recovered by centrifuging at 4500rpm for 10 min at 4°C. 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. Pellet was re-suspended by swirling or gentle vortexing in 30 ml of ice-cold $MgCl_2$-$CaCl_2$ solution. Cells were recovered by centrifuging at 4500rpm for 10min at 4°C. 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 re-suspended 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.

6. Transformation Using $CaCl_2$

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. The tube was transferred to a rack and kept in preheated 42°C water bath and incubated exactly for 90 sec without shaking. The tube was rapidly transferred to an ice bath to chill for 2 min. About 400-800 µl of LB medium was added accordingly to the tube and incubated for 45 min at 37°C. Appropriate volume of cells was plated onto pre-warmed LB plates with appropriate antibiotic. The plates were incubated at 37 °C for 16-18 hours.

7. Triparental Mating

Triparental mating was carried out using *E.coli* DH5α with pHS724 (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 as per the procedure described by Hoekema et al. 1983.Four days prior to 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. *E. coli* DH5α harboring pRK2013 & *E. coli* DH5α harboring the plasmid to be mobilized were streaked before one day to obtain a single colony on LB agar with 50µg/ml of Kanamycin. On the day of the triparental mating, a plate of Luria agar without any antibiotic was prepared. 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. 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. 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}. 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.

8. Results and Discussion

8.1. 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).

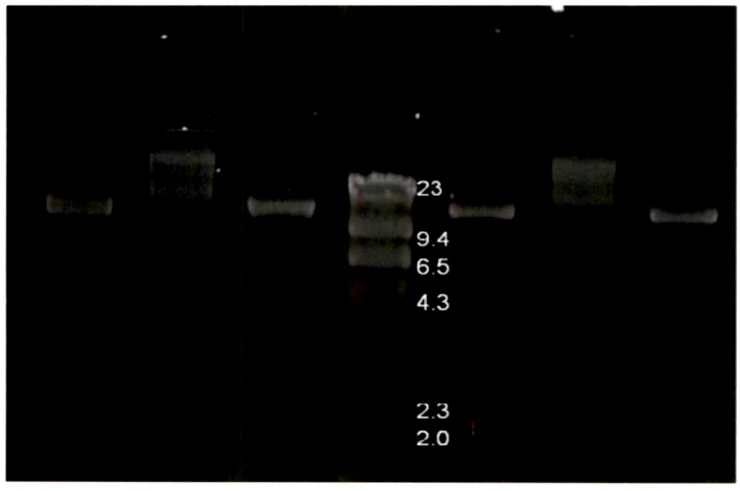

Fig. 1 : Confirmation of the plasmid by agarose gel electrophoresis.

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

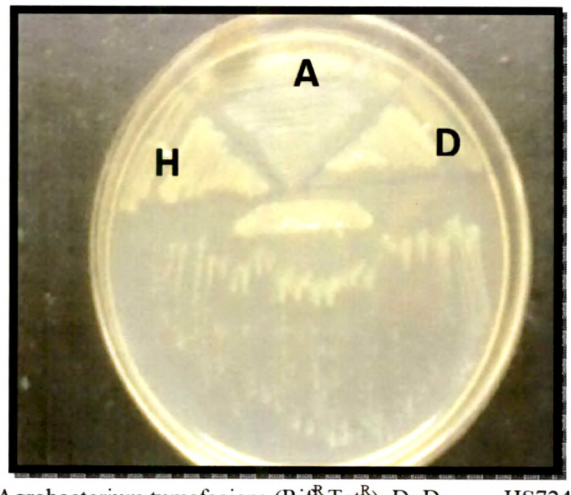

Fig. 2: Triparental mating (A: Agrobacterium tumefaciens (Rif^R Tet^R); D: Donor pHS724 (Kan^R)H: Helper strain (Kan^R)

8.2. Transformation of pHS724 into *Agrobacterium tumefaciens* LBA4404

Table 1: Resistance of plasmids to antibiotics

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

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 survived because kanamycin present in the medium did 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 tetracycline did not allow the growth of both *E.coli* DH5α pHS724 transformants & *E.coli* helper strain as they are sensitive to rifampicin and tetracycline though resistant to kanamycin.

