Studies on Development of Saline Tolerance in plants

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STATEMENT UNDER O. Ph.D. 8/ (iii) OF

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The work presented in this thesis is original and was obtained from the studies undertaken by me under the supervision of Dr. Pushpa Robin.

Place: Vadodara Ashutosh V Vadawale

Date:

This is to certify that the work presented in the form of a thesis in fulfillment of the requirement for the award of the Ph. D. degree of The M. S. University of Baroda by Mr. Ashutosh V Vadawale is his original work. The entire research work and the thesis have been built up under my supervision.

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.....Dedicated to My Parents

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LIST OF SYMBOLS

α	Alpha
β	Beta
µ	Micro (10^{-6})
^{0}C	Degree Celsius
%	Percent (age)
g	Gram
h	Hours
K	Kilo
l	Littre
m	Milli ($10^{-3)}$
mg	Milli gram
M	Molar
min	Minutes
s	Second
U	Unit
Fwt	Fresh Weight

LIST OF ABBREVIATIONS

ABA	Abscisic acid
ATPase	Adenosine triphosphatase
APX	Ascorbate peroxidase
ROS	Reactive oxygen species
NaCl	Sodium Chloride
SOS	Salt Overly Sensitive
GR	Glutathione reductase
OH^-	Hydroxyl radical
$^1O_2$	Singlet oxygen
O_2^-	Super oxide radical
H_2O_2	Hydrogen peroxide
DNA	Deoxyribose nucleic acid
Pro	Proline
GB	Glycine betaine
BADH	Betaine aldehyde dehydrogenase
COD	Choline oxidase
COM	Choline monooxygenase
CDH	Choline dehydrogenase
GST	Glutathione S-transferase
GPX	Glutathione peroxidase
GG20	Gujarat Groundnut 20
NaOCl	Sodium Hypochloride
$HgCl_2$	Mercuric Chloride

MS	Murashige and Skoog
B5	Gamborg vitamins
BAP	N6-benzylaminopurine
NAA	α-naphthalene acetic acid
kn	Kinetin
CN explant	Cotyledon explants
2,4 D	2,4, Di-chloro phynoxyacetic acid
CAT	Catalase
FT	Ferritins
MT	Metal lothioneins
GSH	Reduced glutathione
IAA	Indole-3-acetic acid
PCD	Programmed cell death
PTP	Permeability transition pore
EDTA	Ethylene Di-amine tetra acetic acid
Kan^R	Kanamycin resistance
Rif^R	Refampicne resistance
Tet^R	Tetracyclene resistance
NOS	Nopaline synthase
X-gluc	(5-bromo-4-choloro-3-indolyl) ß-D-glucuronic acid
NRCC	National Research Council of Canada

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REVIEW OF LITERATURE

1.0 Introduction

Salinity is one of the major constraints that limit agricultural production world-wide (Boyer, 1982). It is one of the most serious environmental factors limiting the productivity of crop plants (Ashraf, 1999). Nearly 40% of world's surface has salinity problems (Jadhav et al., 2010). At present, out of 1.5 billion hectares of cultivated land in the world, about 77 million hectares is affected by excess salt content (Evelin et al., 2009) of which 45 million hectare is irrigated and 32 million hectare land is non irrigated land (Munns, 2002). The affect of soil salinity on agricultural yield is enormous as it affects the establishment, growth and development of plants leading to huge losses in productivity (Mathur et al., 2007).
Also, Salinization is further spreading to irrigated land due to improper management of irrigation and drainage.

Salinity reduces the ability of plants to absorb water, causing rapid reductions in growth rate (Borsani et al., 2001a). High salt concentration in the soil decreases the osmotic potential of the soil solution and creates water stress for plants. Secondly, it causes severe ion toxicity since Na^+ is not readily sequestered in to vacuole. Eventually, the interactions of salts with mineral nutrition results in nutrient imbalance and deficiencies. Due to high salt concentration, processes such as seed germination, seedling growth and vigor, vegetative growth, flowering and fruit-set get adversely affected leading to plant demise as result of growth arrest and molecular damage (Sairam RK, 2011). Thus salinity affects most aspects of plant physiology.

1.1 Perception of salt stress

The ability of plants to combat environmental stress is determined by the efficiency of the plant to sense the environmental stress and activate its defense machinery. Plants perceive stress as ionic and osmotic stress. Excess Na^+ and Cl^- induced conformational changes in protein structure and

membrane depolarization can lead to the perception of ionic toxicity. Plasma membrane proteins such as Sln1 and Sho1 (Serrano et al., 2001), ion transporters, and/or Na+ sensitive enzymes such as SOS1 (Zhu, 2003) are the sensors of toxic Na+ concentrations in extra and intracellular cites.

1.1.1 Secondary Messengers involved in salt stress signaling

Ca^{+2} acts as signaling molecule in salt stress signaling and prevents Na^{+} entry in to cells (Knight et al.,1997). Thus Ca^{+2} plays important role in maintaining plant growth under saline condition inducing ion channel discrimination against Na^{+} (Schroeder et al., 2001). Cytocsolic Ca^{+2} oscillations during salt stress are regulated through activities of mechanosensitive and ligand-gated Ca^{+2} channels on plasma membrane, endoplasmic reticulum, and vacuole (Zhu, 2003). Excess Na^{+} induced membrane depolarization activate Ca^{+2} channels to generate Ca^{+2} signatures under salt stress. Salt stress also cause, an increased biosynthesis and also accumulation of abscisic acid (ABA) (Koornneef et al, 1998; Taylor et al, 2000) which plays very important role in osmotic stress tolerance (McCourt, 1999; Rock, 2000; Zhu, 2002). ABA is also involved in the control of ion homeostasis. For example, ABA content was higher in the leaves of the salt tolerant rice cultivar versus the susceptible cultivar. This increase in ABA content was accompanied by an improved K^{+} / Na^{+} ratio (Maathuis & Amtamann 1999). Also, the transport and accumulation of K^{+} in higher plant roots were regulated by ABA (Roberts, 1998). Recent reports indicate that ABA regulates K^{+} channel activity in maize and Arabidopsis roots, suggesting that ABA regulation of K^{+} transport in roots is, at least in part, ion channel-mediated (Roberts and Snowman, 2000).

Salt induced water stress leads to reduction in chloroplast stromal volume and generation of reactive oxygen species (ROS) which also plays an important role in inhibiting photosynthesis (Price and Hendry, 1991). ROS can be generated in the chloroplast by direct transfer of excitation energy from chlorophyll to produce singlet oxygen, or by univalent oxygen reduction at Photosystem I, in the Mehler reaction (Foyer et al., 2003)

1.1.2 Ion Homeostasis

High salt concentration in the external solution of plant cells produces several deleterious consequences. Salt stress causes an ionic imbalance (Niu et al.,1995; Zhu et al., 1997). When salinity results from an excess of NaCl, which is by far the most common type of salt stress, the increased intracellular concentration of Na^+ and Cl^- ions becomes deleterious to cellular systems (Serrano et al, 1999). In addition, the homeostasis of not only Na^+ and Cl^- , but also K^+ and Ca^{+2} ions is disturbed (Serrano et al, 1999; Hasegawa et al, 2000a, b).As a result, plant survival and growth will depend on adaptations that re-establish ionic homeostasis, there by reducing the duration of cellular exposure to ionic imbalance. Plants achieve ion homeostasis by restricting the uptake of toxic ions and maintaining the uptake of essential ions and by compartmentalization of toxic ions into the vacuole of specific tissue type. In most plants Na^+ is the primary cause of ion toxicity and therefore management of Na^+ concentration is critical for salt tolerance. Na^+ is kept below toxic level in the cytosol by 1) restriction of Na^+ entry at the root cortex cells, 2) excretion of Na^+ from root cells into the soil solutions 3)retrieval of Na^+ from the transpirational xylem stream to re-circulate in to root cells. 4) storage of Na^+ into vacuole of the mature cells 5) Na^+ excretion (Zhu, 2000). Among these mechanisms, Na^+ excretion through glands is significant only in halophytes.

Salt stress is sensed by putative salt stress sensors such as Salt Overly Sensitive 1 (SOS1) or two component Histidine kinase (HK1) proteins (Serrano, 2001). These sensors induce elevation of cytosolic Ca^{+2} concentration. A calcium sensor protein, Salt Overly Sensitive 3 (SOS3) perceives this Ca^{+2} signal and activates SOS2 protein kinase. Activated SOS2 phosphorylates SOS1, a plasma membrane Na^+ /H^+ antiporter, which then transports Na^+ out of the cytosol. The SOS3 dependent SOS2 kinase pathway positively regulates SOS1 transcript level and negatively regulate the Na^+ transporter HKT1. The SOS2 kinase, probably through SOS3 like Ca^{+2} Binding Protein (SCaBPs) dependent pathways, activates the tonoplast Na^+ /H^+ antiporter (NHX1) and vacuolar H^+/Ca^+ antiporter (VCX1), (Chinnusamy et al., 2005).

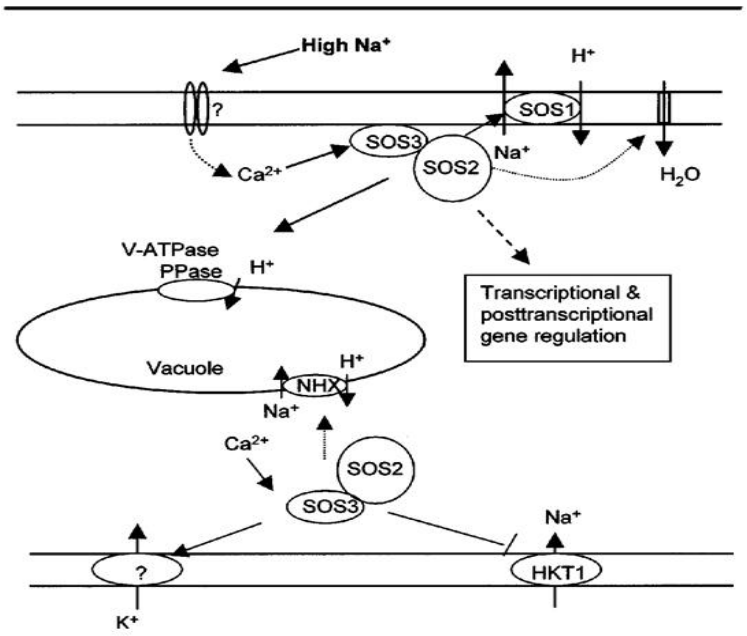


Fig.1.1 Regulation of ion (e.g., Na^+ and K^+) homeostasis by the SOS pathway. High Na^+ stress initiates a calcium signal that activates the SOS3-SOS2 protein kinase complex, which then stimulates the Na^+ /H^+ exchange activity of SOS1 and regulates transcriptionally and posttranscriptionally the expression of some genes. SOS3-SOS2 may also stimulate or suppress the activities of other transporters involved in ion homeostasis under salt stress, such as vacuolar H^+ -ATPases and pyrophosphatases (PPase), vacuolar Na^+ /H^+ exchanger (NHX), and plasma membrane K^+ and Na^+ transporters.

Figure taken from Zhu JK (2002) Salt drought stress signal transduction in plants.

1.1.3 Sodium uptake

Restriction of Na^+ entry in to root cell and then into the transpirational stream is very important to prevent the increase in the toxic level of salt in the shoot. Both glycophytes and halophytes must exclude about 97 % of Na^+ present in the soil at the root surface in order to prevent toxic levels of Na^+ accumulation in shoot (Munns, 2002). Sodium entry into transpirational stream depends on amount of Na^+ uptake by Na^+ and non specific cation transporter and the percentage of water entry in the apoplastic/bypass pathway in the xylem tissue. Na^+ from the soil solution gains initial entry in the root epidermis and the cortex. The casperian strip in the endodermis plays a crucial role in preventing apoplastic Na^+ influx into the root stele (Karahar et al., 2008)

1.1.4 Sodium Efflux

Sodium efflux from root cells is a frontline defense that prevents accumulation of toxic levels of Na^+ in the cytosol and Na^+ transport to the root. Plasma membrane Na^+/H^+ antiporters pump out Na^+ from root cells. In *Arabidopsis*, the plasma membrane Na^+ /H^+ antiporter, SOS1, mediates Na^+ efflux and its activity is regulated by SOS3-SOS2 kinase complex during salt stress (Zhu, 2003). Molecular analysis led to the identification of targets of the SOS3-SOS2 regulatory pathway. One of the target of the SOS pathway is SOS1. The SOS1 has significant protein sequence homology and conserved domains similar to that of plasma membrane Na^+ /H^+ antiporter from bacteria, fungi and animals. Expression of SOS1 is ubiquitous but stronger in epidermal cells surrounding the root tip and in parenchyma cells bordering the xylem. Expression of SOS1-GFP fusion protein and anti-SOS1 antibody confirmed that SOS1 is localized in the plasma membrane of root and leaf cells (Qiu, 2003). The SOS1 mutant plants show hypersensitivity to salt stress (100mM Na^+) and accumulate more Na^+ in roots than do the wild type plants (Shi, 2000).The Na^+ /H^+ exchange activity of SOS1 is regulated by SOS3-SOS2 complex under salt stress. Isolated plasma membrane vesicles from *sos3* and *sos2* mutants showed significantly less Na^+ /H^+ exchange activity than that of wild type plants.

1.1.5 Sodium compartmentation

Soil salinity decreases soil water potential which leads to osmotic stress. To maintain water uptake during osmotic stress, plants have evolved a mechanism known as osmotic adjustment. Osmotic adjustment is active accumulation of solutes such as inorganic ions(Na^+ and K^+) and organic solutes (proline, betaine, polyols and soluble sugars) (Binzel et al., 1987). Vacuolar sequestration of Na^+ is an important and cost effective strategy for osmotic adjustment as it enables at the same time to reduce the cytosolic Na^+ concentration during salinity. Vacuolar Na^+ /H^+ antiporters use the protein

gradient generated by vacouler H^+-adenosine triphosphate (H^+-ATPase) and H^+-inorganic pyrophosphate (H^+-PPase) for Na^+ sequestration in to the vacuole. Hence coordinated regulation of Na^+ /H^+ antiporters, H^+-ATPase and H^+- PPase is crucial for salt tolerance.

1.2 Oxidative Stress Management

High salt concentration also increases the production of reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$), super oxide radical (O_2^-), Hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH). Plants possess both enzymic and non enzymatic mechanism for scavenging of ROS. The enzymic mechanisms are designated to minimize the concentration of O_2^- and H_2O_2. The enzymes over produced include super oxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR) and glutathione synthesizing enzymes.

The super oxide radical is regularly synthesized in chloroplast (Elstner et al., 1991) and mitochondria (Rich et al., 1978), although some quantity is also reported to be produced in microbodies (Lindquist et al., 1991). Scavenging of O_2^- by SOD results in production of H_2O_2 , which is removed by ascorbate peroxidase (Asada, 1992) or catalase (Scandalios et al., 1990). However both O_2^- and H_2O_2 are not as toxic as the hydroxyl radical (OH^-), which is formed by combination of O_2^- and H_2O_2 in the presence of trace amount of Fe^{2+} and Fe^{3+} by Haber-Weiss reaction (Haber et al., 1934). The hydroxyl radical can damage chlorophyll, protein, DNA, lipids and other important macromolecules, thus fatally affecting plant metabolism and ultimately growth and yield.

1.3 Synthesis of osmolytes

In plants, a common response to osmotic stress is the accumulation of compatible osmolytes such as proline (Pro), glycine betaine (GB) and sugar alcohols. It has been suggested that compatible osmolytes do not interfere

with normal biochemical reactions and act as osmoprotectans during osmotic stress. Proline is the most common osmoprotectant that accumulates in plants in response to water stress and salinity (Hanson and Hitz, 1982; Yoshiba et al., 1995).

1.4 Halophytes

The halophytes characteristically posses the capacity to grow under high concentrations of NaCl. The biochemical mechanisms leading to salt tolerance in halophytes are regulated in such way that allow a more successful response to salt stress than in other plants (Hasegawa et al., 2000a, b). However, it remains to be tested that whether the mechanisms employed in salt stress tolerance by halophytes can be employed by glycophytes without a loss in productivity or not. The halophytic land plant M. crystallinum has been frequently used as model plant in salt tolerance studies. This plant is now being used in the identification of ESTs differentially expressed after plant salt exposure (Bohnert et al., 2001). A problem in the use of most halophytes is the identification of gene by the use of a genetic approach (i.e. searching for salt hypersensitive mutants). The halophyte plant-*Thellungiella halophila* can survive at seawater-level salinity and its DNA sequence have a similarity of more than 90% of Arabidopsis (Zhu, 2001). Thus Arabidopsis, a closely related species can also be easily transformed allowing insertion tag mutagenesis (Bressan et al., 2001).

1.5 Glycophytes

All major crop plants are glycophytes and the study of these plants as model organisms would uncover processes related to salt tolerance that are specific to these plant species. Of all the glycophytes, Arabidopsis has most often been useful in the determination of processes involved in salt tolerance. Genetic analysis using Arabidopsis as a model is leading to a deeper knowledge of a key signal transduction pathway in salt tolerance, such as the SOS pathway, critical for salt tolerance (Hasegawa et al., 1994; Zhu, 2000).

Another glycophyte employed in genetic analysis using mutagenesis is tomato (Borsani et al., 2001a). Tomato is a widely distributed annual vegetable crop adapted to a large variety of climates. However, in spite of its broad adaptation, production is concentrated to a few warm and rather dry areas (Cuartero and Fernandez-Munoz, 1999). In these areas with an optimal climate for tomato production, salinity is a serious problem (Szabolcs, 1994). For this reason, a large number of physiological studies on salt stress have been performed using tomato as a model plant (Cuartero and Fernandez-Munoz, 1999). Unlike Arabidopsis, direct studies on salinity, adaptation, and molecular changes in this plant can be assessed also for crop yield.

1.6 Identifying determinants for salt tolerance

The first approach employed to ascertain salt tolerance determinants in plants was to identify the metabolic processes critical to tolerate NaCl. Establishment of ion homeostasis is an essential requirement for plants to survive under salt stress conditions. Plant cells respond to salt stress by increasing Na^+ efflux at the plasma membrane and Na^+ accumulation in the vacuole. Therefore, proteins, and ultimately, genes involved in these processes can be considered as **salt tolerance determinants**. Salt tolerance requires not only adaptation to Na^+ toxicity but also the acquisition of K^+ whose uptake by the plant cell is affected by high external Na^+ concentration. The uptake of K^+ is affected by Na^+ due to the chemical similarities between both ions. Potassium is an essential nutrient being the major cationic inorganic nutrient in most terrestrial plants. Therefore, K^+ transport systems involving good selectivity of K^+ over Na^+ can also be considered as an important **salt tolerant determinant** (Rodriguez-Navarro, 2000).

Another metabolic response to salt stress is the synthesis of compatible osmolytes. These organic compounds are thought to mediate osmotic adjustment, protecting sub-cellular structures and oxidative damage by their free radical scavenging capacity (Hong et al., 1992; Smirnoff, 1993; Hare et

al.,1998). Thus, expression of genes regulating the accumulation of these organic compounds can be considered as **salt tolerant determinants**.

1.7 Mechanism of salt tolerance

A common approach used to determine mechanisms of salt tolerance is the identification of cellular processes and genes whose activity or expression is affected by salt stress (Bray, 1993; Botella et al., 1994; Zhu et al., 1997; Hasegawa et al., 2000a, b). Identification of these salt-regulated genes has allowed a better understanding of the complexity of salt tolerance in higher plants (Bray, 1993, Zhu et al., 1997, Serrano et al., 1998, Hasegawa et al., 2000a, b). The generalized assumption was that a gene regulated by salt stress would probably be important in tolerance. Many of these salt-regulated genes have been reported but the most direct procedure to demonstrate their importance in salt tolerance is through functional genetic analysis. This approach is not easily accomplished in crop plants. The genetic identification of mechanisms involved in K^+ nutrition as a process that is critical for salt tolerance in Arabidopsis and tomato suggests that despite the different degree of salt tolerance among these plant species, they share common mechanisms.

These mechanisms include, in addition to K^+ nutrition, synthesis of compatible osmolytes, Na^+ and Cl^- exclusion and compartmentation, increase of toxic radical scavenging capacity and increase in the content of the phytohormone ABA. Besides these common mechanisms, it is clear that glycophytes have the genes necessary to tolerate salt stress but probably the salt tolerance determinants are properly expressed only after adaptation to stress (Hasegawa et al., 1994; Zhu, 2000).

High concentrations of salt impose a hyperosmotic shock by decreasing the chemical activity of water and causing loss of cell turgor. This negative effect in the plant cell is thought to be similar to the effects caused by drought.

1.8 DEVELOPMENT OF SALT TOLERANCE IN PLANT

Despite the 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. Classic 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 in crop plants by traditional methods (Foolad and Lin, 1997). This situation is complicated by the fact that generally the main character selected in crop plants is productivity, which is also a complex trait. Therefore, the integration of genes required to increase salt tolerance in a specific genotype is difficult without affecting other important multigenic traits like flowering, fruit quality and dry matter production (Flowers et al., 2000).

Plants respond to salt stress at three different levels, i.e., cellular, tissue and whole plant level. Cell-based mechanisms of ion homeostasis and the synthesis of osmoprotectants are essential determinants for salt tolerance. However, as plant cells become specialized during ontogeny, the adaptive mechanisms to tolerate salt stress may be different. Integration and coordination of the responses of cells, tissues, and organs are required for a proper tolerance to salt stress. However, the separate study of each level of response is required to understand the whole picture of salt tolerance.

Moreover, because salt tolerance is regulated throughout the plant development and yet could be a tissue-specific phenomenon, plant tolerance responses at one stage of development may not necessarily be the same at other stages (Johnson et al., 1992; Lauchli and Epstein, 1990). Therefore, the mechanisms of tolerance at specific stages of plant development is required to be studied in order to understand the biochemical events that play important roles in the responses to salt stress (Borsani et al., 2001a).

Therefore, given all the factors determining the pleitropic deleterious effects of salt stress, the adaptation to salinity may involve the modification of a large number of parameters.

