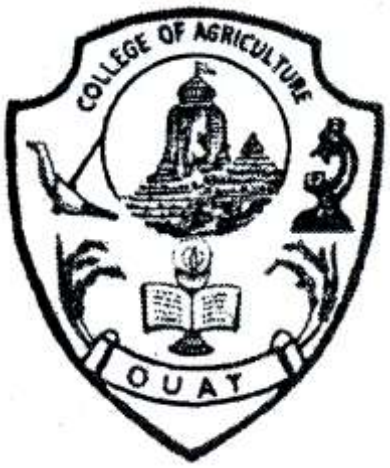
Agrobacterium tumefaciens-MEDIATED GENETIC TRANSFORMATION USING rd29A::DREB1A GENE TO DEVELOP MOISTURE STRESS TOLERANCE IN BRINJAL (*Solanum melongena* L.)

SAGARE DEEPTI BABURAO



DEPARTMENT OF AGRICULTURAL BIOTECHNOLOGY
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A

THESIS SUBMITTED TO
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IN PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
MASTER OF SCIENCE IN AGRICULTURE
(AGRICULTURAL BIOTECHNOLOGY)

By
SAGARE DEEPTI BABURAO

DEPARTMENT OF AGRICULTURAL BIOTECHNOLOGY
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ORISSA UNIVERSITY OF AGRICULTURE & TECHNOLOGY
BHUBANESWAR-751003, ORISSA
2011

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DEDICATED TO MY PARENTS

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CERTIFICATE – I

This is to certify that the thesis entitled "*Agrobacterium tumefaciens* mediated genetic transformation using rd29A::DREB1A gene to develop moisture stress tolerance in Brinjal (*Solanum melongena* L.)" submitted for the degree of Master of Science in Agriculture (Agricultural Biotechnology) embodies a faithful bonafide research work carried out by Sagare Deepti Baburao (205ABT/09) under my guidance and supervision and no part of this thesis has been submitted by her for any other degree or diploma.

It is further certified that she duly acknowledges any help that has been availed of in this connection.

Date:

Place:

Dr. I. C. Mohanty

Chairman

Advisory Committee

CERTIFICATE - II

This is to certify that the thesis entitled "*Agrobacterium tumefaciens* mediated genetic transformation using rd29A::DREB1A gene to develop moisture stress tolerance in Brinjal (*Solanum melongena* L.)" submitted by Sagare Deepti Baburao to the Orissa University of Agriculture & Technology, Bhubaneswar-751003 in partial fulfillment of the degree of Master of Science in Agriculture(Agricultural Biotechnology) has been approved by the students advisory committee after oral examination in collaboration with external examiner.

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ABSTRACT

Moisture stress is one of the major abiotic constraints affecting crop productivity in brinjal (*Solanum melongena L.)*. Water use efficiency under drought conditions is thought to be one of the most promising traits to improve and stabilize crop yields under intermittent water deficit. In the present investigation, genetic improvement for this trait of moisture stress tolerance was carried out through transgenic research in brinjal cv. Utakal Anushree. An efficient and reproducible in vitro regeneration protocol which is a basic need for this research was developed. High frequency indirect somatic embryogenesis was achieved with cotyledonary explants in basal MS salts supplemented with 2,4-D (2.0 mg/l) for callus induction and BAP (2.0mg/l) for shoot induction. Multiple shoot regeneration (20.0 shoot buds/explants) in vitro was also achieved with shoot-tip explants through direct regeneration in MS medium fortified with BAP (2.0mg/l). Direct somatic embryogenesis which has better applicability in the improvement of crop since plant regeneration from callus cultures is often associated with genetic and cytological variations was pursued for genetic transformation. Kanamycin sensitivity to the normal tissues of brinjal plants in vitro was optimized at 100mg/l for selecting putative transformants. A transcription factor *DREB1A* from *Arabidopsis thaliana*, which specifically interacts with the dehydration response element (DRE), a cis–acting promoter element and induces expression of stress tolerance genes, driven by the stress inducible promoter from the *rd29A* gene, was introduced in to the drought-sensitive brinjal cultivar Utkal Anushree by *Agrobacterium tumefaciens*- strain GV3107 with the binary vector pCAMBIA2300. A transformation frequency of 6.4% was reported after molecular analysis with PCR amplification using gene-specific primers.The stress inducible expression of *DREB1A* in these transgenic plants did not result in growth retardation or visible phenotypic alterations. Gene expression studies on the basis of physio-biochemical analysis like membrane stability index, proline content,relative water content suggest that the transgenic brinjal plant can be advanced for further generation and toxicology studies for clearance of biosafety issues for the release of transgenic brinjal.