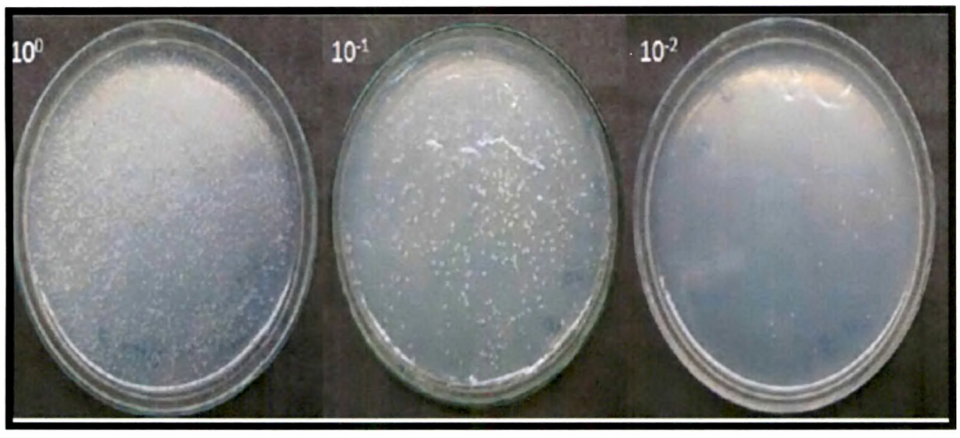

Fig. 3: *Agrobacterium tumefaciens* on LA plate

Table 2: Growth of transformants on Luria Agar 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

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. The *A. tumefaciens* (strain LBA 4404) transformation with the construct (pROK-ITCP17) has also been reported by triparental mating using a helper plasmid pRK 2013 by Raj et al. (2005).

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

The *A. tumefaciens*, ketolactose colony was confirmed through **Ketolactose/** Benedict's test (Bernaerts et al. 1963) which is a confirmatory test given positive only by *A. tumefaciens* but negative by *E.coli*. Therefore, a positive Benedict's test confirms the culture as *A. tumefaciens*. A culture giving positive ketolactose test showed 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 fig 4, a yellow colour was observed around the colony which confirmed that the culture was *A. tumefaciens*.