1.9 Increasing plant salt tolerance through genetic engineering.

Numerous reports in the literature have shown improvement of salt tolerance via genetic engineering. Genes employed in these studies have been isolated from a number of organisms, ranging from prokaryotic organisms such as E. coli to halophytes or glycophytes (Table 1.1). The improvement of tolerance to salt stress has been analyzed in different plant species, different stages of development and by using different evaluation criteria. These aspects make it extremely difficult to compare the improvement conferred by different genes in salt tolerance. But, the metabolic pathways and mechanisms modified to improve the salt tolerance can be grouped according to the genes employed in transgenic experiments. These genes can be classified into five groups according to their functions:

- Ion homeostasis,
- Oxidative stress,
- Synthesis of osmolytes,
- Protection of cell integrity
-Transcription factors.

1.9.1 Genes involved in Ion homeostasis.

An important approach to generate plant salt tolerance is the introduction of genes that modulate ion transport systems such as *HAL1* and *HAL3*, which are involved in the regulation of K+ and Na+ transport, respectively (Gisbert et al., 2000; Yang et al., 2001). Overexpression of HAL1 from *Sachharomyces cerevisiae* in tomato improved salt tolerance by maintaining a high internal K^+ concentration and decreasing intracellular Na+ during salt stress (Gisbert et al., 2000). The results are similar to those already described when HAL1 when overexpressed in yeast (Serrano et al., 1998) or other plants species

such as Arabidopsis (Yang et al., 2001) and melon (Bordas et al., 1997). This suggests that the mechanisms controlling the positive effects of the HAL1 gene on salt tolerance are conserved among plants and yeast. The yeast HAL3 gene encodes a FMN-binding protein. Also overexpression of AtHal3, the *A. thaliana* HAL3 gene orthologue, in Arabidopsis improves salt tolerance (Albert et al., 2000).

Abundant experimental evidence has shown the involvement of cytosolic Ca^{+2} in salt stress signaling (Lynch et al., 1989). The PP2B phosphatase calcineurin (CaN) is a critical component of a Ca^{+2} dependent signal transduction pathway that mediates Na^{+} , Li and Mn tolerance in S. cerevisiae. A truncated activated form of the catalytic subunit and the regulatory subunit of yeast CaN were coexpressed in transgenic tobacco plants to reconstitute an active phosphatase *in vivo* (Pardo et al., 1998). The enhanced capacity to survive Na^{+} shock of plants expressing this gene was similar when the evaluation was conducted either on seedling in tissue culture raft vessels or plants in hydroponic cultures (Pardo et al., 1998). The importance of calcium in salt tolerance was also determined by the use of transgenic rice overexpressing the calcium-dependent protein kinase, OsCDPK7. These plants were able to avoid the wilting phenotype observed in control plants exposed to 200 mM NaCl (Saijo et al., 2000). Overexpression of OsCDPK7 enhanced the induction of RAB16 and WSI18 genes that encode group 2 and group 3 LEA related proteins respectively (Saijo et al., 2000). Plant cells are structurally well suited for the sequestration of ions because of the presence of large, membrane-bound vacuoles. Overexpression of a vacuolar Na^{+} /H^{+} antiporter from *A.thaliana* (AtNHX1) in Arabidopsis plants promoted sustained growth and development in soil watered with up to 200 mM NaCl (Apse et al., 1999). Tomato plants overexpressing the same gene were able to grow, flower, and produce fruit in the presence of 200 mM NaCl (Zhang and Blumwald, 2001). Surprisingly, these plants showed high accumulation of Na^{+} in leaves but low Na^{+}content in the fruit. There is also a report on transgenic *Brasssica napus* plants overexpressing the AtNHX1 gene which were able to grow, flower, and produce seeds in the presence of 200

mM of NaCl (Zhang et al., 2001). The seed production and seed oil quality were reportedly unaffected by the soil salt content.

1.9.2 Genes involved in Oxidative Stress Management.

Salinity generates an increase in reactive oxygen species that can induce deleterious effects on cell metabolism (Polle, 1997; Borsani et al., 2001b). Various groups have developed plants that overexpress several oxidative-stress-related genes, with varied results, depending on the tests used to evaluate these transgenic plants (Bajaj et al., 1999). Transgenic plants overexpressing ROS-scavenging enzymes such as superoxide dismutase - SOD (Alscher et al.,2002) ascorbate peroxidase (APX) (Wang et al., 1999) and glutathione S-transferase/glutathione peroxidase (GST/GPX) (Roxas et al., 1997,2000) showed increased tolerance to osmotic, temperature and oxidative stress. The overexpression of the tobacco *NtGST/GPX* gene in transgenic tobacco plants improved salt- and chilling-stress tolerance because of enhanced ROS scavenging and prevention of membrane damage (Roxas et al., 1997,2000).

The potential role of SOD in the protection against salt stress was examined using transgenic rice plants (Tanaka et al., 1999). At high salinity, the transgenic plants had an ascorbate peroxidase activity about 1.5-fold higher than control plants. Total SOD activity was maintained at a high level and ascorbate peroxidase increased upon salt stress. It was found that the PS II activity and the electron transport in the chloroplast were higher in the transgenic plants compared to the wild type plants under salt stress. These results suggest that an increase in the levels of ascorbate peroxidase and chloroplastic SOD are important factors for salt resistance in rice (Tanaka et al., 1999).

1.9.3 Genes involved in Synthesis of osmolytes

The common ubiquitous mechanism in plants to tolerate salinity is the accumulation of certain organic metabolites of low molecular weight that

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). The major role of these metabolites is to serve as organic osmolytes with compatible properties at high concentrations; such osmolytes increase the ability of cells to retain water without disturbing normal cellular functions (Yancey et al., 1982). Genetic engineering to increase levels of these compatible solutes is the promising approach in efforts to increase the ability of plants to tolerate environmental stress.

One of the important molecule that can act as osmoprotectant is the polyalcohol mannitol. Tobacco plants transformed with the *mtlD* gene from *E. coli*, which encodes a mannitol 1-phophate dehydrogenase accumulated mannitol and showed increased plant growth under salt stress (Tarczynski et al., 1993). However, later studies revealed that the increase in mannitol content was not enough to explain the tolerance solely based on osmotic adjustment and the scientists assigned to this molecule a possible antioxidant function too (Karakas et al., 1997). Arabidopsis plants transformed with the same gene were able to germinate in the presence of a concentration of salt inhibitory to wild type plants. However, unlike tobacco *mtlD* transformants, Arabidopsis transgenic plants did not tolerate prolonged salt stress (Thomas et al., 1995).

Ectopic expression of a gene encoding myo-inositol O-methyltransferase (IMT1) in tobacco resulted in the accumulation of methylated inositol (D-ononitol), that conferred higher salt tolerance by increasing the photosynthetic activity (Sheveleva et al., 1997). The accumulation of this compound reached up to 600 mM in the cytosol, thus osmotically balancing the high external Na^+ concentration. Arabidopsis plants expressing the enzyme that catalyzes the first committed step in inositol biosynthesis, the D-myo-inositol-3-phosphate (*Ins3P*) synthase, exhibit an increased level of free inositol (Smart and Flores, 1997). Despite a slight increase in salt tolerance, the authors could not detect significant

differences in the phenotype of transgenic plants when a number of characteristics linked to functions of inositol and inositol-derived metabolites were analyzed. These results suggest that the engineering of inositol metabolism to generate salt tolerance may require the manipulation of several genes.

Osmoprotectant	**Gene source**	**Enzyme or gene**	**Plant species engineered**	**Promoter used**	**Product level (%) a**	**References**
GlyBet	*E. coli*	CDH	Tobacco	35S	ND	Lilius et al., (1996)
	E. coli	CDH+BADH	Tobacco	RbcS[c]	1	Holmstrom et al., (2000)
	Arthrobacter	COX	Arabidopsis	35S	5	Hayashi et al., (1997)
	Arthrobacter	COX	Rice,Arabidopsis	35S	5-25	Sakamoto et al., (1998)
	Arthrobacter	COX	Canola ,Tobacco	35S	5-10	Huang et al., (2000)
	SpiNa+ h	CMO	Tobacco	35S	1	Nuccio et al.,(1998)
	SpiNa+ h, beet	CMO+BADH	Tobacco	35S	1	Nuccio et al., (2000a)
Proline	Mothbean	P5CS or deregulated P5CS	Tobacco	35S	100-200	Kishor et al., (1995) & Hong et al., (2000)
Ectoine	Halomonas	ectA+ectB+ectC	Tobacco cells	35S	1	Nakayama et al., (2000)
Mannitol	*E. coli*	MtlD	Tobacco, Arabidopsis	35S	16	Tarczynski et al.,(1992) Thomas et al., (1995)
Sorbitol	Apple	Stpd1	Tobacco	35S	1-260	Tao et al., (1995) Sheveleva et al., (1998)
d-Ononitol	Ice plant	Imtl	Tobacco	35S	10-70	Vernon et al., (1993) Sheveleva et al., (1997)
Trehalose	*E. coli*	TPS or TPS+TPP	Tobacco	35S	1-2	Goddijn et al.,(1997) Pilon-Smits et al., (1998)
	E. coli	TPS or TPS+TPP	Potato tuber	Patatin	1-24	
	Yeast	TPS1	Tobacco	RbcS	5-19	Holmstrom et al., (1996)
	Yeast	TPS1	Tobacco	35S	1	Romero et al., (1997)

a :This column reports the level of osmoprotectant synthesis in osmotically stressed transgenic plants as a percentage of the level found in a representative organism that naturally accumulates the osmoprotectant under similar conditions (Nuccio et al., 1999). ND, not determined

Table 1.1: Examples of Metabolic Engineering of Osmoprotectants in Higher Plants (Rontein et al., 2002)

1.10 Synthesis of Glycine Betaine

Out of various compatible solutes mentioned, GB is by far the most common in the plant kingdom. Plants, which naturally accumulate GB to significant levels, are able to survive severe salt (~300 mM NaCl) and drought stress. (Hayashi et al., 1998).

Betaine is a dipolar but electrically neutral molecule at physiological pH. With respect to protection against osmotic stress, betaine is regarded as being a particularly effective compatible solute (Le Rudulier et al., 1984). The action of betaine *in vivo* is not, however, confined to osmoregulation. Numerous experiments *in vitro* have indicated that betaine acts as an osmoprotectant by stabilizing both the quaternary structure of proteins and the highly ordered structure of membranes against the adverse effects of high salinity and extreme temperatures (Gorham, 1995). In photosynthetic systems, for example, betaine efficiently protects various components of the photosynthetic machinery, such as ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) and the oxygen evolving photosystem II (PSII) complex, from salt induced inactivaation and dissociation into subunits

Spp	Enzyme/Gene (Source)	Promoter	Subcellular Targeting	Max. [GB]	Type of Stress	Evaluation of tolerance	Reference
Synechococcus	COD/codA (A.globiformis)	conIIc	Cytoplasm	80 mM	0.4 M Na+ 1/8 d	Growth/Photosynthesis/Chlorophyl	Deshniu m et al., (1995)
	CDH, BADH/	Native	Cytoplasm	50 mM	0.4 M Na+ 1/3 d	Growth	Nomura et al., (1995)
Arabidopsis	COD/codA (A.globiformis)	35S	Chloroplast	1.2 mmol / g fresh wt	0.2 M Na+l/5 d	Growth tolerance	Hayashi et al., (1997)
	COD/cox (A. pascens)	2335 S	Cytosol	19 mmol / g dry wt	0.1 M Na+ 1/23 d	Growth tolerance	Huang et al., (2000)
O. sativa	COD/codA (A.globiformis) fresh wt	35S	Chloroplast	1.1 mmol / g fresh wt	0.15 M Na+ 1/7 d	Growth tolerance	Sakamoto et al., (1998)
	COD/codA (A.globiformis)	35S	Cytosol	5.3 mmol /g fresh wt	0.1 M Na+ 1/23 h	Photosynthesis tolerance evaluated	Sakamoto et al., (1998)
N. tabacum	COD/cox (A. pascens)	2335 S	Cytosol	13 mmol /g dry wt	0.15 M Na+ l	Growth tolerance evaluated	Huang et al., (2000)
	CDH/modified betA (E. coli)	RbcSe	Cytosol	0.035 mmol /g fresh wt	0.15 M Na+ 1/14 d)	Growth tolerance evaluated	Holmstro Èm et al., (2000)
	CMO/not assigned (SpiNa+ ia oleracea)	35S	Chloroplast	0.05 mmol / g fresh wt			Nuccio et al., (1998)
Brassica napus	COD/cox (A. pascens)	35S	Cytosol	13 mmol g21dry wt	0.3 M Na+ 1/10 d)	Growth & Photosynthesis tolerance evaluation under stress	Huang et al., (2000)

Table 1.2. Summary of transgenic cyanobacteria and plants engineered to synthesize GB and evaluation of their stress tolerance (Sakamoto et al., 2001)

The accumulation of betaine in response to salt, drought and cold has been widely recognized in higher plants that are natural accumulators of this compound (Gorham,1995). A positive correlation exists between the accumulation of betaine and the acquisition of tolerance to salt and cold in maize (*Zea mays*) and barley (*Hordeum vulgaris*), respectively (Rhodes et al.,1989; Kishitani et al., 1994) . Genetic evidence also indicates that betaine improves the salt tolerance of these members of Gramineae (Saneoka et al., 1995). Moreover, exogenous application of betaine to leaves or roots has been shown to increase the tolerance to various stresses of several species of plants, including both natural accumulators and non-accumulators (Allard et al., 1998; Hayashi et al., 1998).

In most organisms including plants, betaine is synthesized as a result of the two-step oxidation of choline via betaine aldehyde, a toxic intermediate (Fig. 1.2). In several higher plants from taxonomically unrelated families, the relevant enzymes are choline monooxygenase (CMO), a ferredoxin dependent soluble Rieske-type protein, and betaine aldehyde dehydrogenase (BADH; EC 1.2.1.8), a soluble NAD+-dependent enzyme (Weigel et al., 1986; Brouquisse et al., 1989). These enzymes are found mostly in the chloroplast stroma and their activities, as well as increase levels of betaine, in response to salt stress.

BADH has also been found in several plants that barely accumulate any betaine (Weretilnyk et al., 1990). In mammalian cells and in microorganisms such as *Escherichia coli*, betaine is synthesized by choline dehydrogenase (CDH; EC 1.1.99.1), a membrane-bound oxygen-dependent enzyme, in combination with BADH (Landfald and Strùm, 1986). In contrast to these two pathways that each involve two enzymes, the biosynthesis of betaine is catalysed by a single enzyme, choline oxidase (COD; EC 1.1.3.17), in certain microorganisms, such as the soil bacterium *Arthrobacter globiformis* (Ikuta et al., 1977; Fig. 1.2).

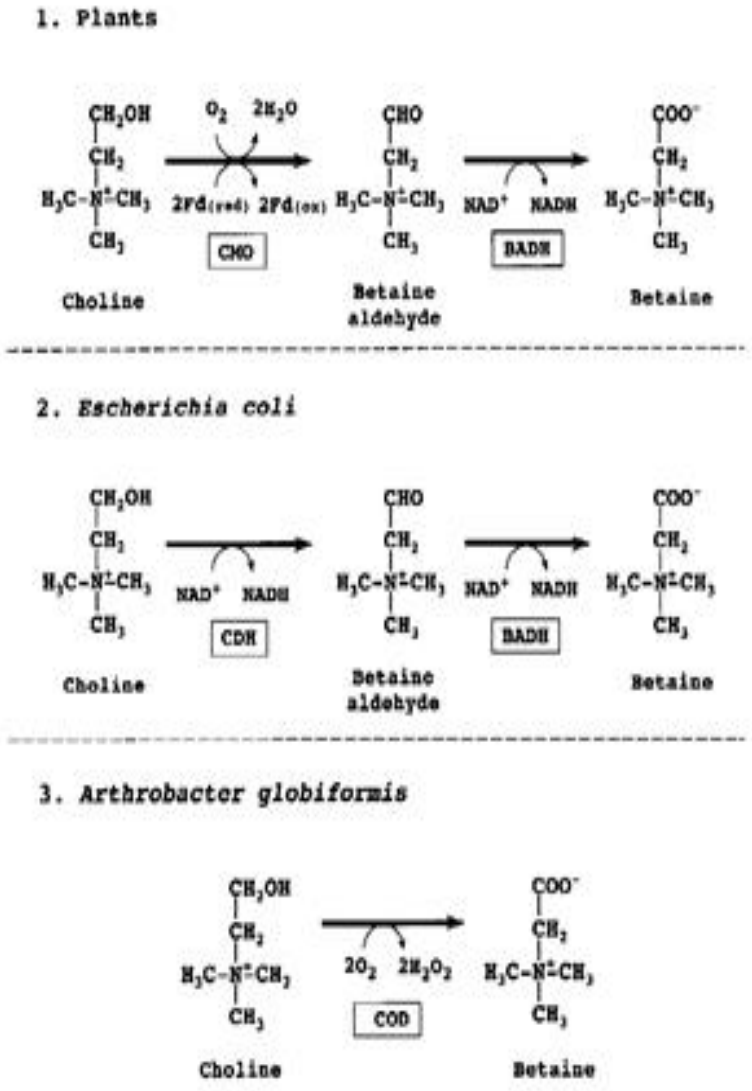


Fig.1.2 Biosynthesis of betaine

The introduction by molecular genetic manipulation of biosynthetic pathways involved in synthesis of betaine has been attempted in non-accumulating species of cyanobacteria (Deshnium et al., 1995; Nomura et al., 1995) and in higher plants (see Table 1.1). Such transgenic plants would hence acquire saline tolerance.

The relevant gene is introduced under the transcriptional control of a strong promoter that ensures high-level expression of the gene in transgenic plants (e.g., the constitutively active promoter of the gene for 35S rRNA from cauliflower mosaic virus or the light-inducible promoter of a gene for the small subunit of Rubisco). For appropriate localization of enzymes of prokaryotic origin, the gene has often been modified so that the encoded polypeptide is transported post-translationally into the chloroplasts of the engineered plants. The transit peptide of the small subunit of Rubisco has been used as a signal for such transport.

1.11 Genetic engineering using the gene for choline oxidase from Arthrobacter

The CODA gene isolated from the soil bacteria *Arthrobacter globiformis*, encodes choline oxidize. The major advantage of using COD, instead of CMO/BADH or CDH/BADH, as a tool for engineering the synthesis of betaine is that the introduction of only a single gene (CODA) for this enzyme is sufficient for the conversion of choline to betaine in transgenic plants. Transformation of *A. thaliana* with codA enabled the plant to accumulate glycinebetaine and enhanced its tolerance to salt stress (Hayashi et al., 1997). Seeds of the transgenic plants were able to germinate in 300 mM NaCl whereas seeds of wild type did not germinate at all. In addition, transgenic plants had retained wild type Photosystem II activity under salt stress conditions. Transgenic rice plants carrying the codA gene with the encoded protein directed to the chloroplast were more tolerant than the transgenic plants with the protein localized in the cytosol. This result suggested that the protective function of glycine betaine is more efficient when produced in a photosynthetic organelle (Sakamoto et al., 1998). An *Arthrobacter pascens* gene encoding a COX enzyme was used to generate transgenic plants in three different species: *Arabidopsis*, *Brassica napus* and tobacco (Huang et al., 2000). Salt tolerance varied among the species and the

authors could not assign this difference in tolerance to the different glycine betaine accumulation levels. Transgenic tobacco plants expressing the BADH gene accumulated a higher amount of glycine betaine in cytosol and chloroplasts and exhibited increased tolerance to salt stress (Holmstrom et al., 2000). These transgenic plants also showed decreased photoinhibition during salt stress and this caused an increase in fresh weight relative to wild type. Similar results were obtained in transgenic tobacco plants over-expressing a BADH from spinach instead of a BADH prokaryote gene (Sakamoto et al., 1998).

While genetic engineering has allowed engineered plants to produce betaine, there are considerable differences in levels of betaine, on a fresh weight basis, between transgenic plants (0.05±5 mmol g-1 FW) and natural accumulators under stress conditions (4 ±40 mmol g-1 FW) (Rhodes and Hanson, 1993). The amounts of betaine accumulated suggest that the enhancement of stress tolerance can hardly be attributed to any action of betaine that involves osmotic adjustment to the external environment. A major role of betaine might be to protect membranes and macromolecules from the damaging effects of stress. It is also possible that betaine might be compartmentalized within cells such that, at certain sites, the concentration of betaine might be high enough to confer substantial protection against stress even when the overall level of accumulation is low. Such a possibility is supported by the observations that betaine is concentrated exclusively in the cytoplasm, and not in the vacuoles, of leaves of salt-grown halophytes (Matoh et al., 1987) and levels of betaine as low as 5 mmol g-1 FW can protect some natural accumulators from the damaging effects of stress (Arakawa et al., 1990; Ishitani et al., 1993). Other roles of betaine in cells under stress have also been studied. Betaine destabilizes double-helixed DNA and lowers the melting temperature of DNA in vitro. Thus, betaine might play a role in promoting transcription and replication under high-salt conditions (Rajendrakumar et al., 1997). The physiological characterization of transgenic plants (Alia et al., 1999) suggested that betaine might accelerate protein synthesis de novo during recovery from stress. Such possibilities require further detailed examination in future studies Glycine

betaine accumulates in cells of a number of halophytes and bacteria as an adaptive response to high salt.