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Finally I bow my head before my parents and my family, who stand tall by me in every moment of my life. I am close to speechless for their love and blessings, I receive.

Date :

(Sagare Deepti Baburao)

Place :

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LIST OF ABBREVIATIONS

BAP	**- 6-Benzyl adenine purine**
Bp	**- base pair**
CaMV	**- Cauliflower mosaic virus**
CCM	**-Cell culture medium**
Dd	**- double distilled**
DMSO	**-Dimethylsulfoxide**
EDTA	**- Ethylene diamine tetra acetic acid**
Gm	**- Gram**
GFP	**- Green Fluorescent Protein**
Gus	**- beta-glucuronidase**
Hrs	**- Hours**
Ha	**- Hectare**
HCl	**- Hydrochloric acid**
Hpt	**- Hygromycin phosphotransferase**
IAA	**- Indole -3- acetic acid**
L	**- Litre**
LB	**- Lauria Bertani**
Luc	**- Luciferase**
M	**- Metric**
Mg	**- milligram**
Ml	**- milliliter**
MS	**- Murashige and Skoog**

M.W	**- Molecular weight**
N	**- Normal**
NAA	**- α- Naphthalene acetic acid**
NaOH	**- Sodium hydroxide**
NOA	**- α- Naphthoxy acetic acid**
Npt	**- Neomycin phosphotransferase**
OD	**- Optical density**
PCR	**- Polymerase chain reaction**
Rpm	**- Revolution per minute**
TE	**- Tris –EDTA**
T-DNA	**- Transferred DNA**
TDZ	**- Thidiazuron**
TSV	**- Tobacco streak virus**
v/v	**- volume/volume**
w/v	**- weight/volume**
Vir	**- Virulence**
YEP	**- Yeast Extract Peptone**
YEMA	**- Yeast Extract Mannitol Agar**
µg	**- microgram**
2,4-D	**- 2,4-Dichlorophenoxy acetic acid**

CHAPTER-I

Introduction

Gateway to the academic creation, it lights the mover with broad pathway ahead to cover. It gives the reader the "starters" for upcoming pleasant behaviour. It is truly said that a gateway shows half of the contents. Introduction gives the entry of the reader to any text. It creates awareness about the subject matter, further creates interest to go deep into the same. Introduction narrates the specific objectives of the study. An attempt was made to introduce the research problem, its need and importance along with the mandatory prints like limitations of the study.

I. INTRODUCTION

Eggplant (*Solanum melongena* L. 2n=24; Family- Solanaceae; Division- Anthophyta; Class- Dicotyledoneae; Order- Solanales) is an agronomically important non-tuberous, solanaceous crop of sub-tropics and tropics. It is also known as, aubergine, guinea squash or brinjal. The name brinjal is popular in Indian subcontinent and is derived from arabic and sanskrit whereas the name eggplant has been derived from the shape of the fruit of some varieties, which are white and resemble in shape to chicken eggs. It is also called aubergine (French word) in Europe.