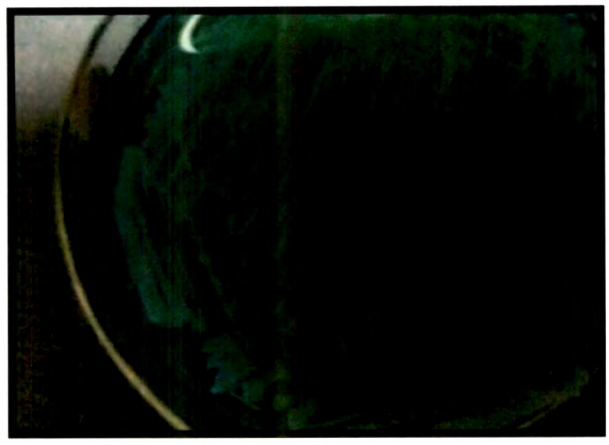

Fig 4: Ketolactose test

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

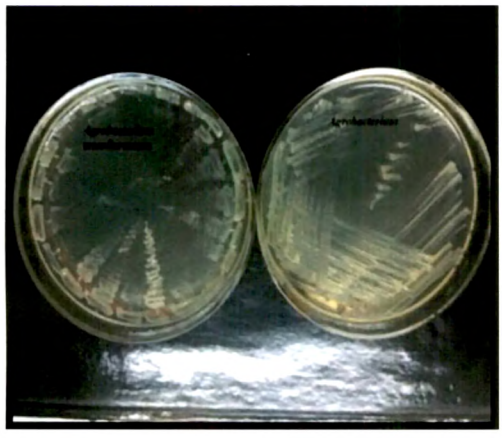

Fig 5a

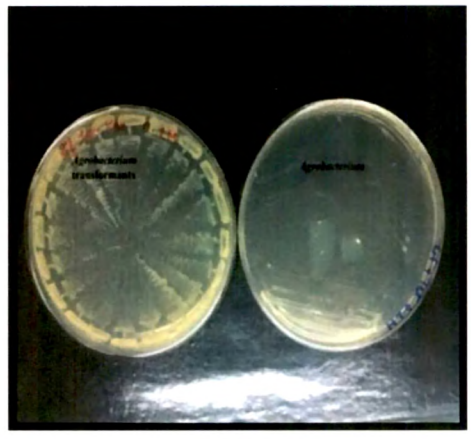

Fig 5b

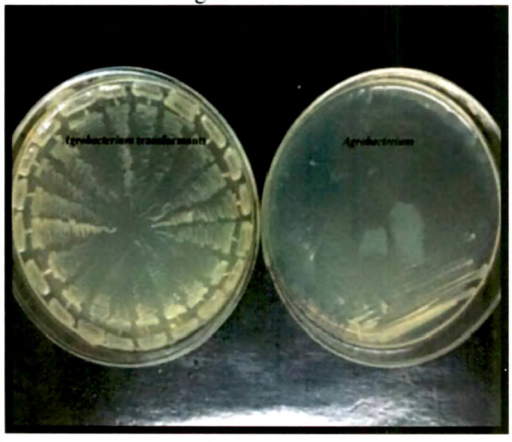

Fig 5c

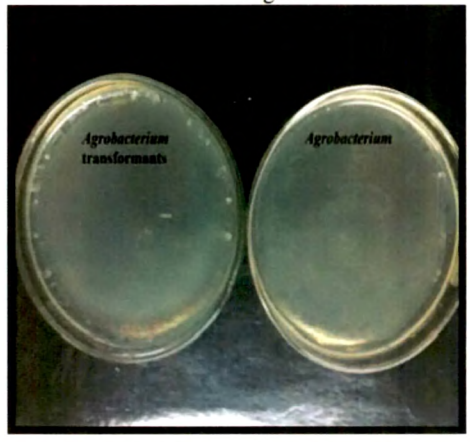

Fig 5d

Fig. 5. *Agrobacterium* and *Agrobacterium* transformants on a medium containing

a: 0.1M NaCl, b: 0.2M NaCl, c: 0.3M NaCl. d: 0.4M NaCl.

8.4. 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

9. Discussion

An attempt was made to incorporate the choline oxidase gene responsible for production of glycine betaine, an osmoprotectant into *Agrobacterium tumefascience*- LBA4404. 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, the expression of *cox* gene in the vector *E.coli* DH5α could not be observed. 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 mobilizing 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 salinity tolerance capacity of the transformants was increased by 200mM as compared to control. Our results matches with Deshnium et al.(1995) who also observed increased salinity tolerance up to 400mM NaCl in Synechococus when transformed with *codA* gene for cholin oxidase. Increased salinity tolerance up to 150 and 300mM NaCl has also been reported by Huang et al. (2000) in *Nicotiana tabacum* and *Brassica napus* when transformed with the choline oxidase gene.

In conclusion, the *Agrobacterium tumefaciens* transformants containing the cholin oxidase gene were successfully generated which can be utilized for further transformation in to variety of crop plants of economic importance for conferring salinity tolerance.

10.Acknowledgements

Authors are thankful to Dr. Gopalan Selvraj, Plant Biotechnology institute, NRCC, Canada for providing the cNDA clone of *cox* (choline oxidase) gene and to the GSFC Science Foundation for funding the project.

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