The possible side effects of the introduction of the gene for COD were examined since the enzyme produces hydrogen peroxide as a by-product of catalysis (Alia et al., 1999). Leaves of transgenic Arabidopsis that expressed COD did have elevated levels of hydrogen peroxide, but the increase were comparable to levels in wild-type plants under stress and non-stress conditions (Alia et al., 1999). Moreover, the activities of enzymes that are responsible for scavenging hydrogen peroxide, namely ascorbate peroxidase and to a lesser extent, catalase, were significantly higher in transgenic plants than in wild-type plants (Alia et al., 1999). These observations suggest that the hydrogen peroxide generated by choline oxidase might have stimulated the expression of scavenging enzymes with the resultant maintenance of intracellular levels of hydrogen peroxide within a certain limited range. Expression of choline oxidase (COD) from Arthrobacter transgenic rice plants were achieved in a fashion similar to the transgenic Arabidopsis plants as described above with COD targeted either to the chloroplasts or to the cytosol. In the former case, betaine accumulated at about 1 mmol g-1 FW in leaves; in the latter plants, it accumulated at about 5 mmol g-1 FW (Sakamoto et al., 1998). Transgenic rice plants with COD targeted either to chloroplasts or to the cytosol exhibited enhanced tolerance to salt or cold induced photoinhibition. Moreover, the photosynthetic machinery was more efficiently protected when COD was targeted to the chloroplasts than to the cytosol of transgenic rice (Sakamoto et al., 1998).This observation indicates that the subcellular site of betaine synthesis might be important in efforts to improve the stress tolerance of plants.

1.12 Aim and scope of the study

In the present thesis, we proposed to undertake studies on the development of saline tolerance in plants with the specific emphasis on synthesis of glycine betaine in the plant of interest through genetic transformation and its subsequent effect on salt tolerance.

The plants of interest for the studies is specifically selected considering regional importance and agro-climatic suitability. The plants under consideration for the studies is Ground nut which is a major cash crop of Gujarat. It is rich source of protein, oil, and fodder and is among the 15 leading food crop of the world. India is the largest producer of groundnut and contributes around 29 % of the total world production. Ground nut has not been specifically transformed for saline tolerance till now.

1.12.1 Work plan for present investigation

1. To study and understand the natural saline tolerances of ground nut.
2. To standardize regeneration protocol using different parts of groundnut plant.
3. To study various biochemical parameters of groundnut under different levels of salinity (NaCl concentrations).
4. To procure gene of interest responsible for synthesis of glycine betaine i.e. COX.
5. Transformation of COX gene into *Agrobacterium tumifaciens.*
6. Co-cultivation of plant parts with *Agrobacterium tumifaciens* containing gene of interest and subsequent regeneration of plants.
7. Selection of successful transformants under appropriate selection pressure and confirmation of transformation.

Chapter 2

TISSUE CULTURE STUDIES FOR DEVELOPMENT OF REGENERATION PROTOCOL FOR GROUNDNUT

2.0 Introduction

Groundnut (*Arachis hypogea* L.) is an important oil, food and fodder crop and plays an important role in the agricultural economies of countries of the semi-arid tropics. It contributes significantly to food security and alleviates poverty (Naidu et al., 1999) and as a legume, improves soil fertility by fixing nitrogen and increases productivity simultaneously.

It is native to South America and forms an important legume in Asia and Africa. It is mainly grown under rain-fed, irrigated, and residual moisture conditions. The crop is cultivated on 24.8 million hectares with an average productivity of 1.32 tonnes/hectare.

It is also one of the major cash crops of the state of Gujarat with highest production and productivity in the country.

There are several constraints to the productivity of the groundnut crop that result in great economic losses annually (Sharma et al., 2000). Conventional breeding has contributed towards the improvement of groundnut (Reddy et al., 1996; Garcia et al., 2006) but strong interspecific barriers, low recovery of hybrids and linkage of undesirable traits limit the introgression of variability from wild species (Halward et al., 1993; Tallury et al., 2005). Genetic transformation approach allows for introducing novel genes that are not accessible normally by conventional cross-breeding, i.e., limited by sexual incompatibility.

Genetic transformation overcomes these limitations and allows introduction of agronomically important genes across taxa (Li et al., 1997; Singsit et al., 1997; Yang et al., 1998; Magbanua et al., 2000; Sharma

and Anjaiah, 2000; Livingstone et al., 2005; Tiwari et al., 2008). The availability of an efficient regeneration system is however an essential prerequisite for utilizing this approach.

For successful genetic modification, production of transgenic plants, effective regeneration system is imperative. Although several reports on efficient regeneration from diverse explants of groundnut have been published, but not much success with genetic transformation of *Arachis* species has been achieved and many of them are genotype dependent (Cheng et al., 1992). This is due to the lack of efficient protocols to regenerate whole plants through *in vitro* regeneration of adventitious shoot buds from the transformed tissues (Lacorte et al., 1991).

2.1 Literature studies

Various regeneration systems reported so far from different explants in groundnut are as given below.

Immature leaflets from young seedlings of groundnut had been utilized to achieve organogenesis (Mroginski et al., 1981; McKently et al., 1991; Cheng et al., 1992; Sukumar and Sree Rangasamy, 1984; Narasimhulu and Reddy, 1983; Akasaka et al., 2000; Chengalrayan et al., 2001). However, among these reports some had shown low regeneration efficiency and excessive time required for the development of the regenerants. For instance, Mroginski et al., (1981) and Cheng et al., (1992) reported bud primordia development that failed to regenerate normal plants. Sukumar and Sree Rangasamy (1984) reported calli development in seven *Arachis* spp. but none of them formed shoots. Narasimhulu and Reddy (1983) reported callus mediated plant development in only 19 % of the explants. Akasaka et al., (2000) reported various abnormalities in shoot development and low conversion rate (34.7%) from shoot buds to shoots. Mature zygotic embryo derived leaflet explants have been deployed by Chengalrayan et al., (2001) to examine the effect of phytohormones on organogenesis and somatic

embryogenesis. Sharma and Anjaiah (2000) have reported efficient regeneration in groundnut from cotyledon explants.

From above it is clear that a perfect protocol for regeneration of groundnut is still elusive. It is also a requirement for the production of transgenic plants.

2.2 Materials and Methods

The pods of peanut cultivar var. GG20 was obtained from the Agriculture University, Junagadh, Gujarat. The methods employed are discussed in subsequent discussion.

2.2.1 Standardization of surface sterilization treatment

A successful tissue culture protocol starts with effective explant sterilization (Dodds and Roberts, 1985). Optimization of the surface sterilization protocol is an important aspect in Tissue Culture study and this ensures that large number of clean explants survive sterilization.

Surface sterilization was followed as per standard procedure with 0.1 % mercuric chloride($Hgcl_2$) for 10 minutes however all seeds turned brown and no growth was observed in the cultures. Reducing the concentration and/or duration of exposure resulted in heavy fungal contamination. Since seeds/pods are underground parts of the plants heavy fungal/bacterial contamination is obvious and careful elimination of this is required for raising the axenic cultures. Various duration & concentrations of $Hgcl_2$ as well as sodium hypochlorite were tried to obtain sterile explants.

The mature 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.

Further procedure was carried out in Laminar Air Flow under sterile condition. Seeds were rinsed in 70% iso propyl alcohol (1 min) followed by 3 to 4 washes with distilled water.

Treatment	**Survival (%) after one week of inoculation**	**Contamination (%)**	**Response pattern**
0.525% NaOCl; 10 min	20	75	Heavy fungal & bacterial contamination
1.050% NaOCl; 10 min	35	60	
0.525% NaOCl; 20 min	30	45	
1.050%NaOCl; 20 min	40	35	Best among NaOCl treatment
0.05% $HgCl_2$, 5 min	40	40	Better survival compared to NaOCl treatment.
0.1% $HgCl_2$, 5 min	55	25	
0.05% $HgCl_2$, 7.5 min	50	35	
0.1% $HgCl_2$, 7.5 min	80	20	Best response with healthy explants
0.05% $HgCl_2$, 10 min	55	45	
0.1% $HgCl_2$, 10 min	95	0	Least contamination Explants turned brown and dead

Table 2.1: Explant contamination and survival rates following surface sterilization treatments with NaOCl as well as $HgCl_2$

Using NaOCl, explants response was better. However it was accompanied with heavy fungal contamination and hence could not be used in further experiments. The best treatments with NaOCl was 1.05% of NaOCl for 20 min indicating efficient sterilization without excessive damage to the explants but it could give only 40 % survival of the explants. In comparison, explant survival rates were generally higher following $HgCl_2$ sterilization

ranging between 80-90 % with least contamination. Among $HgCl_2$ treatment also survival and contamination control was directly related to the concentration of $HgCl_2$ as increased exposure with lower concentration did not result in controlling contamination. Similarly in all the NaOCl treatments also the explants survived after one week developed contamination gradually later during 2nd and 3rd week of incubation. Higher concentration with more exposure to $HgCl_2$ lead to death of the explants which was apparent as they turned brown. However $HgCl_2$ allowed better initiation of regeneration than NaOCl. The optimum treatment for best explant survival and initiation of regeneration was 0.1% $HgCl_2$ for 7.5 min in which around 80% explants could be initiated sterile and there was no aberrant effect.

2.2.2 Culture Medium and growth conditions

In all the experiments, MS (Murashige and Skoog, 1962) medium was used which was fortified with B5 vitamins (Gamborg et al., 1968), 100 mg/l myo-inositol and 30 g/l sucrose. 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 media were solidified with 0.65% agar (colloids). The pH was adjusted to 5.8 before autoclaving at 121°C for 20 min.

All the experiments were performed using Petri dishes (94 mm diameter), test tubes (25 mm diameter) and glass bottles (300 ml). About 4-5 explants were inoculated per Petri dish and bottles, while 2 explants were inoculated per test tube. All experiments were performed in triplicate.

The response was measured in terms of (1). No reaction (explants showing no physical change damage (2). Survival-explants turning green, producing callus or developing shoots. (3) Pathogen contamination.

2.3 Results and Discussion

2.3.1 Direct Organogenesis from young leaflets.

Groundnut leaves are pinnately compound and new emerging leaflets are conduplicate, i.e., folded lengthwise along the middle. The leaflet lamina and leaf rachis elongated extensively before leaflet unfolding.

The development and nodal position of the leaflets used for explanting are noted. Nodes are numbered beginning with the most proximal leaves of the shoot beyond the cotyledons.

Nodes 1 and 2 are usually separated by short internodes, in which case the first two true leaves appear almost oppositely oriented, in contrast to the wider-spaced, alternating leaf pattern at subsequent nodes. Leaflets at the first two nodes are smaller and usually dark green in color than later-formed leaves. The effect of leaflet size from leaves at nodes 1 and 2 was evaluated. To obtain these leaflets, after surface sterilization, seeds were soaked in sterile double distilled water over night and inoculated on MS medium with 2.22 µM BAP for pre-incubation.

After seven days of pre-incubation, the enlarged seeds were taken out aseptically, seed coat was removed, elongated radical was cut apart and the green young leaflets were separated (Fig. 2.1A-2.1B) and used as explants for direct organogenesis.

These leaflets were given superficial cut on the edges and further placed on MS medium with various combinations containing different concentrations of N^6-benzylaminopurene (BAP; 2.22-22.2 µM) and α-naphthalene acetic acid (NAA-2.68 -5.36 µM).

Percentage of explants showing shoot development response was noted. Percent response, mean and standard deviation in each experiment were

calculated from data generated at this stage. Subsequently, shoot clusters were sub-cultured on similar medium every three weeks for enhancing shoot elongation. After four cycles, the number of elongated shoots was recorded. Shoots (3-4 cm) derived from the shoot clusters were excised and rooted on MS medium supplemented with NAA (4.95 µM).

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 response of shoot initiation started with up ward curling of leaf cut edges. Few explants did not respond and turned transparent. The shoot bud cluster formation started and was restricted at 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.

Maximum of 3 elongated shoots were observed per explants directly within a month however, numerous shoot forming buds were found which when transferred to same medium showed further development and elongation. The immature leaflet explants are reported by many researchers to be most responsive in giving direct organogenic response.

The results obtained correlates with Seitz et al. (1987) who reported that immature leaves induced up to 30% shoot formation on the MS medium containing NAA and BAP.

Cheng et al. (1992) and Akasaka et al. (2000) also reported that MS medium containing NAA and BAP was the most promising combination for shoot bud formation from immature leaflet explants.

Fig.-2.1A: Seed coat peeled off from pre-soaked sterilized seed.

Figure-2.1B: Embryonated cotyledon with immature leaflets intact in pre-soaked sterilized seed.

Fig.2.2A: Young green folded leaflets developed during seed germination within 7 day incubation

Fig.2.2B: Young green folded leaflet lamina separated from seeds and used as explant.

Tiwari and Tuli (2009) also reported 81.5 % shoot bud formation on the MS medium with combination of NAA and BAP.

However, the findings differs from such earlier reports in a way that we used green young leaflets and obtained direct shoot formation within one month of incubation or at the most after 2 subculture. 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 the explants.

BAP (µM)	**NAA (µM)**	**Response after 3 weeks of incubation**	**Percentage of explants showing shoot buds**	**Average number of shoots per explants**
2.22	2.68	No change to explant, gradually drying up	-	-
4.44			-	-
6.66		Curling of edges Shoot bud initiation	45	2
8.88			60	3
13.32			70	4
17.76		Thickening of explants, curling of edges, no further development	-	-
22.2			-	-
2.22	5.36	No change to explant, gradually drying up	-	-
4.44	5.36		-	-
6.66	5.36	Curling of edges Shoot bud initiation	50	2
8.88	5.36		40	1
13.32	5.36		40	3
17.76	5.36	Thickening of explants, callus initiation no further development	-	-
22.2	5.36		-	-

Table 2.2: Effect of different concentrations of BAP and NAA on direct organogenic response from young leaves of *A. hypogaea* cv GG-20.

Fig. 2.3A: Shoot bud initiation after 15 days of inoculation

Fig.2.3B: Shoot elongation after 30 days of inoculation

2.3.2 Direct Organogenesis from embryo axis.

Intact zygotic embryo axis is a good source of highly merristematic cells. The connecting tissues of zygotic embryo axis with cotyledonary parts become more active when process of germination starts. To obtain this highly active and meristamatic tissue, 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. Explants were prepared from 3-7-days-old seedlings to evaluate the influence of explant age on regeneration efficiency. Based on the initial responses (data not shown) it was concluded that 3 days old seedlings gave best regeneration efficiency and hence in the subsequent experiments, 3-days-old seedlings were used. 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 with a sterile surgical blade and the cotyledons along with their nodes were embedded into the induction medium in such a way that the wounded meristematic nodal region and the adaxial surface of the cotyledon were in direct contact with the medium. From each seed, two explants were obtained.

Various combinations of growth regulators like 6-benzylaminopurine (BAP), kinetin (KN), 2,4-dichloro-phenoxy acetic acid (2,4-D) and α-napthaleneacetic acid (NAA) were tested with MS basal salts (Murashige and Skoog, 1962), A pH 5.6-5.8 was maintained before autoclaving and 3% sucrose was used as a carbon source. Explants were cultured on agar (0.65%) medium containing 17.76 µM BAP and 0.53 µM NAA that induced maximum number of shoots per explant. Subcultures were done at a15-days interval with 2-weeks duration each on induction media for the development of adventitious shoot buds. Explants producing multiple shoots were transferred to elongation medium comprising 8.88 µM BAP and 0.53 µM NAA. Elongated

shoots were cut at the inter nodal region and transferred to root induction medium comprising of 4.3 µM NAA with MS basal salts.

Three-days old seedlings responded well in terms of regeneration. The percentage regeneration and average number of shoots were highest on MS media fortified with BAP and NAA. Among the various BAP and NAA combinations tested, high frequency regeneration was obtained following culture of explants on MS medium supplemented with 13.32, 17.76 and 22.20 µM BAP along with 0.53 NAA (Table 2.3). BAP at 17.76 µM and 0.53 µM NAA was most effective for multiple shoot bud formation. Both the percent regeneration as well as the average number of shoot buds per explant was found to be higher (76% regeneration, 18 ± 1.5), when the adaxial side of the cotyledon was in direct contact with the medium compared to the abaxial side (58% regeneration, 8.75 ± 2.4). Elongated shoots when cultured on root induction medium developed adventitious roots within 15 days of culture. An average of 5 plants were recovered from each explant.

Plant regeneration is usually a bottleneck for the development of highly reproducible transformation protocols. Adventitious shoot formation via organogenesis becomes more efficient, once a suitable explant has been identified. Previously, leaf discs and leaf-section explants were used to generate transgenic groundnut plants but with low frequencies (Eapen and George, 1994; Cheng et al., 1996).

BAP (µM)	**NAA (µM)**	**Regeneration (%)**	**Average number of shoot buds / explant ± SE**
4.44	0.53	55	14 ± 2.1
8.88	0.53	68	12 ± 2.5
13.32	0.53	66	13 ± 1.75
17.76	0.53	76	18 ± 1.5
22.20	0.53	60	16 ± 2
26.64	0.53	67	14 ± 3.4

Table 2.3. Effect of different concentrations of BAP and NAA on regeneration from 6-day-old CN explants of A. hypogaea cv GG-20.

Each mean value was an average calculated from three experiments ± SE.

In the studies by Venkatachalam et al. 2000, cotyledons were used as a source of explants for achieving high frequency of transformation in groundnut, more particularly in the cultivar JL-24 (Sharma and Anjaiah, 2000). However, the regeneration response from the cotyledonary nodes may be comparatively higher than the cotyledons and other explants reported so far. The junction of the cotyledon and the embryo axes contain axillary meristematic cells that are highly regenerable and hence could be useful as potential targets for gene delivery. Cotyledonary nodes from mature seeds have been proved to be most responsive for the induction of multiple shoots and to generate transgenic plants in other grain legumes studied; viz. Soybean (Olhoft et al., 2003), Pea (Bean et al., 1997), Pigeon pea (Geetha et al., 1999), Mungbean (Jaiwal et al., 2001), Black gram (Saini et al., 2003).

2.3.3 Direct Organogenesis from Cotyledon explants

If Cotyledons can be used as explants it would make them as choice source due to their round the year availability of the healthy explants therefore an efficient protocol for regeneration of plants from the cotyledon explants would be of great use.

After surface sterilization the seed coat were removed and the embryo axis were cut surgically. The seed was then cut vertically along its natural ribs to get the two individual cotyledon halves- Cotyledon node(CN) and the same were used as explants- Cotyledon explants.

The explants were placed on MS medium as said above with different combinations of BAP (4.44 17.76 µM) and NAA (2.68-5.36 µM) for getting direct shoot induction. About 20 explants were inoculated per combination of BAP and NAA.

After 4 weeks of incubation, the cultures were transferred to MS medium with BAP 8.77 µM where it elongated to form plantlets.

CN explants when inoculated on different combinations of BAP and NAA shown direct shoot formation response within one month of incubation.

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 for about 5-6 minutes after surface sterilization as given below in the table 2.4.

Name of Antibiotic	**Concentration (mg/l)**
Ampicillin	100
Gentamycin	100
Ceftazedim	100
Ofloaxacin	100
Refmpicin	50
Total	**450**

Table 2.4 : Combination of antibiotics used to control bacterial contamination of explants

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 2.4A) however not all combinations yielded shoot bud initiation. Maximum of 65 % of explants shown direct shoot bud formation from the halved CN explants.

Fig. 2.4A: Initiation and development of shoot buds at the proximal end of the de-embryonated CN explants.

Fig. 2.4B : Elongation of shoots after two sub culture.

Fig. 2.5 :Rooting of the elongated shoots

The best combination for direct shoot initiation was observed to be MS medium with BAP 17.76 µM and NAA 2.68 µM. As many as 6-8 shoot buds were observed which when transferred to MS medium with BAP 8.77 µM concentration developed and elongated.

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

Table 2.5. Response of CN explant on MS medium supplemented with different combinations of NAA & BAP for direct shoot organogenesis.

The findings of direct shoot regeneration from cotyledon explants is in line with the number of other reports including Sharma et al. (2000), Maina et al. (2010). However they reported use of 2,4- D in combination with BAP. These results with further optimization of subculture stages can be effectively used for agrobacterium- mediated transformation.

2.3.4 Indirect organogenesis from mature leaves.

Regeneration in groundnut using mature leaf explants has not been reported successfully so far. However, an efficient regeneration through mature leaves could be of great use as availability of such explants would not be a limiting factor.

To get the mature green leaflets, seeds after surface sterilization were inoculated in MS medium with 2.22 µM BAP for germination. The seeds

germinated within 7-10 days of incubation and grown in to plantlets within 21-28 days of incubation. After 4 weeks, the grown plantlets were taken out aseptically and individual leaflets were used as explants for indirect organogenesis.

These mature leaflets were given superficial cut on the edges and further placed on MS medium with various combinations of plant growth hormones BAP (1.11 - 4.44 µM), kinetin (1.16 – 4.64 µM) in combinations with 2,4-D and NAA.

The mature leaf explants curled from the edges after 4 weeks of incubation and started showing growth of callus (green). The callus cultures were transferred to the same medium with same concentration of growth hormones where cultures developed different types of callus. White - Greenish friable callus was observed in medium containing 2,4-D while in case of medium containing NAA the callus type was brown and compact. The callus was subcultured further for 2-3 passage on the same medium for about 3-4 weeks.

After, 4^{th} subculture the green compact callus was transferred to MS basal medium where it showed gradual development of shoot buds which elongated to form defined shoots.

Both the types of callus were maintained on the same respective medium and on the MS medium with reduced concentrations for 3 subculture during which the callus mass proliferated. The brownish callus showed development of root initially however this was not accompanied by the shoot development. The white –brownish callus when transferred to MS plain medium, root initials developed in case of white friable callus while in case of green callus in MS medium containing 2.32 µM kinetin, it showed green spots at the center of hard compact callus within one week of transfer which enlarged in the size and showed initiation of shoots. The shoots elongated in the same medium within 4 weeks of incubation. As many as 6 defined shoots were observed

along with numerous developing shoot buds. Upon subculturing on same medium the culture with shoot buds developed in to further plantlets.

Fig. 2.6A: Initiation of callus at the cut edge of the leaf margin

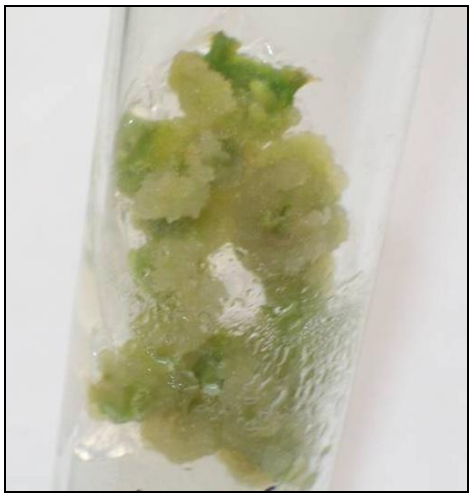


Fig. 2.6B: White-greenish friable callus growth after 3 sub culture on MS medium with BAP & 2,4,D

Fig.2.7A: Brownish-hard compact callus with development of root initial after 3 sub culture on MS medium with kinetin & NAA.