The brinjal is of much importance in the warm areas of Far East, being grown extensively in India, Bangladesh, Pakistan, China and the Philippines. It is also popular in Egypt, France, Italy and United States. Brinjal is of Indian origin and has been in cultivation for long time (De Candolle,1886). In India, it is one of the most common, popular and principal vegetable crops grown throughout the country except higher altitudes. It is a versatile crop adapted to different agro-climatic regions and can be grown throughout the year. It is a perennial but grown commercially as an annual crop. A number of cultivars are grown in India, consumer preference being dependent upon fruit color, size and shape. The varieties of *Solanum melongena* L. display a wide range of fruit shapes and colours ranging from oval or egg-shaped to long club-shaped; and from white, yellow, green through degrees of purple pigmentation to almost black. Most of the commercially important varieties have been selected from the long established types of the tropical India and China. Brinjal fruit (unripe) is primarily consumed as cooked vegetable in various ways and dried shoots are used as fuel in rural areas.

Although lower than tomato, nutritive value of brinjal is comparable to other common vegetables (Grubben, 1997). Its fresh weight is composed of 92.7% moisture, 1.4% protein, 1.3% fibres,

0.3% fat, 0.3% minerals and the remaining 4% consists of various carbohydrates and vitamins (A and C)(Khan, 1999).

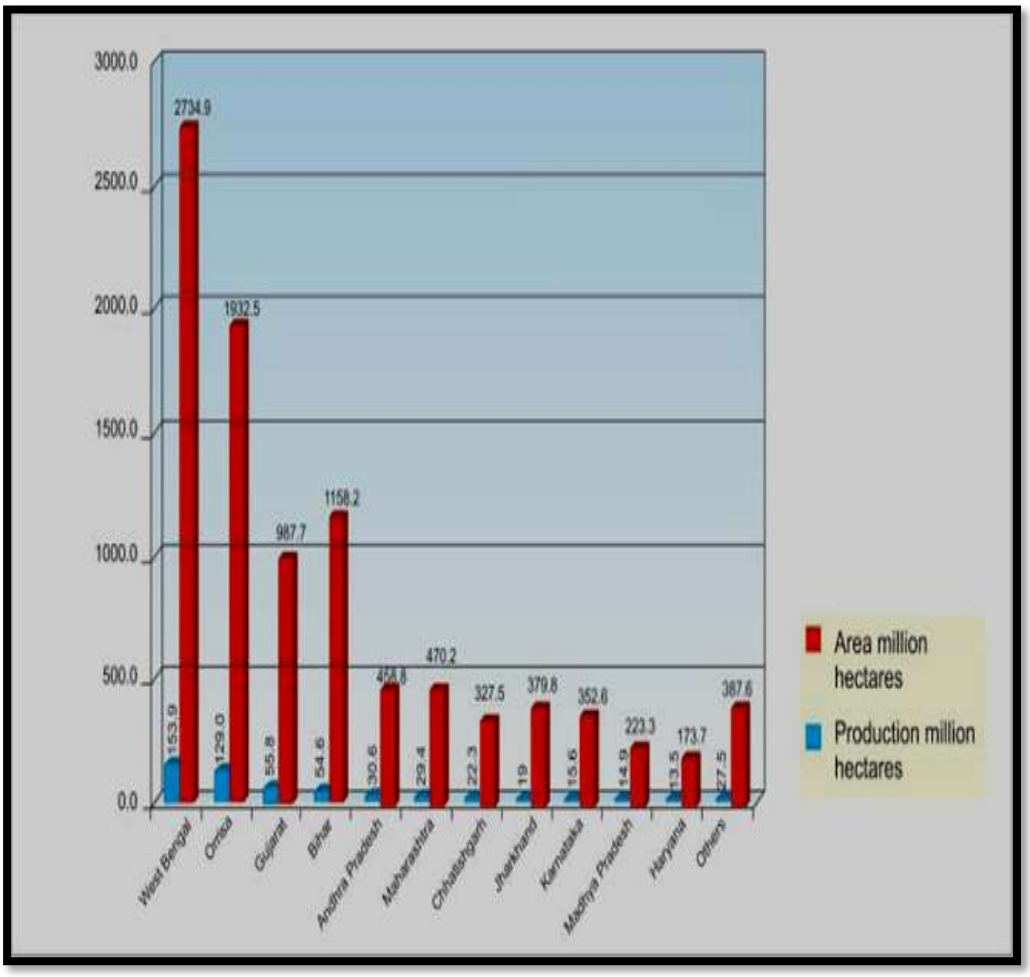
In popular medicine, eggplant is indicated for the treatment of several diseases, including diabetes, arthritis, asthma and bronchitis. In addition, several groups have provided evidence that eggplant extracts have a significant effect in reducing blood and liver cholesterol rates in humans (Khan 1979; Jorge *et al.*1998) and adult rats (Silva *et al.* 1999). Nasunin, a major component of anthocyanin pigment of eggplant, has been shown to inhibit lipid peroxidation (Igarashi *et al.* 1993). More recently, free radical scavenging and iron chelating activities of nasunin were demonstrated by electron spin resonance (Noda *et al.*1998; 2000). Furthermore, anti-mutagenic activity of pheophytin components from eggplant fruit extracts acting against several chemical mutagens was demonstrated by the *Salmonella* microsome assay (Yoshikawa *et al.* 1996).