Fig.2.7B: Development of shoot buds and plantlets from the brownish hard callus on MS medium with reduced kinetin concentration after 5 subcultures.

This findings of shoots regeneration from hard compact greenish callus are in line with opinion of Maina et al. (2010).

Chapter 3

BIOCHEMICAL CHANGES SEEN IN GROUNDNUT DUE TO SALT STRESS

3.0 Introduction

Due to increased environmental stress the balance between the production of reactive oxygen species (ROS) and the scavenging activity of antioxidants is upset resulting in oxidative damage. ROS can be important mediators of damage to cell structures, nuclei acid, lipids and proteins. Generation of ROS in plant tissues, such as superoxide anion radicals (O_2•-), hydroxyl radicals (•OH), singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2), is a common response observed in salt-stressed plants (Vaidyanathan et al., 2003; Azevedo-Neto et al., 2006). So, in addition to its known osmotic and ionic effects, salt stress is also manifested as an oxidative stress (Hernández et al., 1995). These ROS are highly reactive and can alter the normal cellular metabolism through oxidative damage to proteins and nucleic acids, as well as causing peroxidation of membrane lipids (Møller et al., 2007). In order to prevent oxidative damages, plants have evolved a complex antioxidant system, which includes both enzymatic (scavenger enzymes) and non-enzymatic (mainly ascorbate and glutathione) components differentially found in cell compartments (Mittler, 2002). Enzymatic components include superoxide dismutase (SOD), which is a major scavenger of O_2•- free radicals, converting them into O and HO. The HO may be scavenged by a variety of peroxidases; including catalase (CAT), ascorbate peroxidase (APX) which uses ascorbate as electron donor in the first step of the ascorbate glutathione cycle, and guaiacol peroxidase (GPX), that decompose H_2O_2 by oxidation of co-substrates such as phenolic compounds and/or ascorbate. The capacity to scavenge ROS and to reduce their damaging effects on macromolecules appears to represent an important stress tolerance trait (Amor et al., 2005). A close correlation between the antioxidant capacity and NaCl tolerance has been demonstrated in several crops such as rice (Vaidyanathan et al., 2003), tomato (Mittova et al., 2002) and maize (Azevedo-Neto et al., 2006). In view of the above discussion, we investigated the effects of salt stress on growth and development of groundnut (one of the major sources of income for agriculture in Gujarat) seedlings *in vivo* and *in*

vitro, which were associated with changes in antioxidative enzyme system. This knowledge will contribute to improve our understanding on the groundnut's acclimation process to salinity conditions.

3.1 Effect of salt stress on biochemical parameter: H_2O_2, catalase and paroxidase.

Salt stress induces ionic stress and osmotic stress in plant cells. A direct result of these primary effects is the enhanced accumulation of ROS that are harmful to plant cells at high concentrations.

These are small molecules having unpaired valance shell electrons and are produced continuously as byproduct of various physiological metabolic pathways, such as photosynthesis, photorespiration and CO_2 assimilation. They are formed during certain redox reactions and during incomplete reduction of oxygen or oxidation of water by the mitochondrial or chloroplast electron transfer chains.

Apart from salinity, various environmental factors of stresses like air pollution (increased amounts of ozone or sulfur dioxide), oxidant-forming herbicides such as paraquat dichloride (methyl viologen, 1,1 -dimethyl-4,4 -bipyridinium), heavy metals, drought, heat and cold, wounding, UV light, and highly intense light conditions that stimulate photoinhibition also leads to increased ROS production. Due to the unpaired valance shell electrons the ROS are highly reactive and can react readily with neighboring molecules.

ROS were traditionally considered toxic to plants. However, recently it has become apparent that plants actively produce these molecules which may control many different physiological processes such as abiotic and biotic stress response, pathogen defense and systemic signaling.

Under non-stress environmental conditions, ROS can modulate usual events such as DNA synthesis, enzyme activation, selective gene expression and regulation of the cell cycle, hence the indirect control of cellular differentiation, growth, development, and death (Franck et al., 2000).

Thus ROS may act as damaging, protective or signaling factors and the nature of its role depends on the delicate equilibrium between ROS production and scavenging at the proper site and time (Gratão et al., 2005).

3.2 Damages caused by ROS

Higher accumulation of ROS in cell leads to damages in the form of change in membrane permeability, increase in rate of lipid peroxidation and thus spreading of ROS effects like increase in rate of mutation, indirect role in chlorosis etc. These changes could culminate in cell death.

In plants, ROS represent the products of successive single-electron reductions of oxygen (Eqn. 1; see Fig. 3.1). Ground state O_2 is a triplet state molecule and is relatively unreactive, but it can accept a single electron from a variety of reducing agents, particularly if it gets converted to the singlet state (for instance, by the transfer of energy from photochemically excited chlorophyll), if the reducing agent is photochemically excited, or if the oxygen and reducing agents are in the presence of an appropriate enzyme. The product of this reduction is superoxide, O_2^-, which under acidic conditions protonates to the hydroperoxyl radical (Eqn. 2 in Fig. 3.1). Subsequent reductions will form hydrogen peroxide (H_2O_2), hydroxyl radical, and water.

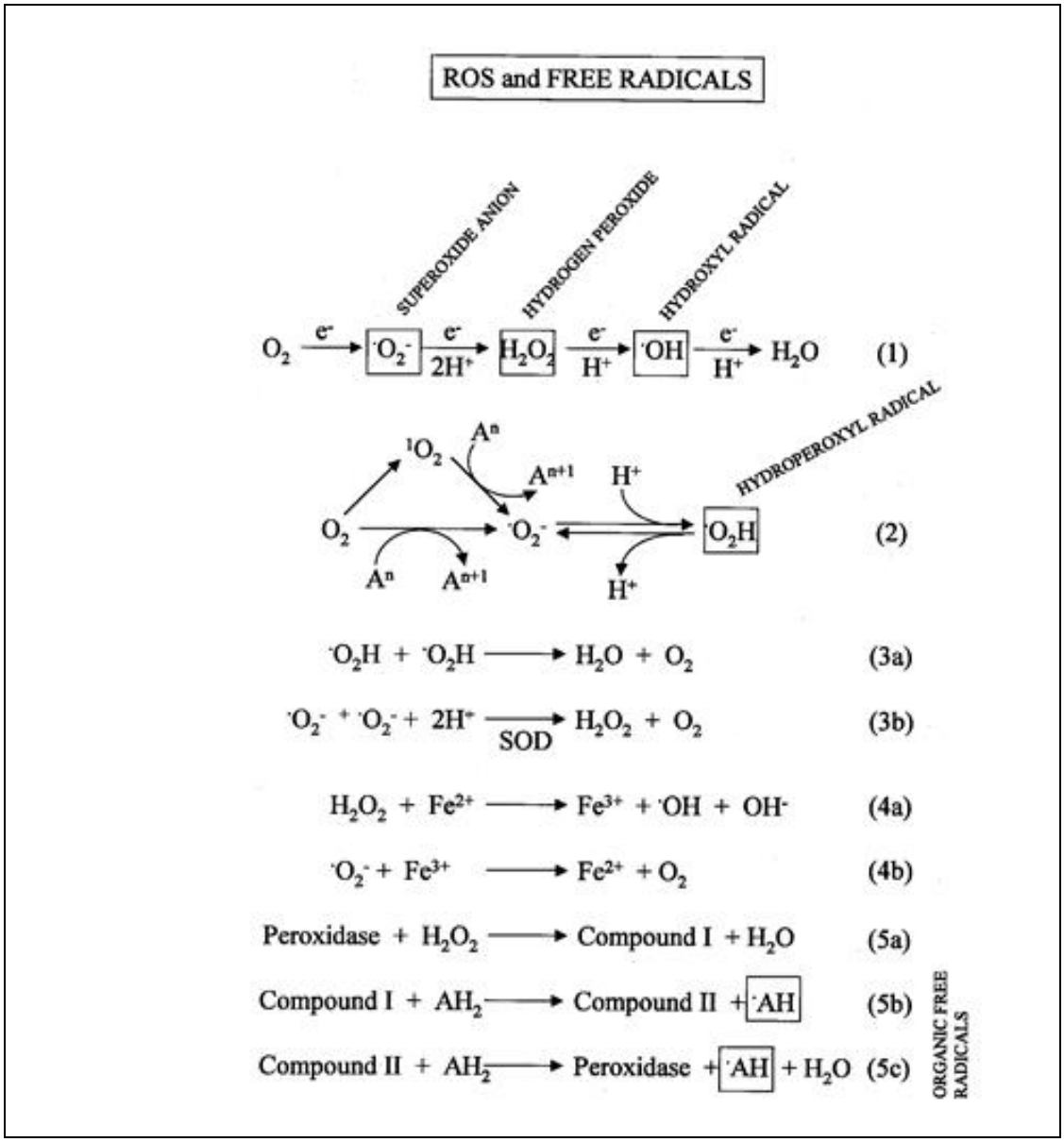


Fig.3.1. Reactions producing ROS and organic free radicals. (Cassells and Curry, 2001)

In cells, the appearance of H_2O_2 and hydroxyl radical generally does not occur through the sequence of reactions shown in Eqn. 2. Instead, once superoxide has been synthesized, interconversions of the various ROS may account for the appearance of the more reduced forms. For instance, disproportionation of hydroperoxyl radical, or of superoxide in the presence of superoxide dismutase (SOD), forms hydrogen peroxide (Eqn. 3a,b in Fig. 3.1). The combination of hydrogen peroxide and superoxide in the presence of iron or other transition metals forms hydroxyl radical (the Fenton reaction - Eqn. 4a,b in Fig. 3.1). Thus, in the oxidative burst, an enzymatic synthesis of superoxide may lead to the appearance of the other ROS. However, alternative mechanisms for the synthesis of H_2O_2 are possible.

Plants scavenge and dispose of the reactive molecules by use of antioxidant defence systems present in several subcellular compartments. There are other ways in which the synthesis of ROS could serve to defend plant cells against pathogens. O_2^- and H_2O_2 are required for lignification. When the apoplastic matrix is modified, peroxidase-catalysed reactions that generate free radicals lead to the cross-linking of cell wall proteins, polyphenolics, and cutin, forming walls that better resist penetration by pathogens (Pedreno et al., 1995; Franck et al., 2000). In certain systems, ROS, including H_2O_2, superoxide, and possibly hydroxyl radicals may serve as extracellular signals, stimulating defense responses in other parts of the plant.

ROS, especially HO^-, are highly destructive to lipids, proteins and nucleic acids. Peroxidation of membrane lipids and other critical cell components can also occur through the production of organic free radicals. Hydroxyl radical is extremely reactive, with a life-time in the cell of nanoseconds. In cells it can oxidise - abstract H^+ from almost any available biological molecule, forming an organic radical. Normally, the production of hydroxyl radical is limited by the presence of SOD, which keeps superoxide concentration low. Catalase or peroxidases, which remove H_2O_2, also restrict the production of hydroxyl radical. On the other hand, free radicals may be formed also by peroxidases, using H_2O_2 as initial oxidant (Eqn. 5a,b,c; Fig. 3.1). The summary of the harmful effects of ROS when the pro- and anti-oxidant balance is perturbed in oxidative stress. (Fig. 3.3).

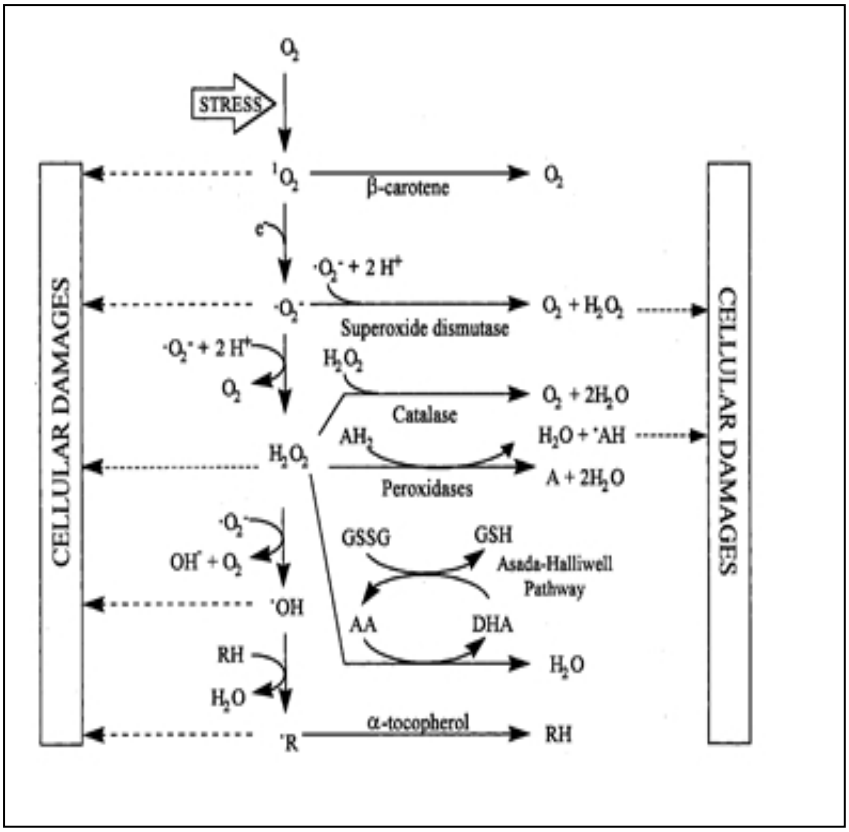


Fig. 3.2. Defense systems (enzymes, antioxidants) against ROS

Source : (Gasper et al., 2002).

Two types of scavenging-pathways / antioxidant defense systems exists in plant cells in protection against oxidative stress and damage caused by ROS 1. Non- enzymatic and 2. Enzymatic (Fig. 3.2 and 3.3). The major antioxidant species in plants are ascorbate (vitamin C), reduced glutathione (GSH), α-tocopherol (vitamin E), and carotenoids; polyamines and flavonoids also may provide some protection from free radical injury (Foyer and Noctor, 2005). Furthermore, the metal chelators, such as metallothionins (MT) and ferritins (FT), due to their metal-binding activity play an important role in metal metabolism and detoxification (Briat et al., 1999).

The ascorbate-glutathione cycle is the major antioxidant pathway in plastids, where ROS are generated during normal biochemical processes that include photosynthetic transfer of electrons. The photosynthetic apparatus receives additional protection from oxidative damage by the exothermic production of the xanthophyll zeaxanthin (Gaspar et al.,2002).

These compounds and enzymes are not distributed uniformly, so defence systems vary among specific subcellular compartments.

ROS are produced in root nodules of nitrogen-fixing plants and are scavenged by enzymatic antioxidants. Regulation of the concentrations of antioxidants and antioxidant enzymes, necessarily in a coordinated manner, constitutes an important mechanism for avoiding oxidative stress.

I

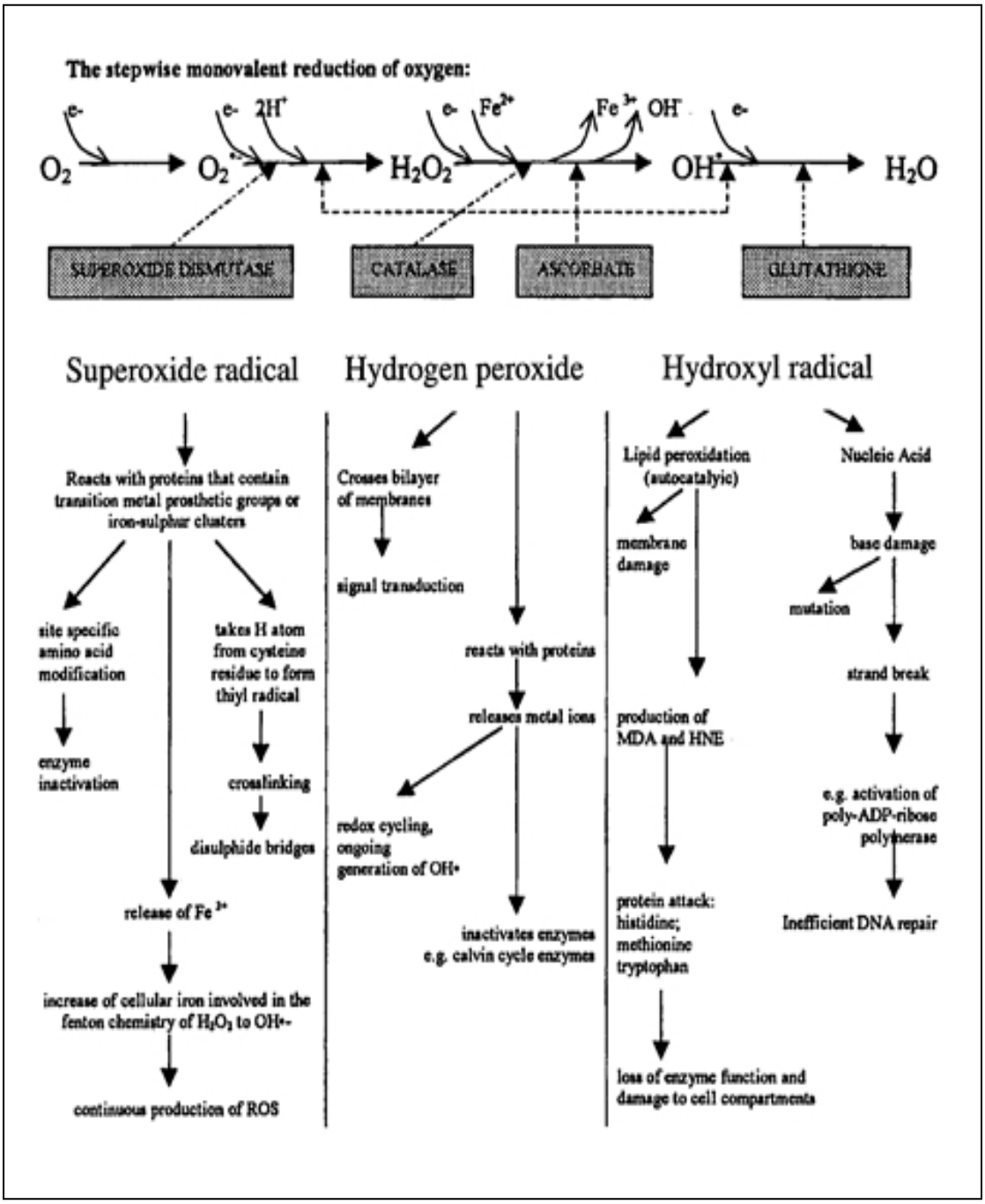


Fig. 3.3. ROS and its harmful effect.

The upper section shows the ROS produced constitutively in the cell, and the natural antioxidants and enzymes used to minimise their toxic effect. The lower section gives selected examples of the harmful effects of ROS when the pro- and anti-oxidant balance is perturbed in oxidative stress. (Gasper et al., 2002)

3.3 Materials and Methods.

3.3.1 Effect of salt stress on seed germination and growth of groundnut *in vivo.*

The pot experiment was planned and carried out to find out the level of natural salt tolerance and to study the effect of different concentrations of salt stress on groundnut germination.

Salinity is a complex parameter and is contributed by number of salts out of which sodium chloride (NaCl) is dominant salt and is major among all followed by Sodium Sulphate which is of secondary importance and then magnesium and calcium chloride which are of third importance. Cooke et al. (1993) stated that, because of the great solubility of NaCl it is taken down slope, and it characterizes the soil nearest to a playa, while the other salts are deposited to higher altitudes in soil.

Therefore, to provide salt stress, different concentrations of NaCl solution were used in all the experiments. Five different concentrations 10 mM, 25 mM, 50 mM, 100mM and 200 mM were taken along with the control.

Mature dry seeds from the pods of groundnut variety GG-20 were used for the experiments.10 seeds each were taken per treatment. Seeds were washed with 0.1 % bavistin and then immersed separately in flask containing different NaCl concentrations as mentioned above for 4 hours. Along with five doses double distilled water was taken as control without NaCl.

These seeds were planted in separate pots containing mixture of normal soil and Farm yard manure in equal proportion.

The pots were kept in dry open space to facilitate normal germination process. Each pot were irrigated separately with the particular NaCl solution twice a day. Control pot was irrigated with normal double distilled water.

Various parameters like days required for germination, % germination, shoot height, no. of leaves, no of days required for flowering etc. were noted.

3.3.2 Effect of salt stress on *in vitro* seed germination and growth of cultures.

The effect of NaCl stress was also tested with different concentration for the germination of seeds and its growth *in vitro.*

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.

Seeds were inoculated in test tubes containing MS medium fortified with B5 vitamins (Gamborg et al., 1968), 100 mg/l myo-inositol, 30 g/l sucrose, BAP 8.88 µM. Based on the results obtained in *in vivo* germination experiment, NaCl 25mM , 50mM, 75mM , 100mM , 125mM , 150mM were taken as treatment along with control. Respective amount of NaCl was separately weighed and added in medium before autoclaving.

The media were solidified with 0.65% agar. The pH was adjusted to 5.8 before autoclaving at 121 C for 20 min. The cultures were incubated at 25 ± 28 ^{o}C under 80 mmol photon m^{2}/s light intensity with a photoperiod of 16/ 8 h.

Two seeds were inoculated per test tubes and ten test tubes per treatment. The experiment were performed in triplicate.

3.3.3 Role of Hydrogen Peroxide in Plants

Hydrogen Peroxide is one of the major reactive oxygen species (ROS) in plant tissues. It is produced in chloroplast and mitochondria via electron transport chain, where oxygen is reduced to super oxide which is further dismutated into H_2O_2 spontaneously or catalyzed by super oxide dismutase (SOD) (Asada, 1999; Moller, 2001). The dismutation of superoxide into hydrogen peroxide and oxygen constitute the first line of cellular defense to

prevent undesirable biological oxidation by oxygen radical generated during cellular metabolism.

H_2O_2 production in plant cells is also catalyzed by glycollate oxidase in peroxisomes (Noctor et. al., 2002) membrane bound NADPH oxidase (Jiang and Zhang, 2003) and oxalate oxidase. When plants are subjected to environmental stress, it accumulates and leads to oxidative damage (Asada, 1999).

Accumulating evidences suggests that H_2O_2 is a key signalling molecule involved in plant response to biotic and abiotic stresses, such as pathogen attack, extreme temperature, drought, excessive radiation, ozone and wounding.

Therefore it is important to determine H_2O_2 concentration in plant tissue.

3.3.3.1 H_2O_2 Estimation: (Patterson et al., 1984)

Reagents required:

5% TCA

Activated charcoal

17M Ammonia solution

colorimetric reagent

10 mg 4- Aminoantipyrin

10 mg phenol

5 mg peroxidase dissolved in 100 ml of glucose solution.

Method:

0.5 gm fresh tissue was weighed and rinsed with distilled water. Tissue was then grinded to powder in a mortar with pestle together with 5ml 5% TCA and .15gm activated charcoal and glass powder in dark condition. The mixture was centrifuged at 10,000g for 20 min at 4^0C. Supernatant is adjusted to pH-8.4 with 17M Ammonia solution and filtered. 1ml of colorimetric reagent was added to 0.5ml sample extract and incubated for 10 min at 30 ^{0}C. The absorbance was taken at 505nm by spectrophotometer.

3.3.4 Role of catalase in plants

Catalase is one of the most potent catalysts known. The reactions it catalyses are crucial to life. It is an antioxidant enzyme that converts H_2O_2 to water in the peroxysomes (Fridovich, 1989; McCord and Fridovich, 1969). In this organelle, H_2O_2 is produced from ß-oxidation of fatty acids and photorespiration (Morita et al., 1994). Higher activity of CAT and APX decrease H_2O_2 level in cell and increase the stability of membranes and CO2 fixation because several enzymes of the Calvin cycle within chloroplasts are extremely sensitive to H_2O_2. A high level of H_2O_2 directly inhibits CO2 fixation (Yamazaki et al., 2003).

Thus Catalase protects the cell from the deleterious effects of hydrogen peroxide accumulation. Multiple isoenzymes of CAT have been studied in higher plants, three main CAT isoforms have been characterized (Scandalios, 2005).

Catalase also uses Hydrogen Peroxide to oxidise toxins including Phenols, Formic Acid, Formaldehyde and Alcohols.

The reaction catalyzed is:

$2H_2O_2$ to $2H_2O + O2$,

H_2O_2 is a powerful oxidizing agent and is potentially damaging to cells. By preventing excessive H_2O_2 build up Catalase allows important cellular processes which produce H_2O_2 as a byproduct to take place safely.

3.3.4.1 Catalase Estimation: Aebi (1984)

Reagents required:

1) Extraction buffer

Na-PO_4 buffer 50mM of pH 7.0

DTT 1mM

EDTA 0.1mM , 1% PVP

2) Assay buffer:

Tris HCl buffer 75mM; pH – 8.2

Method

0.5 gm of fresh tissue was weighed and rinsed with distilled water and kept it in deep fridge for 30 min. The tissue was grinded to powder with 5 ml of extraction buffer and a pinch of glass powder in chilled condition with precooled mortal and pistel. Centrifuged at 10,000 rpm for 15 min. at 4◦c. The supernatant was removed and used as crude enzyme extract. 100µl of enzyme extract was added with 875µl of assay buffer along with 25µl of H_2O_2. The absorbance was taken at 240 nm for I min.

Catalase activity was measured according to Aebi (1984) as described above. Reaction started by adding H_2O_2 and decrease in absorbance recorded at 240 nm for 1 min. Enzyme activity was computed by calculating the amount of H_2O_2 decomposed.

3.3.5 Role of Peroxidases in plants

Peroxidases are haem-containing enzymes that use hydrogen peroxide as the electron acceptor to catalyse a number of oxidative reactions. Most haem peroxidases follow the reaction scheme:

Fe3+ + H_2O_2--> [Fe4+=O]R' (Compound I) + H_2O

[Fe4+=O]R' + substrate --> [Fe4+=O]R (Compound II) + oxidised substrate

[Fe4+=O]R + substrate --> Fe3+ + H_2O + oxidised substrate

In this mechanism, the enzyme reacts with one equivalent of H_2O_2 to give [Fe4+=O]R' (compound I). This is a two-electron oxidation/reduction reaction where H_2O_2 is reduced to water and the enzyme is oxidised. One oxidising equivalent resides on iron, giving the oxyferryl intermediate, while in many peroxidases the porphyrin (R) is oxidised to the porphyrin pi-cation radical (R'). Compound I then oxidises an organic substrate to give a substrate radical.

Peroxidases are found in bacteria, fungi, plants and animals and can be viewed as members of a super family consisting of three major classes.

Of these, Class III comprises the secretory plant peroxidases, which have multiple tissue-specific functions e.g., removal of hydrogen peroxide from chloroplasts and cytosol; oxidation of toxic compounds; biosynthesis of the cell wall; defence responses towards wounding; indole-3-acetic acid (IAA) catabolism; ethylene biosynthesis; and so on Hiraga et al., 2001). The wide spectrum of peroxidase activity, coupled with the participation in various physiological processes, is in keeping with its relative lack of specificity for substrates and the occurrence of a variety of isozymes.

Plant peroxidases are monomeric glycoproteins containing 4 conserved disulphide bridges and 2 calcium ions. The 3D structure of groundnut peroxidase has been shown to possess the same helical fold as class I and II peroxidases.

3.3.5.1 Peroxidase Estimation

Reagents required:

1M NaOH

0.1M Phosphate buffer

5.33% Pyrogallol solution

Sudstrate H_2O_2 solution

Enzyme solution

Method

2.4 ml of 0.1M Phosphate buffer, 0.33 ml of Pyrogallol solution and 0.20 ml of H_2O_2 solution was pipetted into a 10 mm cuvette and it was equillibrate in water bath until temperature reaches 20 ^{0}C. The enzyme was added at zero time and mixed properly. The total reaction volume was made to 3ml and the recording of increase in absorbance was started immediately at 420 nm.

3.4 Result and Discussion

3.4.1 Effect of salt stress on seed germination and growth of ground nut *in vivo*

As shown in table 3.1, average 3 days were required for germination of seeds in control as well as low NaCl treatment. While the germination was delayed for the seeds under 100 mM treatment. There was no germination at all for the seeds under 200 mM treatment. All other parameters were more or less similar for initial 3 NaCl treatments i.e. 10, 25 and 50 mM concentrations.

After subjecting plants with different salt concentrations height of plant treated with 25mM were not affected. Whereas in case of plants treated with 50mM, 100mM and 125mM shows significant decrease in plant height with respect to control. It is known that salinity induces a change in the signals of root origin, which changes the hormonal balance of the plant, and this affects root and shoot growth(Munns et al., 1986) . Another reason for that is, at higher salt concentration especially Na+ becomes toxic to plant by inactivating the enzymes like rubisco and disturbs the ion homeostasis that leads to cytotoxic effect in cell In turn leading to death of plant cells and thus decrease in shoot length.

Observations	**Control**	**10 mM**	**25 mM**	**50 mM**	**100 mM**	**200 mM**
Pretreatment	4 hrs presoaking in 10 ppm GA3 with respective salt concentration					
Sowing date	28/01/2010	28/01/2010	28/01/2010	28/01/2010	28/01/2010	28/01/2010
No. of replicates	10	10	10	10	10	10
Avg. no. of Days required for germination	3	3	4	6	8	
Avg. 'no. of seeds germinated	10	9	9	7	4	0
% seed germination	100.00	90.00	90.00	70.00	40.00	0.00
Avg. No. of compound leaves after 10 days	3-4	3-4	2-3	2	2	0
Avg. no. of Days required for 1st flowering	19	20	21	21	60	-
Avg. No. of flowers after 3 weeks	8	6	4	1	0	0
Ave. No. of compound leaves after 21 days	9	9	6	5	3	0

Table 3.1: Effect of different level of NaCl stress on seed germination and plant growth & development.

Fig .3.4: Ground nut plants under different level of NaCl stress Control, 10mM to 200 mM from L to R.

Fig. 3.5 : Ground nut plants under NaCl stress Control and 10mM on 22nd day.

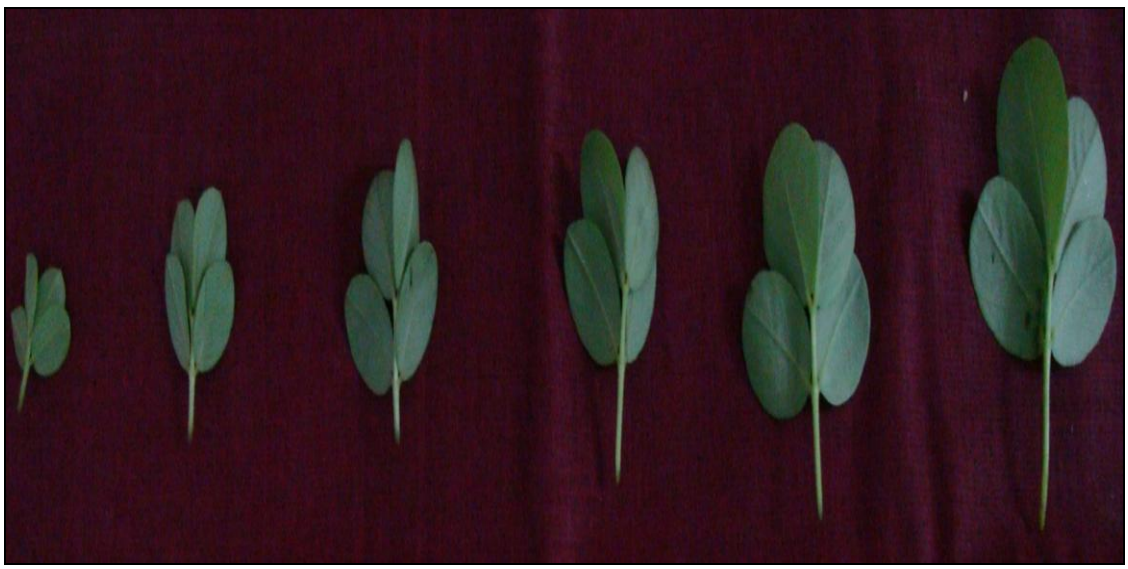
There was no significant difference in the germination rate of plants treated with 10 mM, 25mM and 50mM of NaCl. However the germination rate was retarded at concentration 100mM.There was no germination at concentration 200 mM as compared to control. Abscisic acid in plant plays major role in germination (Leung et al.,1998). It is known that at higher salt concentration there will be increase in abscisic level that will lead to decrease in activity of α-amylase. Thus inhibiting the germination of seedlings.

A significant decrease of leaf area was observed in plants under high salt stress. Higher concentration of salt leads to accumulation of Na+ in the cells. That leads to toxicity to the cell, in turn reduction in leaf area exposed for photosynthesis. With the increase of NaCl concentration there was reduction in the number of leaves and yellowing of leaves.

These parameters were also more or less same for the treatment up to 50 mM NaCl treatment compared to control however germination, number of leaves, morphological growth and days required to flower were severely affected at the 100 mM treatment. Hardly a flower was observed in any plant that to after 60 days of germination which took 3 times more duration than the normal control plants.

Based on these observations it was concluded that ground nut can naturally withstand salinity up to 50 mM without any aberration.

Fig. 3.6 : Ground nut plants under NaCl stress 25mM and 50mM on 22nd day.



150 mM 100 mM 50 mM 25 mM 50 mM Control

Fig. 3.7: Leaves size of plants under different level of NaCl stress.

Fig. 3.8 - Ground nut plants under NaCL stress 100mM on 22nd day.

3.4.2 Effect of salt stress on *in vitro* seed germination and growth of cultures.

The germination of seeds in *in vitro* condition was observed to be severely more affected compared to *in vivo* condition. The germination started on third day of incubation. Within 12 days of incubation seedlings grown to the height of 4 cm with 3 functional compound leaves each in control as well as 25 and 50 mM NaCl treatments. However, for higher dose 75 and 100 mM, the germination was delayed by 4-5 days. Shoot height also was shorter with no germination at all in 125 mM treatment for up to 10 days.

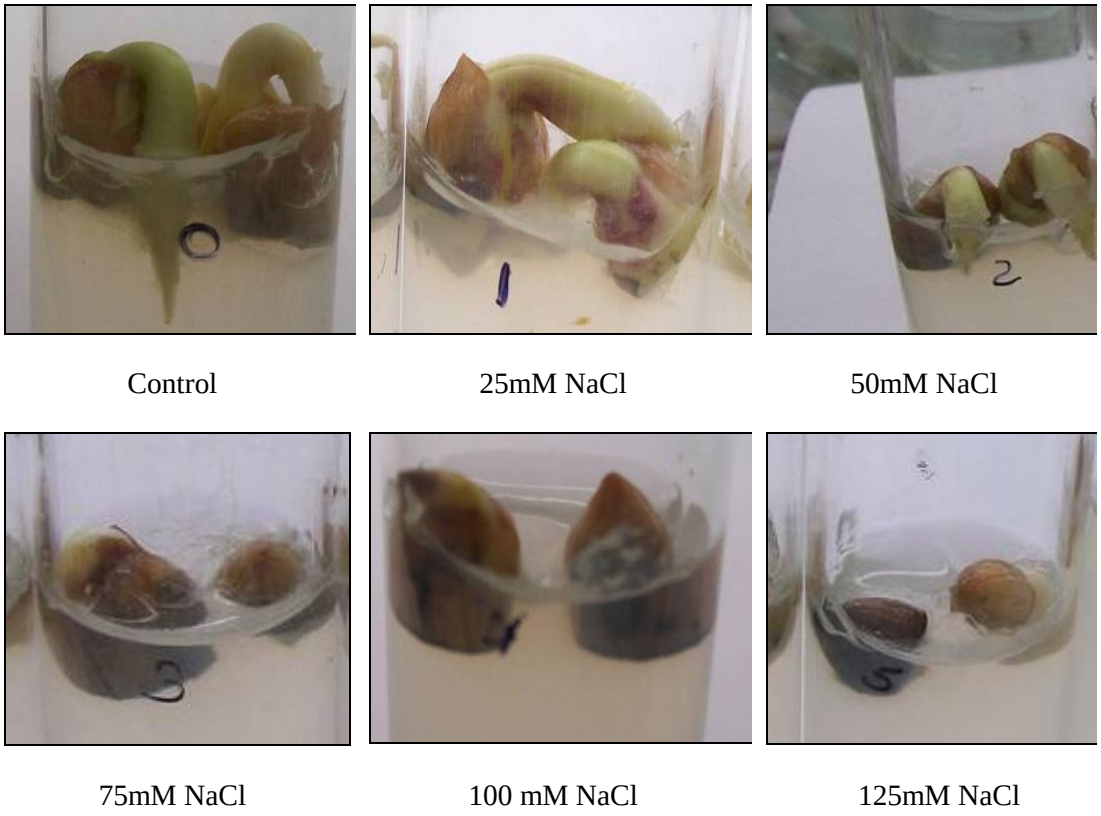


Fig. 3.9: *In vitro* germinating seeds on MS medium on 3rd day of inoculation with different level of NaCl stress.

3.4.3 Effect of salt stress on H_2O_2 release

H_2O_2 level was measured by Zhu et al.2006.The results of hydrogen peroxide estimation showed an increase in H_2O_2 level under high salt stress. At the increasing concentrations of salt many reactive oxygen species are produced including hydrogen peroxide as result of free radical chain process in plants.

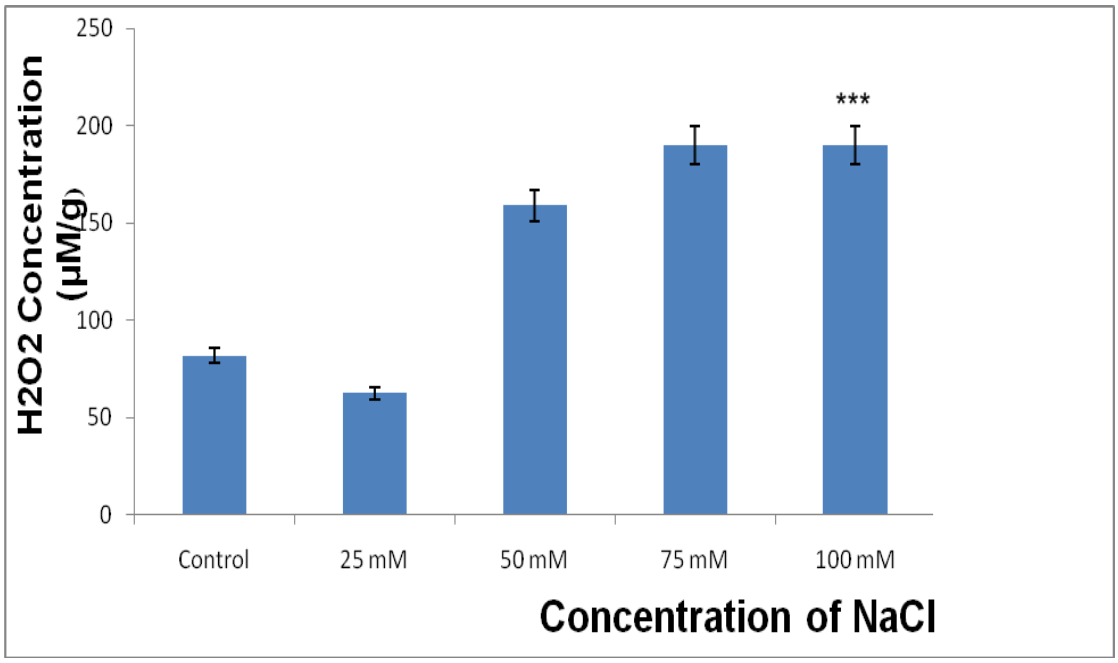


Fig. 3.10: Release of H_2O_2 in different concentration of NaCl stress.

Values are reported as Mean + SEM, *** indicates significantly different at $p<0.05$ and $p<0.001$ respectively as compared to the corresponding control. N=5

3.4.4 Effect of salt stress on catalase release

The activities of catalase increased with the increase in the concentrations of NaCl. The increase in the activities of POD and CAT with the increase of NaCl concentration has been shown in *S. sieb* by Li (2009).

Catalase is involved in scavenging of reactive oxygen species. Results show that increasing activity with increased concentration of salt. At 75 and 100 mM NaCl the CAT activity increases 2-3 folds compared to that of control. The plant combat salt stress by increasing catalase activity. But at higher concentration of salt as 75mM and 100mM there was no significant change in activity and It seems that, CAT activity was not adequate for the complete scavenging of H_2O_2 and thus to combat salt stress. Although its activity increased compared to control.

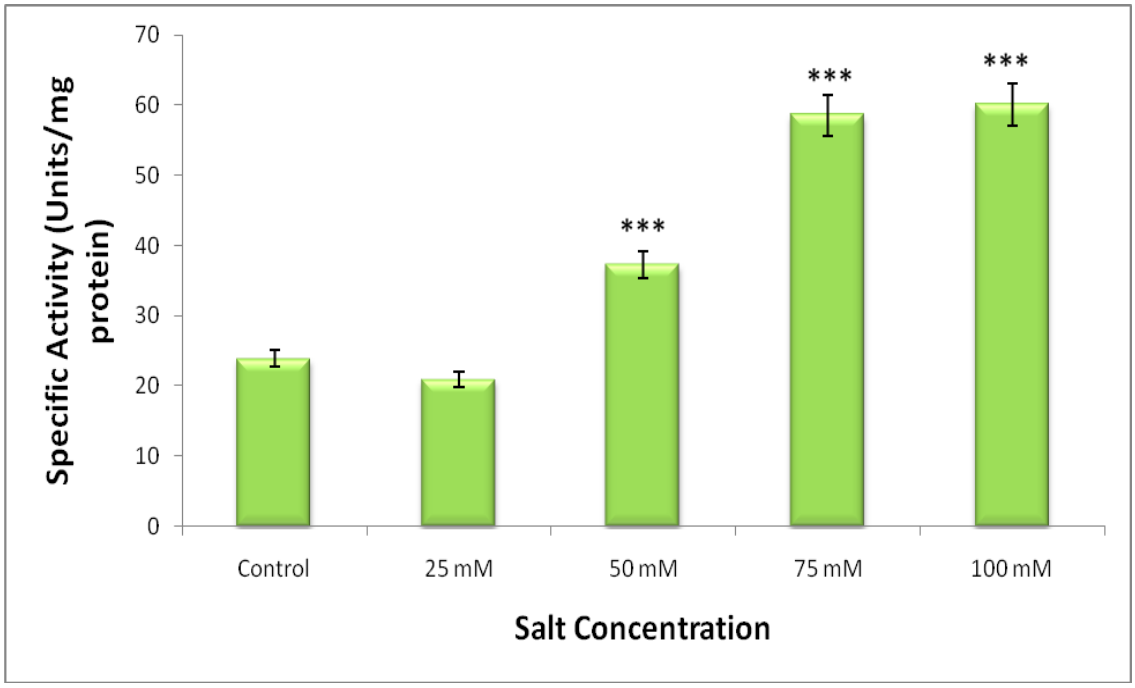


Fig. 3.11: Catalase activity at different concentration of NaCl stress.

Values are reported as Mean ± SEM, *,** and *** indicates significantly different at $p<0.05$ and $p<0.001$ $p<0.001$ respectively as compared to the corresponding control N=5

3.4.5 Effect of salt stress on Peroxidase release

Peroxidase activity increased with increased exposure to salt indicating that the hydrogen peroxide produced during salt stress could be effectively removed by it. Peroxidase activity increase with respect to salt concentration. As compared to catalase, there was increase in peroxidase activity at 75mM and 100mM. So it can be concluded that at very high salt concentration peroxidase rather than catalase plays major role in combating salt.