At present brinjal is the third, after potato and tomato, most important crop from *Solanaceae* family. Brinjal is cultivated throughout the world (Daunay, *et al.,* 2001).The greatest brinjal producer is China (17 mln tons per year) followed by India (8 mln tons), Egypt (1 mln tons) and Turkey (0.9 mln ton) (FAOSTAT Data 2006). In Poland, like in many Central European countries, brinjal is still an exotic vegetable but in Asia and the Mediterranean it is an important and valuable nourishment component, the so-called ‘the king of vegetables’.

In India brinjal is grown in almost all parts except in higher altitudes all the year round. A number of cultivars are grown throughout the country depending on the yield, consumer’s preference about the colour, size and shapes of the fruit. There is huge diversity in Indian germplasm ranging from long to round fruits; white fruited to black fruited, thorny to non thorny and bushy plant habit to erect plant habit. It is a versatile crop adapted to different agro-climatic regions. Brinjal cultivation in India is estimated to cover about 8.14% vegetable area with a contribution of 9% to total vegetable production. In India alone,

25 million farmers cultivate brinjal on over 5.5 lakh hectares with an annual production of about 8.5 million tonnes (Choudhary and Gaur, 2009). The crop is largely grown in small plots or as inter crop both for cash and domestic consumption by farmers all over India. The major brinjal producing states are West Bengal, Orissa, Bihar, Gujarat, Maharashtra, Karnataka, Uttar Pradesh and Andhra Pradesh.

State wise area and production of brinjal in India for the year 2007-2008



Source: National Horticulture Board

The production of brinjal is severely affected by several components of abiotic stress. Abiotic stress is the primary cause of crop loss worldwide, reducing average yields for most major crop plants by more than 50% (Mayra Rodriguez *et al*., 2005). Abiotic stress like drought,

salinity, extreme temperatures and oxidative stress affect the water relations of a plant on the cellular as well as whole plant level causing specific as well as unspecific reactions (Beck *et al*. 2007). This leads to a series of morphological, physiological, biochemical and molecular changes that adversely affect plant growth and productivity (Wang *et al*. 2001). Among the various abiotic stresses, drought is the major factor that limits crop productivity globally. In brinjal, the high night and day temperature condition of 22-24°C to 33-35°C markedly reduce fruit set and yield (Kalloo *et al*, 1990;Kumar *et al*., 2000; Mohanty and Prusty, 2000; Thapa, 2002). Therefore there is urgent need to improve this crop plant by introducing stress tolerance as the gap between the population growth and the food production is enlarging (Rajam *et al*.1998). Cultivated *S. melongena* genotypes often have insufficient levels of resistance to biotic and abiotic stresses. There are also many wild species of eggplant that carry many economically important genes. Genetic resources of this species have been assessed for resistance to its most serious diseases and pests. The traditional method of developing improved crop varieties through crossing eggplant with its wild relatives has limited success because of sexual incompatibilities due to deleterious gene linkages, followed by difficulty in getting fertile progenies, besides, the process takes a long time.

Nevertheless, conventional breeding programs alone may be valuable, but to include molecular methods to introduce the genes for stress-associated mechanisms will give an added advantage by surpassing limitations of the former (Rajam 1997). Genetic engineering provides the eggplant breeders with new tools to complement and supplement sexual hybridisation for improvement of existing varieties or creation of totally new germplasm, by insertion of genes encoding for useful agronomic traits. Genetic engineering, especially the engineering of biosynthetic pathways associated with stress responses has emerged as a promising way to improve tolerance in crop plants (Rotino *et. al*.,1990). Crop improvement for drought tolerance through genetic engineering can be achieved by using several functional or regulatory genes which activate or repress specific or broad pathways

related to drought / salinity tolerance in plants (Trujillo *et al*., 2009). Transcription factors (TFs) are critical regulators of the changes in gene expression and environmental stress responses. Overexpression of the genes that regulate the transcription of a number of down-stream drought responsive genes seems to be a promising approach in the development of drought resistant/tolerant transgenic plants when compared to engineering individual functional genes (Bartels and Hussain, 2008). In fact, major breakthrough in stress technology comes when DREB/CBF genes from *Arabidopsis thaliana* and other plants were identified. The DREB1A/CBF3 genes, which are rapidly induced in response to abiotic stresses, encode transcriptional activators that control the expression of genes containing C-repeat/Dehydration responsive element (DRE) (regulatory element in their promotors). Several cDNAs encoding the DRE binding proteins, DREB1A and DREB2A isolated from *Arabidopsis thaliana* have shown to specifically bind and activate the transcription of genes containing DRE sequences (Liu *et al*., 1998). In fact, in many studies over-expression of stress inducible DREB transcription factor was found to activate the expression of many target genes having DRE elements in their promoters and the resulting transgenic plants showed improved stress tolerance. Constitutive expression of CBF3/DREB1A in transgenic *Arabidopsis* plants has also been shown to induce the expression of target cold regulated genes (COR) and enhance freezing tolerance in non-acclimated plants (Gilmour *et. al*,.). DREB genes from other plants have also been shown to be functional, for example transgenic rice over-expressing *Oryza sativa* DREB1A has demonstrated improved tolerance to drought, high salt and low temperature stresses and also caused accumulation of elevated levels of osmoprotectants such as free proline and various soluble sugars (Ito *et al*., 2006). But over-expression of DREB1A/CBF3 also causes severe growth retardation (Dwarf phenotype) under normal growth conditions. Use of stress inducible promoter rd29A instead of the constitutive CaMV35S promoter for over-expression of DREB1A/CBF3 minimizes the negative effect on plant growth (Kasuga *et. al*.,1999). Interestingly the rd29A

promoter contains ABRB (ABA Responsive elements) regulatory elements that mediate activation of bZIP protein during ABA Signaling.

For the improvement against biotic and abiotic stresses as well as quality through genetic transformation, standardization of plant regeneration protocol is the prerequisite. Eggplant tissues present a high morphogenetic potential, useful for developmental studies as well as establishing biotechnological approaches to produce improved varieties, such as embryo rescue, *in vitro* selection, somatic hybridization and genetic transformation (Collonnier *et al*. 2001, Magioli and Mansur 2005). The nature and concentrations of a given growth regulator in association with specific genotype and explants can cause significant differences in morphogenetic response of brinjal (Matsuoka and Hinata). The adventitious shoot regeneration capacity of cells or tissues to be used in transformation studies affects the success of genetic transformation significantly and frequency of explants responding after co-cultivation is severely affected (Chen *et al.*,). Therefore, as successful application of *in vitro* techniques for crop improvement rests upon a reproducible plant regeneration protocol, standardization of reproducible regeneration protocol is a basic need. Besides, the standardization of *in-vitro* regeneration system as a prerequisite for genetic transformation, standardization of Kanamycin/Hygromycin based selection system is also very important for selection of transformants as, most of the gene constructs contain selectable marker genes such as, antibiotic resistant genes.