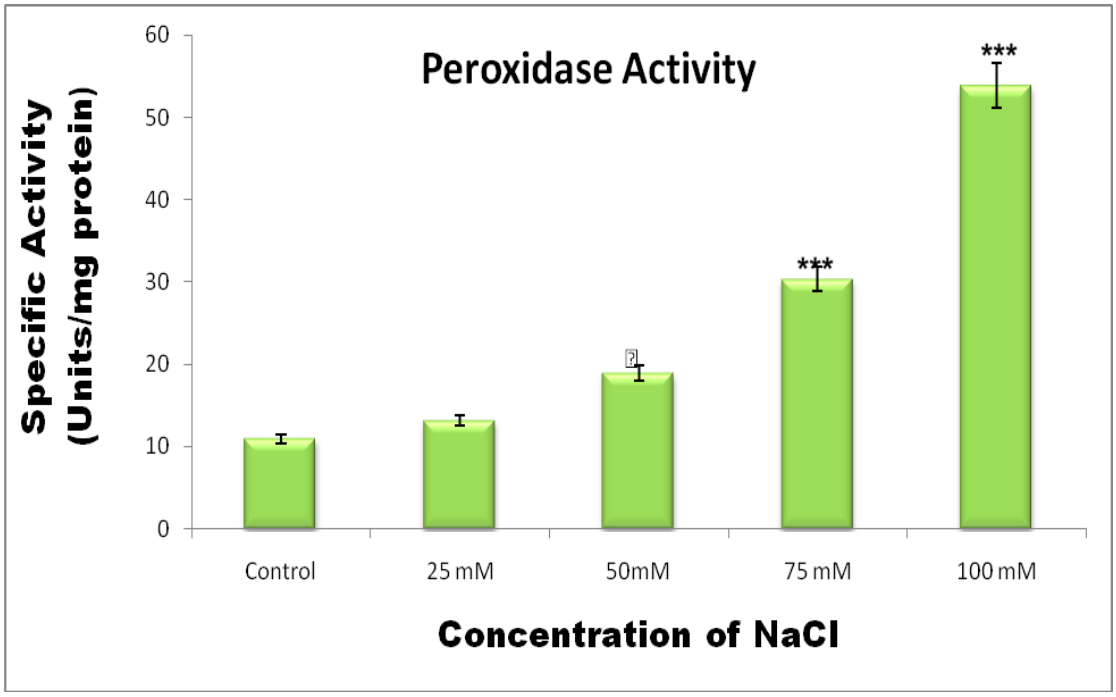


Fig. 3.12: Peroxidase activity at different concentration of NaCl stress.

Values are reported as Mean + SEM, *** indicates significantly different at $p<0.05$ and $p<0.001$ $p<0.001$ respectively as compared to the corrosponding control. N=5

It has been shown, that high salinity leads to programmed cell death (PCD) in higher plants which could be regarded as a salt adaptation mechanism (Huh et al., 2002). The status of mitochondrial permeability transition pore (PTP) and levels of reactive oxygen species (ROS) play key roles in regulating apoptosis in animal cells. Similar role of mitochondria has been observed in plants mediating animal like apoptosis cell death. It is known that at higher salt stress there will be increase in generation of reactive oxygen species that lead to change in mitochondrial transition pore permeability, and in turn release of cytochrome C leads to apoptosis like cell death. It has been shown that at higher salt concentration of salt stress ROS species will damage biomolecules like DNA resulting in oligonucleosomal fragements generation (DNA laddering) (Lin et al., 2006). But when plants are exposed to very high salt concentration that causes severe damage to cells, necrosis will occur, and this gives DNA smear on gel. Using the above mentioned method of DNA isolation, smeared DNA was obtained. Hence, there is a need of improvement of isolation technique in order to be able to study DNA fragmentation.

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^{o}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).

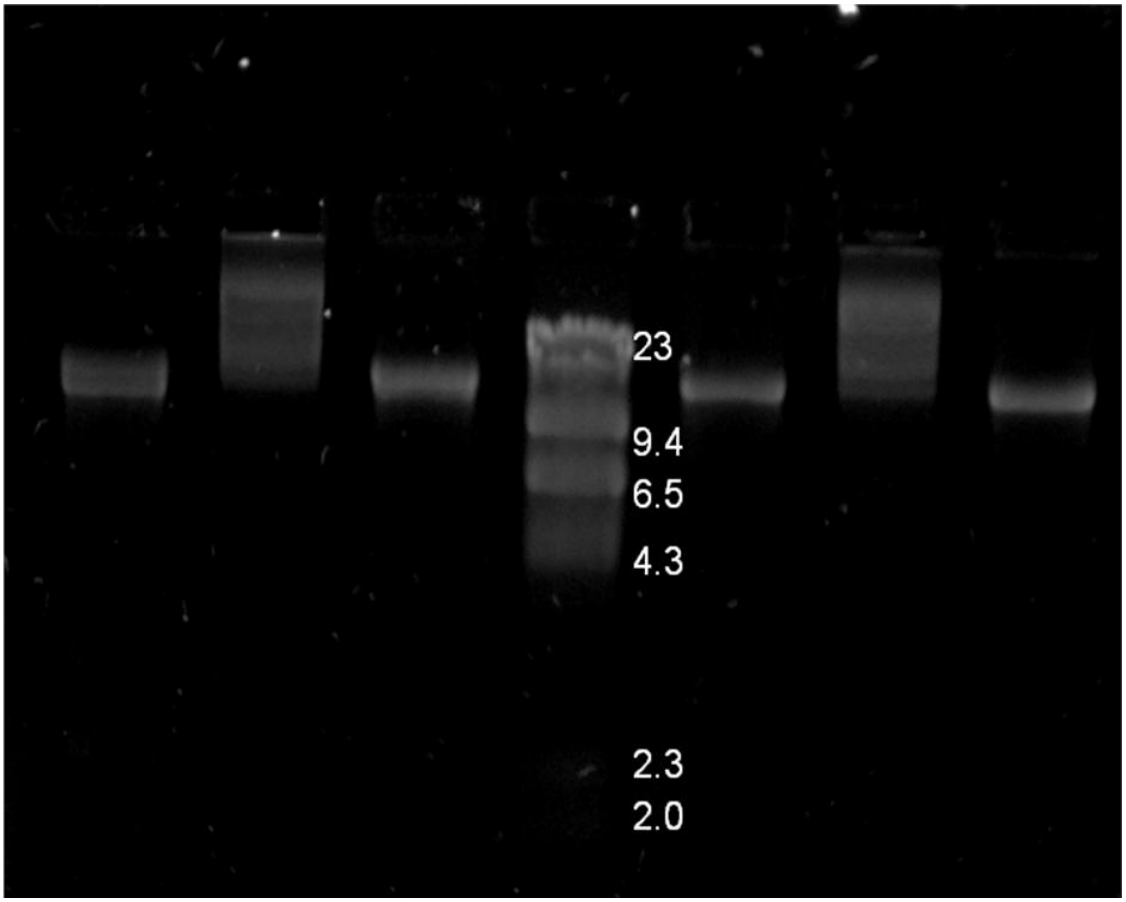


Fig.4.1 : Confirmation of the plasmid by agarose gel electrophoresis.

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

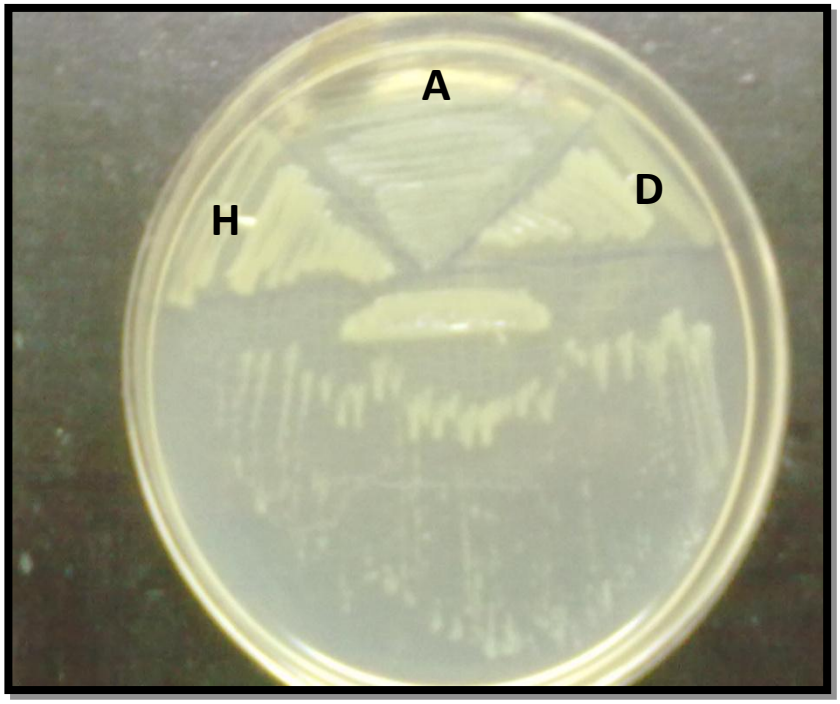


Fig. 4.2: Triparental mating (A: *Agrobacterium tumefaciens* (Rif^R Tet^R); D: Donor pHS724 (Kan^R); H: Helper strain (Kan^R)

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.

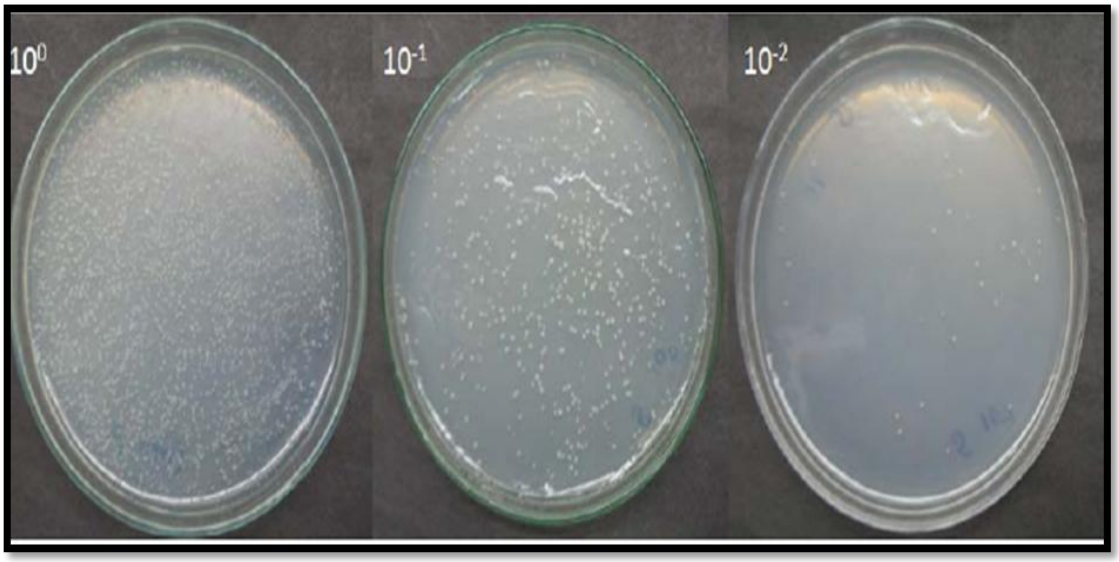


Fig. 4.3: *Agrobacterium tumefaciens* on LA plate

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

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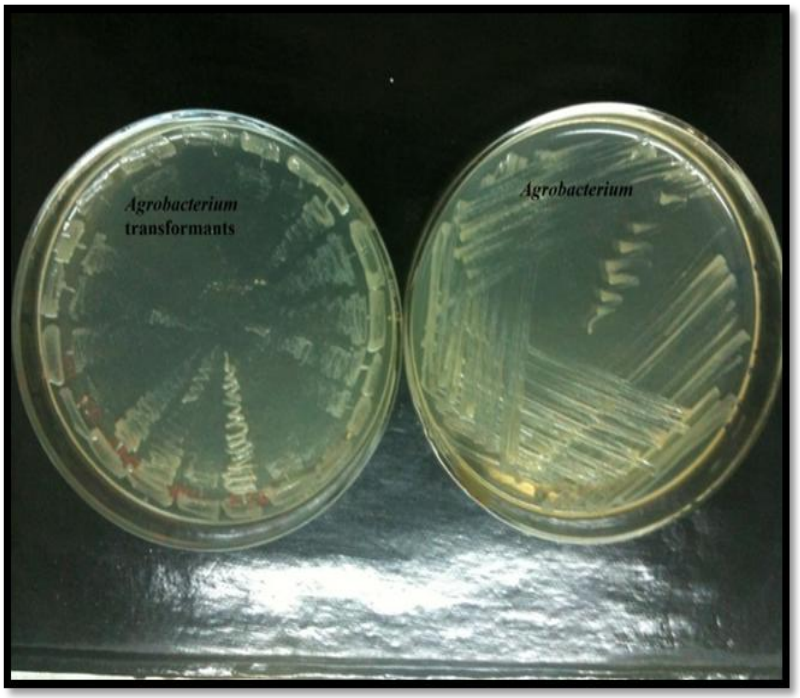

Fig 4.6: *Agrobacterium* and *Agrobacterium* transformants on a medium containing 0.1M NaCl

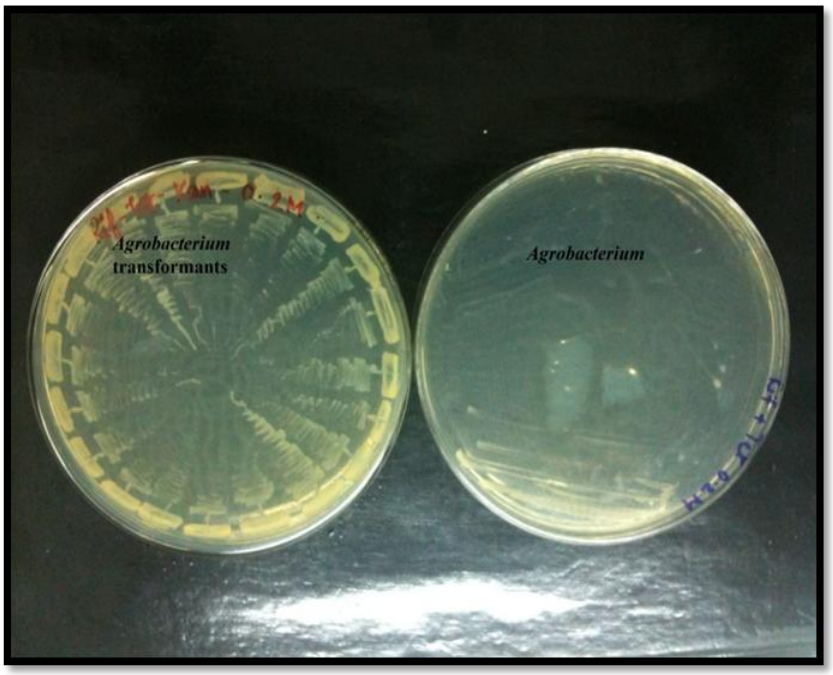

Fig 4.7: *Agrobacterium* and *Agrobacterium* transformants on a medium containing 0.2M NaCl

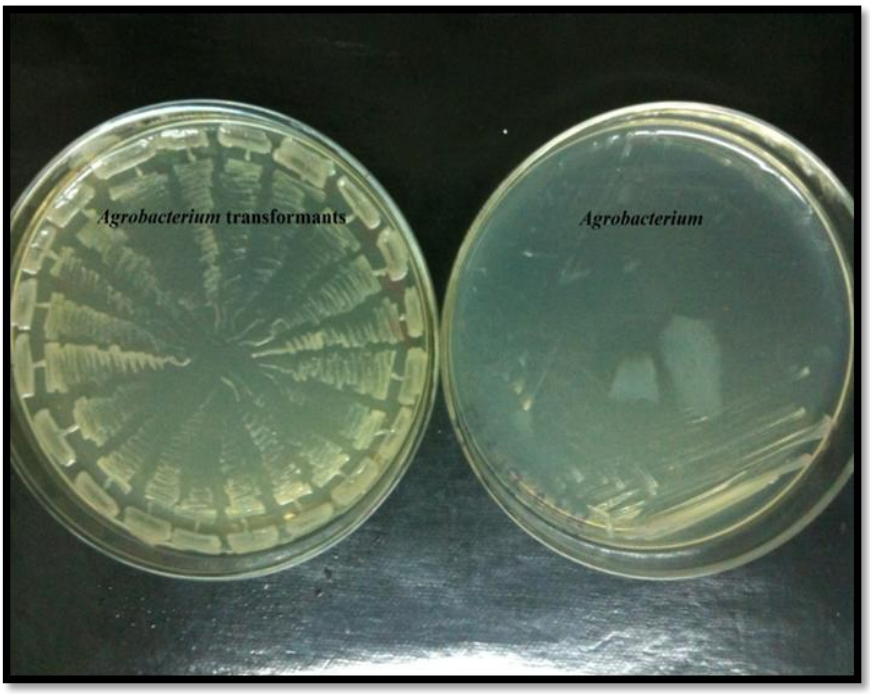

Fig 4.8: *Agrobacterium* and *Agrobacterium* transformants on a medium containing 0.3M NaCl

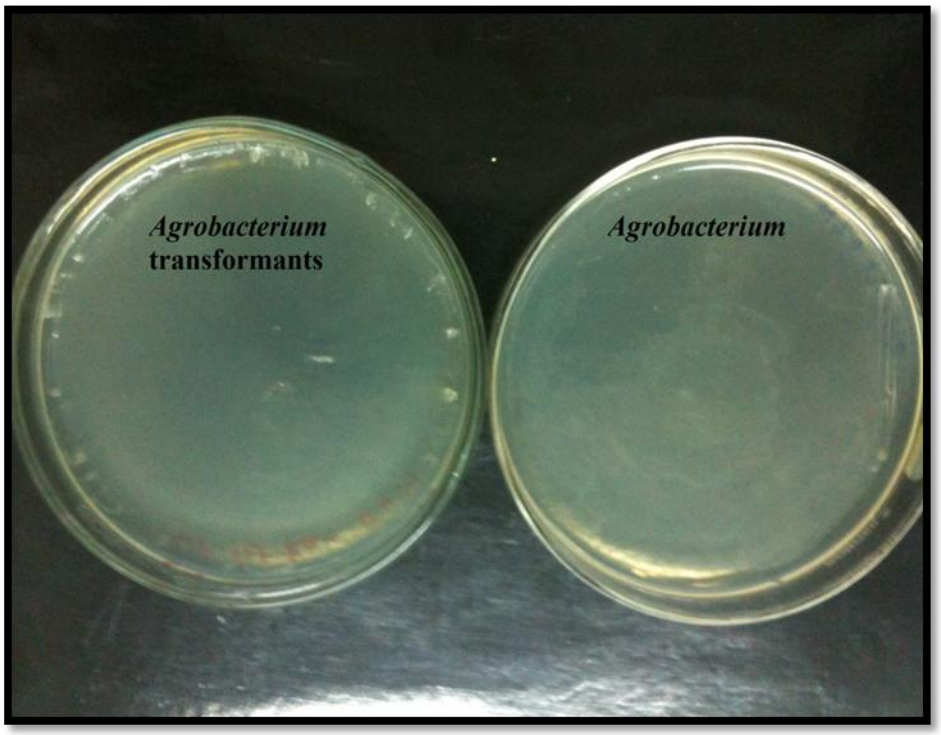

Fig 4.9: *Agrobacterium* and *Agrobacterium* transformants on a medium containing 0.4M NaCl

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).

Chapter 5

TRANSFORMATION OF PLANTS FOR SALINE TOLERANCE

5.1 Strategies of generating transgenic plants

The following are the major strategies for creating transgenic plants:

5.1.1 Electroporation:

In this technique, protoplasts are incubated in buffer solution containing DNA and subjected to high-voltage electrical pulses. DNA migrates through pores in the membrane and integrates into the plant genome (Sorokin et al., 2000)

Advantages- This technique is a Simple, fast and inexpensive way for transient and stable transformation of different tissues.
Disadvantages- It has a low efficiency and requires careful optimization, also the range of tissues that can be transformed is narrow as compared to particle bombardment.

5.1.2 Microprojectile Bombardment:

In this technique Tungsten or gold particles are coated with DNA and accelerated towards target plant tissues using a special apparatus called a particle gun. These accelerated particles punch holes in the plant cell wall and usually penetrate only1-2 cell layers. The DNA-coated particles goes either near or in the nucleus, where the DNA comes off the particles and integrates into plant chromosomal DNA (Klein et al., 1987).

Advantages- It is one of the most versatile and effective technique. It is also environmentally friendly.

Disadvantages-low efficiency of transformation, low survival of bombarded cells caused by mechanical damages, DNA is not protected during bombardment, high number of transgene copies, random intracellular target.

5.1.3 Microinjection:

Using a micromanipulator and an inverted microscope, DNA is injected into cells or protoplasts by a syringe. (Holm, 2000)

Advantages-Possibility for optimization of amount of DNA delivered to a single plant cell, precision and predictability of the DNA delivery place.

Disadvantages - A single cell receives DNA during a single injection event. It is an expensive technique and requires exceptional manual capabilities.

The above methods have been used widely in plants but have the following disadvantages:

1. A relatively low efficiency of transformation
2. Integration of many transgene copies into the plant genome
3. Rearrangements of the transgene copies and chromosomes
4. Transgene silencing

5.1.4 *Agrobacterium tumefaciens* mediated transformation:

This has become the method of choice for plant molecular biologists. In this method, co-cultivation of plants or plant explants with *Agrobacterium* is done and the gene of interest is transferred by T-DNA mediated transfer from bacterial cell to the plant genome.