During the last decade, *Agrobactreium* mediated transformation has become well established in several transformations. The advantages of this method include high transformation efficiency, minimal rearrangement of the transgenic plants that harbour a single copy of the transgene. Development of marker free transgenic plants using super binary *Agrobacterium* based vectors is another promising utility of this methodology. The possibility of transferring large DNA fragments in the 100 kb range is now possible but requires additional

experimentation (Veluthambi *et al*., 2003). After the transformation process the confirmation of transformed plants at molecular, biochemical and physiological basis is necessary to study the gene expression and its association with other genes.

Therefore, it is proposed to carry out *Agrobacterium tumifaciens* mediated transfer of rd29A:: DREB1A/CBF3 gene to the brinjal cultivar to improve abiotic stress tolerance without affecting plant growth. The present study was undertaken with the following objectives:

1. To develop a protocol for In-vitro regeneration system (both via Direct and Indirect method) of locally improved Brinjal cultivar-Utkal Anushree.
2. Development of Kanamycin based selection system
3. Standardization of parameters for high efficiency *Agrobacterium*-mediated transformation
4. To transfer rd29A::DREB1A to Brinjal cultivar by *Agrobacterium*-mediated transformation.
5. Selection of putative transformants on kanamycin containing selective medium.
6. Detection of trans-genes by molecular analysis of transformants.
7. Study of gene expression by morpho-physiological and biochemical analysis of transformants.

CHAPTER-II

Review of literature

This is a "small articulation of previous research" highlight. Past, golden, enthusiastic, and eminent works vibrated and finally selected for the current piece of work for guiding in times of necessary reference. Related reviews by eminent authors and scholars had found their place in this research work to establish necessary relationship with the current research. Pertinent reviews by authors and research scholars are a guiding force for any researcher to form various parameters of research outline. The researcher is deeply grateful to those unseen personality for their necessary reviews from their research work. The help is deeply acknowledged by the researcher.

II. REVIEW OF LITERATURE

Rapid progress in recombinant DNA technology and elucidation of molecular basis of tumorigenesis due to *Agrobacterium* infection provides an opportunity to transfer, achieve stable integration and express useful genes in plants. The application of *in vitro* methodologies to eggplant has resulted in considerable success. Eggplant tissues present a high morphogenetic potential that is useful for developmental studies as well as for establishing biotechnological approaches to produce improved varieties, such as embryo rescue, *in vitro* selection, somatic hybridization and genetic transformation. Taken together, these characteristics also make eggplant a complete model for studies on different areas of plant science, including control of gene expression and assessment of genetic stability of somaclones derived from different morphogenetic processes.

The literature pertaining to the various aspects of *in vitro* regeneration and *Agrobacterium*-mediated transformation are reviewed under the following heads.

2.1 *In vitro* regeneration studies

2.1.1 Direct regeneration

2.1.2 Regeneration through callus (Indirect regeneration)

2.1.3 Somatic embryogenesis

2.1.4 Rooting

2.1.5 Hardening and establishment of the regenerated plants

2.2 Transformation studies

2.2.1 Direct transformation

2.2.2 *Agrobacterium*-mediated DNA transformation

2.3 Eggplant as a model plant

2.4 rd29A::DREB1A gene for abiotic stress tolerance

2.5 Biochemical and morpho-physiological studies of transformants

2.1 REGENERATION STUDIES IN EGGPLANT

Regeneration is the process by which a cell or a group of cells differentiates to form organs. This is commonly induced by manipulation of exogenous phytohormone levels and occurs either directly from explanted tissue or callus. Most often direct regeneration occurs through shoot proliferation from pre-existing meristems instead of *de novo* formation of a meristem. In general, even in callus mediated regeneration organ forming capacity limited to primary callus indicates the potential existence of meristems embedded in the original explant. Several protocols for plant regeneration via direct and indirect organogenesis have been developed from different eggplant tissues. In those protocols, the regeneration efficiency has been reported to be affected by different factors, such as combination of growth regulators, explant type and genotype.