The phytopathogenic bacterium *Agrobacterium tumefaciens* genetically transforms plants by transferring a portion of its resident Ti plasmid, the T-

DNA (Transferred DNA) to the plant cells (Gelvin, 2000). It naturally infects wounded plant tissues and causes the formation of crown gall tumors (de la Riva, 1998). Virulent strains of *A.tumefaciens* harbor large plasmids of size 140-235 kb (Zaenen et al., 1974)

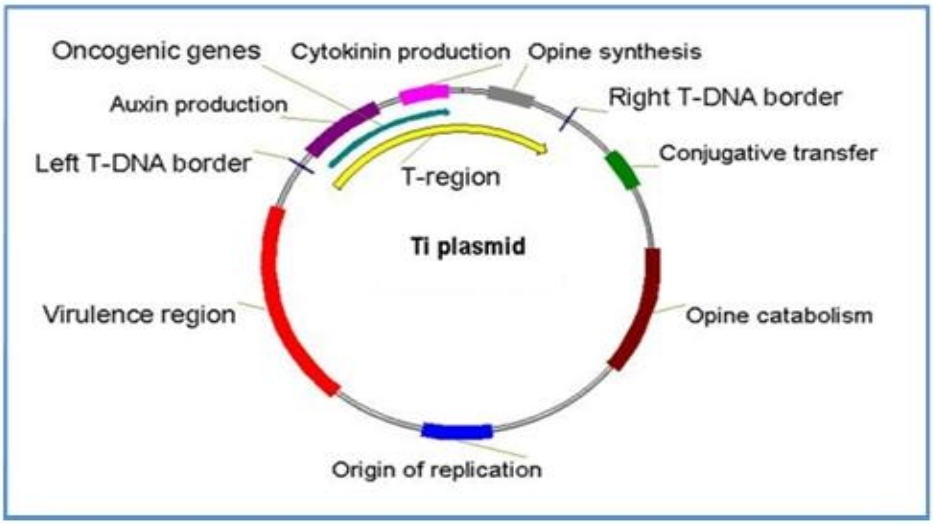

Fig. 5.1: Ti Plasmid (Chilton MD, 1978)

5.1.4.1 Ti Plasmid

The Ti plasmid consists of the following regions:

1) Right T-DNA border sequence and Left T-DNA border sequence. These are imperfect direct repeats flanking the T-DNA region. They are not transferred to the plant genome but are essential components in the transfer process.

2) Origin of replication which lies outside the T-DNA and allows the Ti plasmid to be stably maintained in *A. tumefaciens*.

3) Gene for opine catabolism which lies outside the T-DNA region. Opines are unique and unusual condensation products of an amino acid and keto acid.They are synthesized within the crown gall and then secreted. They can be used as a carbon and nitrogen source by an *A. tumefaciens* carrying a Ti plasmid bearing the gene for catabolism of that particular opine. This is therefore a unique mechanism that has evolved where each strain of *A.*

tumefaciens genetically transforms only those plant cells which produce a compound that it alone is able to utilize (Glick, 2003).

4) T-DNA region which consists of oncogenic genes namely those for auxin and cytokinin production. These phytohormones are responsible for the tumorous growth which results due to a disturbance in the levels of the phytohormones. It also contains gene for opine synthesis. All genes of the T-DNA have promoter sites and polyadenylation sites that are eukaryotic in nature (Ooms et al., 1981).

5) The vir (virulence) genes that lie outside the T-DNA region encodes virulence (Vir) proteins process the T-DNA region from the Ti-plasmid, producing a 'T-strand'. After the bacterium attaches to a plant cell, the T-strand and several types of Vir proteins are transferred to the plant through a transport channel. Inside the plant cell, the Vir proteins interact with the T-strand, forming a T-complex. This complex targets the nucleus, allowing the T-DNA to integrate into the plant genome and express the encoded genes as shown below.

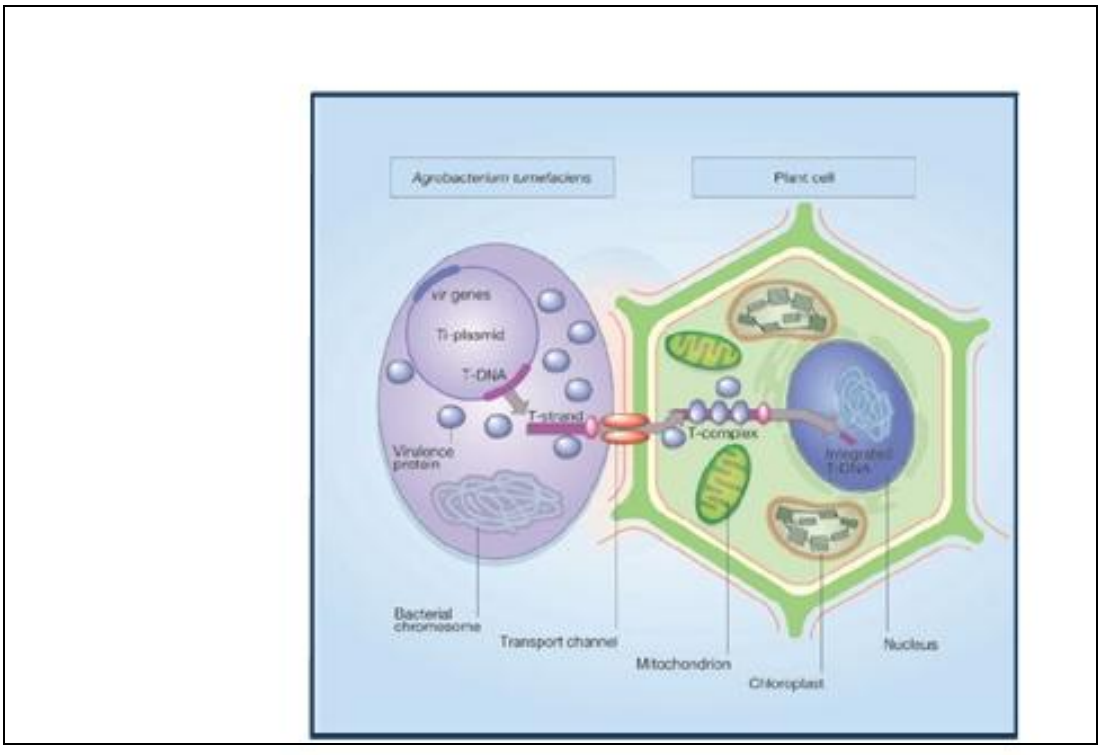

Fig. 5.2: Transformation of plant cells by Agrobacterium (Gelvin, 2005)

As the T-DNA was found to be the only part of the Ti plasmid to be transferred to plant cells, it was proposed that Ti plasmids could be used as a cloning vector for introducing foreign genes to plants where the gene of interest could be inserted in the T-DNA region and thus use this plasmid for transferring the gene of interest to plant cells. However there were certain limitations noticed when the natural Ti plasmid was used as a vector which are as follows:

1) The natural Ti plasmids being large molecules are extremely difficult to handle.
2) The natural Ti plasmid cannot replicate in *E. coli* as it lacks an *E. coli* replication origin and cannot be manipulated in *E. coli*
3) This plasmid lacks unique restriction endonuclease sites which are a prerequisite for any cloning experiment.
4) The production of phytohormones by transformed plant cells which is undesirable as it prevents transformed plant cells from being regenerated into mature plants.

Taking into consideration these limitations, following modifications were made in the Ti plasmid so that it became suitable as a vector:

1) The genes for synthesis of phytohormones were deleted so that tumors could not be generated in transformed plant cells and caused a decrease in the size of the plasmid.
2) Other dispensable regions were also removed causing a considerable decrease in the size of the plasmid.
3) An *E. coli* replication origin was introduced outside the T-DNA.
4) A bacterial selectable marker gene was added outside the T-DNA.
5) A multiple cloning site (MCS) was added in the T-DNA region to facilitate cloning the gene of interest into the T-DNA region.
6) A plant selectable marker gene was introduced in the T-DNA region so that it facilitated selection of transformed plant cells.

These modifications did not prevent the T-DNA transfer to the plant cells.
When the T-DNA of a Ti plasmid is deleted, it is termed as a disarmed Ti plasmid. In order to use the modified Ti plasmid as a vector two different types of vector systems were developed:

5.1.4.2 Co integrative vector

Cointegrative vector system was developed where it consisted of the cointegrate vector which contained the *E. coli* replication origin, a bacterial selectable marker gene operative in both *E. coli* and *A. tumefaciens*, the cloned gene of interest, the right border sequence, plant selectable marker gene and a region homologous to a segment of the disarmed plasmid contained in *A. tumefaciens*. The disarmed plasmid contained the *A. tumefaciens* replication origin, the left border sequence and the vir gene cluster. When the cointegrate vector was transformed in an *A. tumefaciens* bearing the disarmed plasmid, recombination occurred between the two plasmids to form the recombinant Ti plasmid (cointegrated plasmid). This was now capable of transferring the gene of interest to the plant cells when infected as the gene of interest was now located between the left and right border sequences. In this case, the T-DNA was cis with respect to the vir genes. The cointegrate vector could not replicate in *A. tumefaciens* as it lacked *A. tumefaciens* replication origin (Gelvin, 2003)

5.1.4.3 Binary vector

Binary vector system was a further advancement. It was constructed on the basis that there was no special advantage in retaining the T-DNA cis with respect to the vir genes (Hoekema et al., 1983). The observation that the T-DNA need not be physically linked to the vir genes for the transfer of the T-DNA into plant cells resulted in the development of the binary vector system (de Framond et al., 1983). In this system, the binary cloning vector contained both E. coli and *A. tumefaciens* replication origins outside the T-DNA making it a shuttle vector, a bacterial selectable marker gene operative

both in E. coli and *A. tumefaciens*, a plant selectable marker gene and the gene of interest cloned between the left and right border sequences. Cloning of the gene was performed in *E. coli*. The cloned plasmid was transferred to an *A. tumefaciens* strain bearing a disarmed Ti plasmid. The disarmed Ti plasmid provided the Vir proteins in order to enable the T-DNA of the binary vector to be transferred to plant cells (Gelvin, 2003).

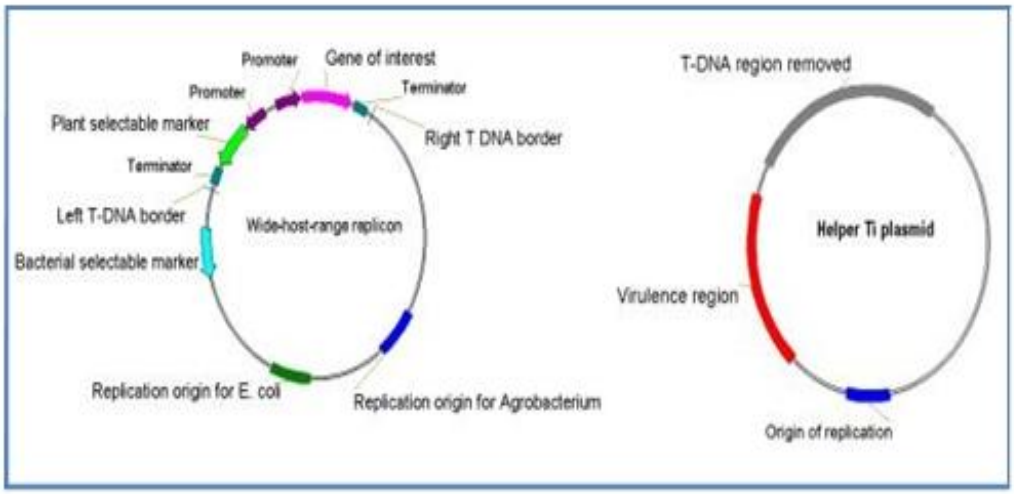

Fig. 5.3: Binary Vector System (Hoekema *et al*.1983),

The binary vector system has advantages over the cointegrative vector system which are as follows:

1) Use of binary vector system obviates the need for in vivo recombination.
2) The binary vector exists as a separate replicon and its copy number is not linked strictly to the copy number of the Ti plasmid.
3) Use of binary vector system is more efficient and quicker (Walkerpeach and Velten, 1994).

5.2 Materials and Methods

Agrobacterium mediated transformation of groundnut.

5.2.1 Plant Material, culture initiation and maintenance

Ground nut var. GG-20 obtained from Junagadh Agricultural University was used as the source of planting material.

The mature seeds were washed under running tap water for 25 min., treated with a solution of detergent Extran for 10 min, then subjected to treatment of

antifungal agent Bavistin (2%) for 10 min and finally washed thoroughly in sterile double distilled water.

Further procedures were carried out in Laminar Air Flow under sterile condition. Seeds were rinsed in 70% iso propyl alcohol (1 min) followed by 3 to 4 washes with double distilled water. The explants were then treated with 0.1% $HgCl_2$ for 7.5 min followed by three washes of 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.

Transformation was attempted using three different explants and regeneration protocol as explained below.

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 a test tubes. In case of embryoid axis and cotyledon explants, the explants were collected from 3-days-old seedlings. While in case of young immature leaves the explants were taken from 7 days 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.

In case of young green immature leaflet explants, seven days pre-incubated seeds on MS medium with 2.22 µM BAP were taken out aseptically, elongated radical was cut apart and the green young leaflets were separated and used as explants.

5.2.2 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 Nos T, nopaline synthase gene terminator sequences served as reporter gene. The neomycin phosphotransferase II (nptll) gene driven by the nopaline synthase (NOS) promoter and Nos T, nopaline synthase gene terminator sequences was used as the selectable marker gene. Plasmid pHS724 contains the gene of interest - *cox* for choline oxidase gene and kanamycin resistance gene for bacterial selection. Plasmid pHS724 carries gene of interest driven by a double 35S promoter, and terminated by 35S poly A signal and the *nptII* and *uidA* genes.

5.2.3 Vector Map:

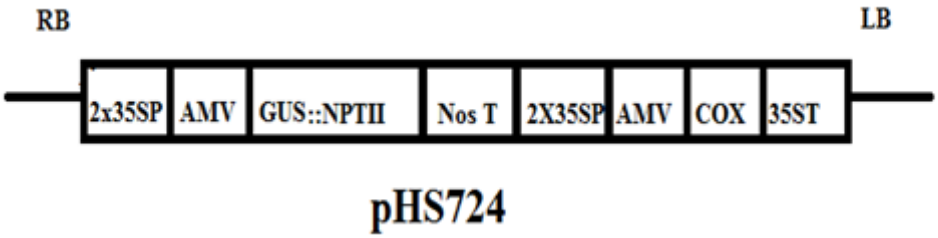

Fig. 5.4 : Vector map

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.

5.2.4 Co-cultivation

5.2.4.1 Preparation of *Agrobacterium* suspension for co-cultivation

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^{o}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 µM acetosyringone. The suspension was kept at 28^{o}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 on to co-cultivation medium for 5 days at 21^{o}C in the dark. The co-cultivation medium for different explants was different as mentioned in the table-5.1.

5.2.4.2 PROTOCOL USED FOR *AGROBACTRIUM* MEDIATED TRANSFORMATION

Isolate single Colony of *Agrobacterium* strain

↓

Incubate in 50ml YEP medium and keep over night/2 days on shaker @ 200 rpm at 28 ^{o}C

↓

Growth of the bacterial suspension should be to an OD 600 of 1.4-1.6

↓

Centrifuge the suspension at 5000 rpm and re-suspend pellet in liquid MS (MS salts + B_5 vitamins + 100 mg/L myo-inositol + 100 µM acetosyringone + 2% sucrose)

↓

Keep suspension at 28 ^{o}C for about half an hour and use it for explant infection

↓

Infect the surface sterilized explants with above suspension for about 20 min at RT, continuously shaking

↓

Transfer explants ascetically on to co-cultivation medium and incubate at 28 ^{o}C for 2-5 days in dark.

(MS salts + B5 vitamins + 100 mg/L myo-inositol + PGR + 3% sucrose)

↓

After co-cultivation, rinse explants 5-6 times with sterile DD water, remove excess bacterial suspension by blotting and inoculate in induction medium with the distal end in direct contact of media and incubate for 3-4 weeks.

The cotyledonary explants and the embryo axis with cotyledon were placed in such a way that adaxial surface of the cotyledon were in direct contact with the medium. The immature leaf explants were given superficial cuts on the margin and placed on the medium.

After 5 days of incubation the *Agrobacterium* infection was conspicuously seen in ring fashion on cotyledons and the leaf explants (Figure-5.5). After co-cultivation for 5 days, the explants were rinsed 5–6 times with sterile water separately under laminar hood. All explants were blotted on sterile paper to remove excess bacterial suspension and further placed on different shoot induction medium as shown in table 5.1. Cefotaxime (Alkem, India) (200 mg/l) was added in all shoot induction medium 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.

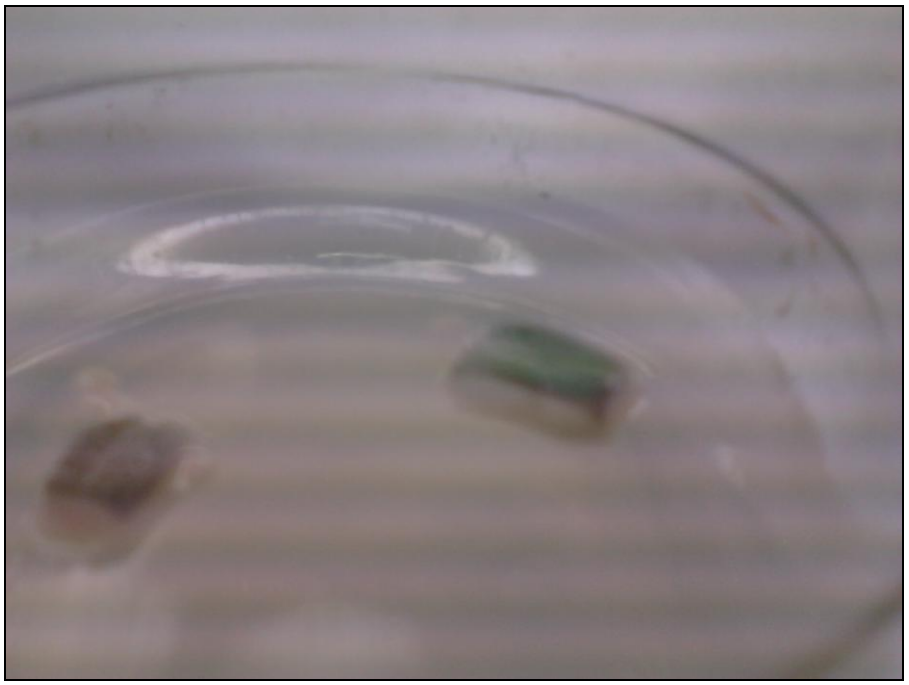

Fig. – 5.5A

Fig. 5.5B

Fig.5.5A: Infection of leaf explants after 5 day co-cultivation with *Agrobacterium*.
Fig.5.5B:Infection of cotyledon explants after 5 day co-cultivation with *Agrobacterium*.

Explants	**Embryo axis**	**Cotyledon explants**	**Young leaves**
Co-cultivation medium	MS salts, B5 vitamins, 100 mg/l myo-inositol, 30 g/l sucrose, 6.5 g/l agar 17.76 µM BAP and 0.53 µM NAA	MS salts, B5 vitamins, 100 mg/l myo-inositol, 30 g/l sucrose, 6.5 g/l agar 17.76 µM BAP and 2.68 µM NAA	MS salts, B5 vitamins, 100 mg/l myo-inositol, 30 g/l sucrose, 6.5 g/l agar 13.32 µM BAP and 2.68 µM NAA
Shoot induction medium	MS salts, B5 vitamins, 100 mg/l myo-inositol, 30 g/l sucrose, 6.5 g/l agar 17.76 µM BAP, 0.53 µM NAA , 200 mg/l cefotaxime	MS salts, B5 vitamins, 100 mg/l myo-inositol, 30 g/l sucrose, 6.5 g/l agar 17.76 µM BAP, 2.68 µM NAA , 200 mg/l cefotaxime	MS salts, B5 vitamins, 100 mg/l myo-inositol, 30 g/l sucrose, 6.5 g/l agar 13.32 µM BAP and 2.68 µM NAA, 200 mg/l cefotaxime
Shoot multiplication and Selection medium	MS salts, B5 vitamins, 100 mg/l myo-inositol, 30 g/l sucrose, 6.5 g/l agar 8.88 µM BAP, 0.53 µM NAA, 200 mg/l cefotaxime 60 mg/l kanamycin	MS salts, B5 vitamins, 100 mg/l myo-inositol, 30 g/l sucrose, 6.5 g/l agar 8.88 µM BAP, 200 mg/l cefotaxime 60 mg/l kanamycin	MS salts, B5 vitamins, 100 mg/l myo-inositol, 30 g/l sucrose, 6.5 g/l agar 13.32 µM BAP and 2.68 µM NAA, 200 mg/l cefotaxime 60mg/l kanamycin
Rooting medium	MS salts, B5 vitamins, 100 mg/l myo-inositol, 30 g/l sucrose, 6.5 g/l agar 4.30 µM NAA,	MS salts, B5 vitamins, 100 mg/l myo-inositol, 30 g/l sucrose, 6.5 g/l agar 4.30 µM NAA,	MS salts, B5 vitamins, 100 mg/l myo-inositol, 30 g/l sucrose, 6.5 g/l agar 4.30 µM NAA,

Table – 5.1: MS medium with different combinations used during different stages of production of transformed plants using three different explants of groundnut var. GG20.

5.2.5 Selection of transformants, multiplication and growth conditions

After 4 weeks of incubation on shoot induction medium, shoot forming buds were observed growing directly on embryo axis explants as well as proximal end of the cotyledons explants. However, immature young leaf explants

could not grow properly on the selection medium. The bud forming region from the embryo axis explants and cotyledon explants were transferred to respective 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 8.88 µM BAP, 200 mg/l cefotaxime and different concentrations of kanamycin (Macleods, India) for both the type of explants.

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.

5.2.6 GUS analysis (Jefferson et al., 1987)

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*.1987 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.