2.1.1 DIRECT REGENERATION

Most of the organogenic systems reported are based on supplementing culture media with auxins and cytokinins, either alone (Kamat & Rao 1978;Matsuoka & Hinata 1979; Alicchio *et al.* 1982; Gleddie *et al.* 1983; Sharma & Rajam 1995) or in combination (Sharma & Rajam 1995; Kamat & Rao 1978; Matsuoka & Hinata 1979).

Different sources of explant have been used for the induction of organogenesis in eggplant, including hypocotyl (Sharma & Rajam 1995; Kamat & Rao 1978; Matsuoka & Hinata 1979; Alicchio *et al.* 1982; Magioli *et al.* 1998), leaf (Gleddie *et al.* 1983; Alicchio *et al.* 1982; Mukherjee *et al.* 1991; Sharma & Rajam 1995; Magioli *et al.* 1998), cotyledon (Sharma & Rajam 1995; Alicchio *et al.*1982; Magioli *et al.* 1998),epicotyl (Magioli *et al.* 1998), stem nodes (Magioli *et al.* 1998) and roots (Franklin & Sita 2003). The regeneration efficiencies reported in those systems were relatively low (approximately 7 shoots/explant) (Gleddie *et al.* 1983; Mukherjee *et al.* 1991; Sharma & Rajam 1995), except in the one described by Sharma & Rajam (1995),

who achieved the production of 20 shoots/explant in one of the four cultivars studied. The use of low concentrations (100-200nM) of thidiazuron (TDZ) was also reported to induce efficient organogenesis in five cultivars (around 20 shoots/explant) from leaf and cotyledon explants (Magioli *et al.*1998; 2000).

Sarker *et al.* (2006) studied on multiple shoot formation in Eggplant (*Solanum melongena* L.) and reported that cotyledonary leaf was found to be the best for multiple shoot regeneration. High frequency direct organogenesis of shoots was achieved from cotyledonary leaf in MS supplemented with 1.0 mg/l BAP and 1.0 mg/l Kinetin. They obtained 3.5 no. of shoots/explant after 25 days of inoculation.

Carmina Gisbert *et al.* (2006) developed protocol for efficient regeneration in two potential new crops for subtropical climates, the scarlet (*Solanum aethiopicum*) and gboma (*S. macrocarpon*) eggplants and they observed that media containing the cytokinin thidiazuron (TDZ) induced the greatest regeneration response from both cotyledonary and true leaf explants. They also observed differences in regeneration ability among varieties, TDZ concentrations of 0.1 or 0.2 µM gave the best results, with 70–100% explants with shoots and a mean of 2–7 shoots per explant.

Sharmin *et al*. (2008) studied on *in-vitro* propagation of eggplant through meristem culture for developing an efficient protocol of production of eggplant clones. For primary establishment of isolated apical meristem in MS liquid medium containing 2.0 mg/l BAP was found the best. Subsequent development of meristem derived shoot was achieved in MS semisolid medium containing either 2.0 mg /l BAP, maximum number (2.20 ± 0.21) of shoots/explant was found.

Shivaraj G and Srinath Rao (2011) developed a protocol for rapid and efficient plant regeneration of eggplant (*Solanum melongena*) from cotyledonary leaf explants. The highest number of shoots (23.3±0.10) was obtained on MS medium containing 2.0 mg/BAP+0.5 mg/L Kinetin. The *in vitro* regenerated small shoots were further elongated on MS medium supplemented with gibberellic acid (GA3) at 1.5 mg/L.

Mohinder Kaur *et al* (2011) studied on *in vitro* plant regeneration in Brinjal from cultured seedling explants and reported that cotyledon explant gave cent percent regeneration on MS medium fortified with 2.0 mg/l BAP, 2.5 mg/l BAP or 2.5 mgl/l BAP + 1.0 mgl/l kin, while the highest numbers of buds on 2.5 mg/l BAP (24.90), followed by 2.0 mg/l BAP (17.90). Leaf explant also induced cent percent regeneration on MS medium fortified with 2.0 mg/l BAP and maximum number of buds (9.53) regenerated with 2.5 mg/l BAP. Hypocotyl had the maximum regeneration (66.53%) and maximum buds (3.96) on MS with 2.5 mg/l BAP. Maximum bud elongation (58.73%) was obtained on ½ MS medium supplemented with 0.3 mg/l BAP + double agar.