5.2.6.1 Preparation of GUS assay solution.

X-gluc solution to assay for GUS activity:

1 mM X-Gluc (5-bromo-4-choloro-3-indolyl) ß-D-glucuronic acid was prepared in 0.1% of 50 mM Na_2HPO_4 at pH 7 along with Triton X-100. The aliquots of the solution were stored in the dark in a refrigerator. 0.01 g of X-Gluc was weighed out and dissolved in about 15 ml of sterile distilled water. 1 ml of 1 M sodium phosphate stock solution and 0.02 ml of the detergent Trition X-100 was added and after proper mixing the total volume made to 100 ml with distilled water. The solution was stored in the dark in the refrigerator.

1M sodium phosphate stock solution:

14.2 g of Na2HPO4 (dibasic sodium phosphate) and 13.8 g of NaH2PO4**.**H2O (monobasic sodium phosphate) was dissolved in approximately 90 ml of sterile, distilled water. pH was adjusted to 7.0 using NaOH. And the total volume was adjusted to 100 ml with distilled water.

5.3 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. Co-cultivated explants swelled and developed shoot buds with little callus after 3 weeks on selective shoot regeneration medium (Fig. 5.6). Shoot clumps which survived this selection step were sub-cultured to shoot multiplication medium. Differences in explant response on transformation frequency were apparent in culture (Table 5.2). Two weeks after culturing in selective 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 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 (Fig. 5.7). 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.

Fig 5.6A

Fig 5.6B

Fig –5.6 : Cotyledon explants after 3 weeks of incubation on regeneration medium
A: Swelling of explants with growing buds and callus at the proximal end **cotyledon explants**.
B: Shoot bud producing plants from the proximal end of cotyledon explants.

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	-

Table 5.2. Effect of co-cultivation period on shoot bud regeneration on three explants of groundnut on shoot induction medium with 60 mg/l kanamycin sulfate.

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

5.3.1 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 compare 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. These young leaves explants were transferred to the respective medium as per experimental design till rooting.

Fig.5.7 A

Fig. 5.7 B

Fig. -5.7 C

Fig. 5.7 Comparison of growth performance of transformed and control plants in kanamycin rich MS medium.

A: Untransformed plants in MS medium without kanamycin.

B: Untransformed plants in MS medium with 60 mg/l kanamycin.

C: Transformed plants in MS medium with 60 mg/l kanamycin.

5.3.2 Effect of Induction medium.

The excised multiple shoot buds from cotyledon and embryo axis explants were sub-cultured for two to three cycles on respective MS medium as shown in table 5.1. The transformation experiment was conducted with 30 explants each. The induction medium has profound effect on the number of shoots buds development and subsequent regeneration. Out of various combinations of BAP and NAA used for all the kinds of explants, the concentrations mentioned in table 5.1 could only yielded considerable number of shoots. Following two cycles of kanamycin selection, 32 shoots were recovered from the embryo axis explants while 20 shoots were recovered from cotyledon explants.

5.3.3 Effect of co-cultivation periods on transformation

Co-cultivation period had influence on the transformation frequency of different explants. Transformation frequencies of embryo axis and cotyledon explants co-cultivated on respective shoot regeneration medium for different periods are shown in Table 5.1. After two cycle 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 could be explained by the fact that the concentration of *Agrobacterium* was higher after 5 days of cultivation and this could increase considerably 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 *et al.,* 1991). Co-cultivation for 48 h has been generally adopted, although longer periods have been reported to enhance the transformation efficiency in other legumes (Chabaud *et al.,* 1988, Geetha *et al.,* 1997).

Fig.5.8: Transformed multiple shoot from embryo axis explants.

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.

5.3.4 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 5.3 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 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/l kanamycin also caused chlorosis and death of the explants. Thus, transformation efficiency was decreased when there was an increase in the kanamycin level beyond 60 mg/I. 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/I kanamycin was used for selecting transformants in subsequent subculture to prevent possible escapes. Similar findings were also reported by Kar et al. (1996) in chickpea and Venkatchalam et al. (1998).

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

Table 5.3: Transformation frequency of groundnut var. GG20 as influenced by explant type and level of kanamycin sulfate.

5.3.5 Confirmation of transformants

5.3.5.1 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 (Fig.5.9), 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 room 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-5.11) 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 developing leaf burning and curling from the leaf margins while the transformed plants did not show such sign of burning of leaf edges 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* (CODA).

Fig. 5.9 Comparison of transformed plant with the control plants growing on MS medium without stress.

Fig. 5.10 Transformed plants which survived 2 cycle selection under kanamycin rich medium growing on MS medium with 100 mM NaCl .

Fig. 5.11 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: Transgenic plant in MS medium with 100 mM NaCl.

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. 5.12). Both leaves and cotyledon parts of the transformants showed dense GUS positive sectors and not only GUS spots which indicate uniform transgene integration.

Fig. 5.12B Blue colored X – gluc staining in the leaves of transformed plants.

Fig. 5.12A Comparison of X – gluc staining in the leaves of control & transformed plants.

L : control plant

R : Transformed plant

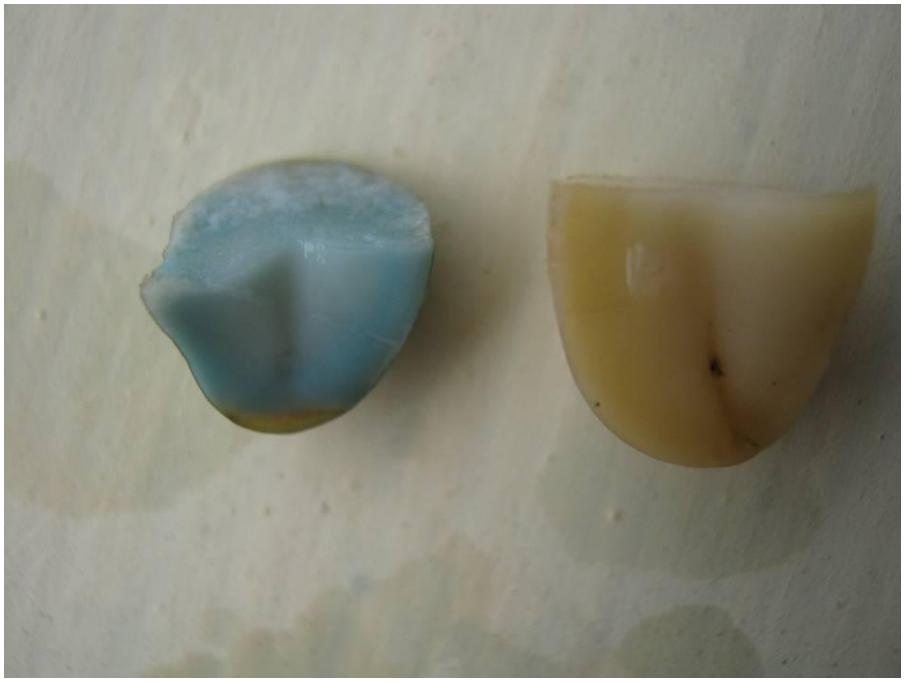

Fig. 5.13 Comparison of X – gluc staining in the cotyledon tissues of control & transformed plants

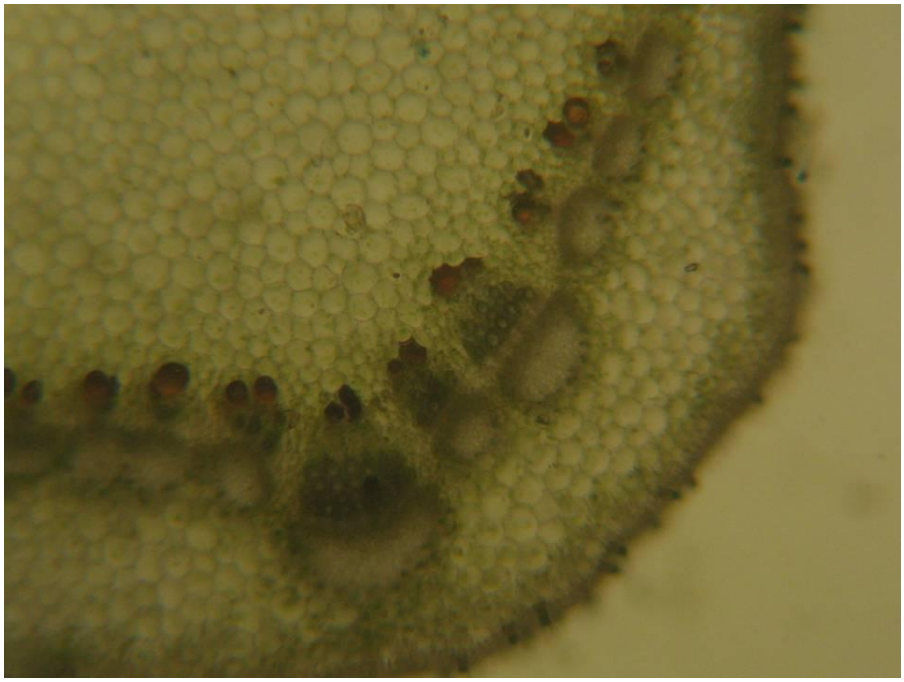

Fig.5.14 Blue spots of X – gluc staining in the cells of stems

5.3.5.2 GUS Expression:

This is a typical expression pattern of the CaMV 35S promoter regulated uidA gene in young tissues. Leaf tissues from untransformed control plants did not show GUS activity. These results and the extensive GUS expression in tissues, clearly demonstrate the stability of the inserted genes in the transformed plants. GUS expression has been reported previously by many workers in the groundnut (Eapen and George, 1994; Cheng et al., 1996).

However, further acclimation of these transgenic plants and growth performance under different salt concentrations in pots or soil is required to be performed.

SUMMARY

- The optimum surface sterilization treatment for best explant survival and initiation of regeneration was standardized for groundnut seeds explants. The treatment of 0.1% $HgCl_2$ for 7.5 min followed by three double distilled water wash gave around 80% sterile explant initiation without any aberrant effect.

- Direct organogenesis was achieved from the young, immature leaflets on MS medium with 13.32 µM BAP and 2.68 µM NAA which gave 70 % direct shoot formation within 30 days of incubation.

- Direct organogenesis was achieved from the cotyledon explants on MS medium with 17.76 µM BAP and 2.68 µM NAA which gave 65 % direct shoot bud initiation within 30 days of incubation.

- Direct organogenesis was achieved from the embryo axis explants on MS medium with 17.76 µM BAP and 0.53 µM NAA which gave 76 % direct shoot formation within 30 days of incubation.

- Indirect organogenesis was achieved from the mature leaf explants on MS medium with 2.32 µM kinetin after 4-5 cub cultures on the same medium within 5-6 months.

- *In vitro* rooting of the plantlets produced from various explants was achieved on MS medium with 4.30 µM NAA within 30 days of transfer.

- In the pot experiments, it was observed that groundnut plants could withstand salinity up to 50 mM NaCl concentrations beyond which all the morphological growth parameters were observed to be affected.

- In the *in vitro* seed germination experiments also it was observed that the seeds could germinate normally in the medium containing 50 mM NaCl without delay compared to control.

- The release of H_2O_2 was observed to be increasing with increase in exposure of NaCl stress.

- The catalase activity was observed to be increasing with increasing level of NaCl up to 75mM. However, there was no significant change in activity at NaCl concentration of 75mM and 100mM indicating that catalase activity was not adequate for the complete scavenging of H_2O_2 and thus to combat salt stress. Despite its increased activity compared to control.

- The peroxidase activity was observed to be increasing with increasing level of NaCl stress up to 100 mM NaCl linearly. Hence at very high salt concentration peroxidase rather than catalase plays major role in combating salt.

- Transformation of the binarry vector **pHS724** containing gene of interest i.e COX was carried out through $CaCl_2$ mediated transformation 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).

- 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).

- *Agrobacterium tumefaciens* LBA4404 was transformed with the COX gene through triparental mating using *E.coli* DH5α with pHS724 (kan^R) as the donor strain, E.coli DH5α with pRK2013 (kanR) as the helper strain under appropriate antibiotic selection.

- Confirmation of *A.tumefaciens* transformants was carried out through ketolactose test (also known as Benedict's test).

- The phenotypic expression of COX gene in *A.tumefaciens* was carried out by growing in various NaCl concentration where transformants were able to survive in presence of 300mM NaCl.

- Co-cultivation of *A.tumefaciens* containing COX gene with different groundnut explants was carried out for 5 days under dark condition. After co-cultivation, the explants were washed and inoculated on MS medium containing respective growth hormones for shoot induction.

- The cultures were sub cultured on respective medium containing (cefotaxime 200 mg/l) and kanamycin (60 mg/l) for selection.

- The transgenic plants so raised were rooted on MS medium with 4.30 µM NAA.

- The transformation success was confirmed through GUS assay.

- The transgenic plants so raised were shown to tolerate higher salt levels. However, plants could not be grown beyond 150mM NaCl concentration.

Conclusion

- Transgenic groundnut plants expressing COX gene for synthesis of glycine betaine were produced through of *A.tumefaciens* mediated transformation.
- However, detail analysis of glycine betaine synthesis in transgenic plants and its co-relation with the increase saline tolerance is required to be established.
- This study and results can be used as a stepping stone to produce saline tolerant ground nut plants.

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Presentations and Publications

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

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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.

Genetictransformationovercomestheselimitationsandallowsintroductionofagronomically 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

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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.

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 .

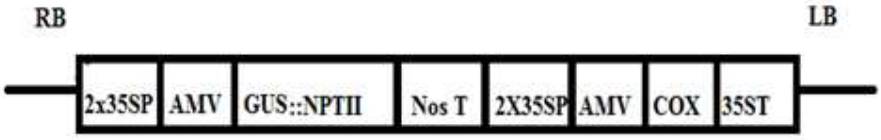
Mature dry seeds were imbibed in sterile double distilled water for 6-h after surface sterilization and kept for germination on autoclaved filter paper bridges in sterile double distilled water in test tubes. In case of embryo axis and cotyledon explants, the explants were collected from 3-day-old seedlings. 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^{o}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 *uid*A gene and *npt*II 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

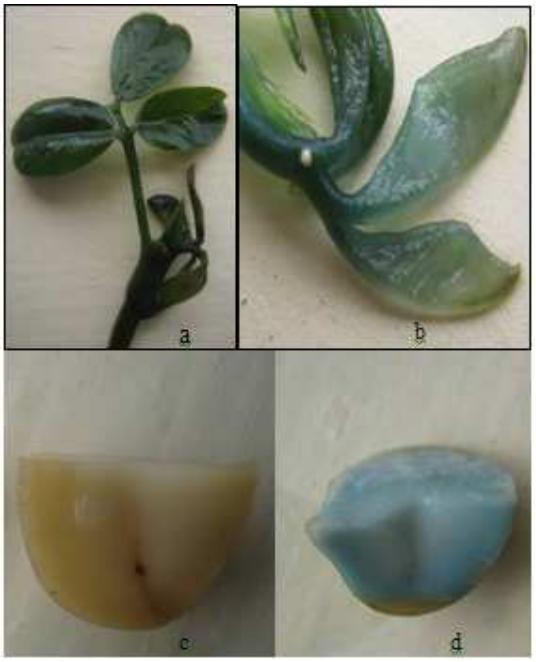


Figure -2.
X – gluc staining in the control and transformed tissues
a: control leaves with no blue staining, b: Transformed leaves with blue staining.c: control cotyledon with no blue staining, d: Transformed cotyledon with blue staining.

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

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

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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^{o}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 $CaCl_2$

1*0*0-200 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).

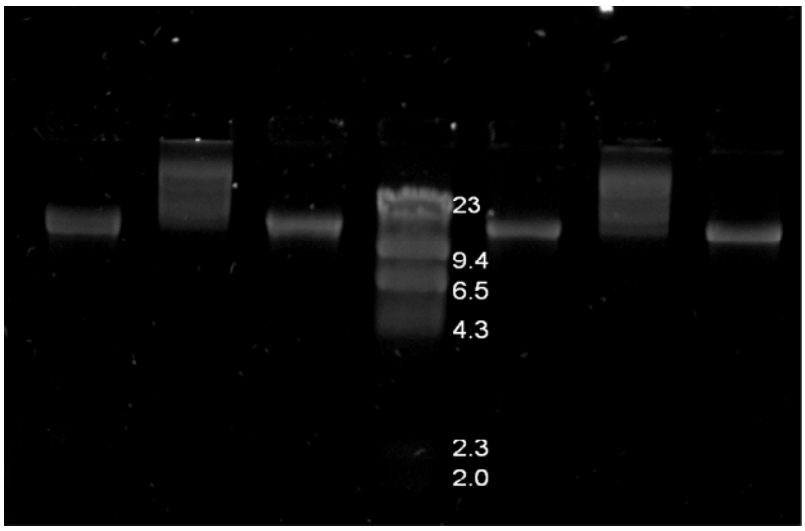

Fig. 1 : Confirmation of the plasmid by agarose gel electrophoresis.

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

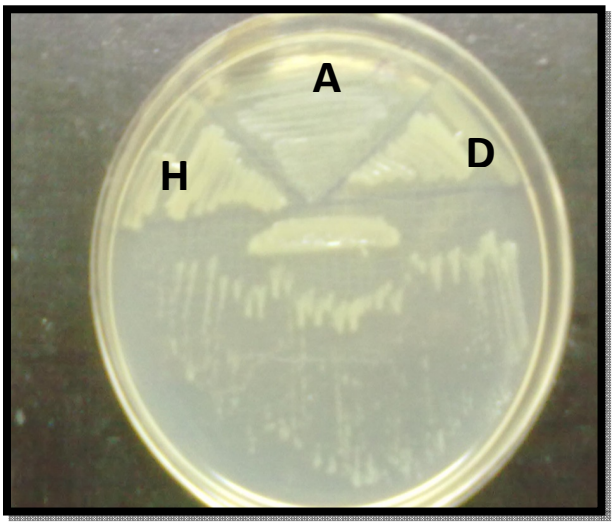

Fig. 2: Triparental mating (A: Agrobacterium tumefaciens (Rif^R Tet^R); D: Donor pHS724 (Kan^R)H: Helper strain (Kan^R)

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.

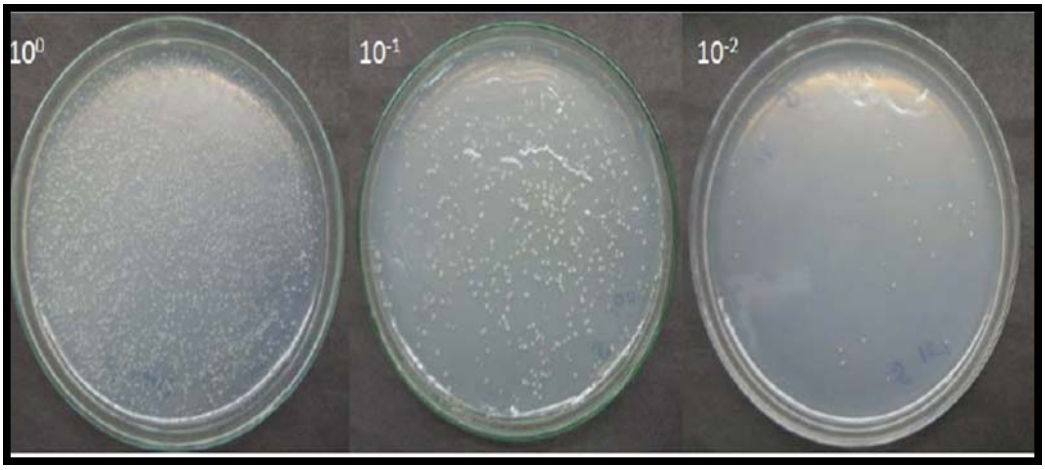

Fig. 3: *Agrobacterium tumefaciens* on LA plate

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.

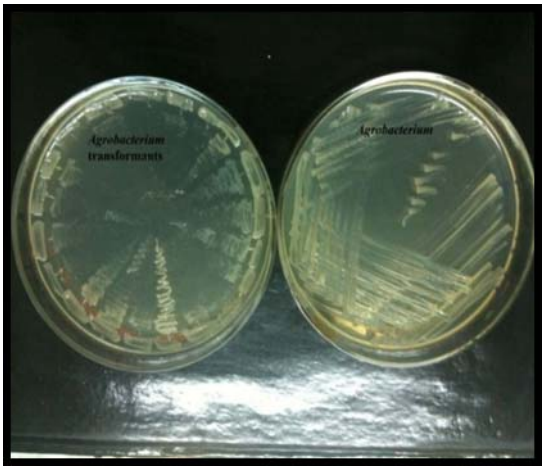

Fig 5a

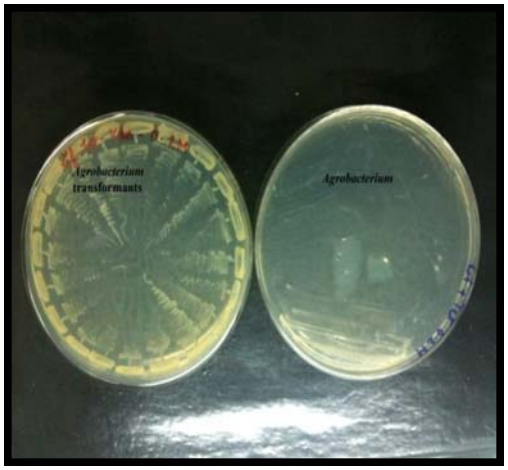

Fig 5b

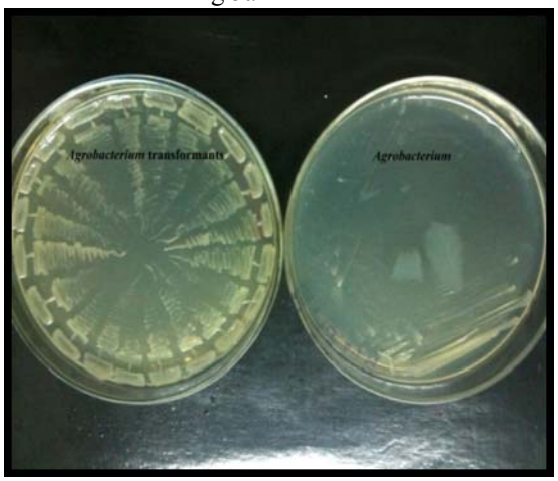

Fig 5c

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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