2.1.2 REGENERATION THROUGH CALLUS (INDIRECT REGENERATION)

Early studies of *in vitro* regeneration of eggplant were based on culturing cell suspensions (Fassuliotis *et al.* 1981; Gleddie *et al.* 1983), anthers (Isouard *et al.*1979; Dumas de Vaulx & Chambonnet 1982; Tuberosa *et al.* 1987) and protoplasts (Jia & Potrykus 1981; Saxena *et al.* 1981; Bhatt & Fassuliotis 1981; Guri & Izhare 1984; Gleddie *et al.* 1986; Nishio *et al.* 1987; Li & Zhang 1988; Clark *et al.* 1988). Successful development of morphogenic calli was obtained from isolated microspores, resulting in the production of putative spontaneously doubled haploids (Miyoshi 1996).

The first report was published by Yamada *et al.* (1967), who induced somatic embryogenesis from zygotic embryos cultured on MS supplemented with indole-3-acetic acid (IAA). Similarly to the observed in the organogenic process, the efficiency of somatic embryogenesis depends on several factors, including genotype, explant type and growth regulators. In general, high frequencies of somatic embryogenesis induction are obtained in response to the auxins α-naphthaleneacetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D) (Matsuoka & Hinata 1979; Gleddie *et al.* 1983; Ali *et al.* 1991; Saito & Nishimura, 1994; Magioli *et al.* 2001).

However, conversion of somatic embryos into plantlets is usually limited due to abnormalities such as hyperdricity, lack of apical meristem, cotyledon fusion and inefficient maturation (Gleddie *et al.* 1983; Saito & Nishimura 1994; Magioli *et al.* 2001). Nevertheless, conversion rates can reach up to 92% by culturing mature embryos on MS solidified with 1% phytagel (Saito & Nishimura 1994; Magioli *et al.* 2001). Studies on the morphological aspects of somatic embryogenesis have been performed in a number of species and showed that somatic embryos can have different origins according to the species and the explant type. The type and concentration of a given growth regulator in association to specific genotypes can cause significant differences in the morphogenetic responses of Eggplant.

Kazumitsu Miyoshi (1996) studied on callus induction and plantlet formation through culture of isolated microspores of eggplant *(Solanum melongena* L.) and observed that NLN medium containing 2% sucrose and phytohormones (NAA 0.5 mg /l, BA 0.5 mg /l) gives rise to callus 4 weeks after inoculation and the microspores which were inoculated on sucrose free medium for 3 days gives better result. Then shoot regeneration from Small calli derived from microspores was observed on MS medium containing 4 mg/l zeatin and 0.2 mg/l IAA.

M Rahman *et al.* (2006) developed a protocol for efficient plant regeneration from cotyledon and midrib derieved callus in Eggplant (*Solanum melongena L.*). The best callusing 83-85% was obtained from both of the explants cultured on MS medium containing 2.0 mg/l NAA + 0.05 mg/l BAP. Somatic embryogenesis and shoot regeneration was achieved after transferring the calli to MS medium supplemented with BAP, GA3, NAA and Zeatin. Cotyledon derived calli showed better performance (87%) for regeneration than that of midrib (82%) when sub cultured on MS medium having 2.0 mg/l Zeatin + 1.0 mg/l BAP.

Hossain *et al*. (2007) developed an efficient protocol for establishment of cell suspension culture and plantlet regeneration through cell culture from the cotyledonary explants of eggplant and they observed that, MS medium supplemented with 2 mg/l NAA gives maximum callus. When the obtained callus gives micro-calli and from

this micro-calli shooting was obtained on MS medium supplemented with 1mg/l BAP and 0.05 mg/l GA3.

Huda *et al*. (2007) has undertaken a research programme with a view to develop an efficient and reliable method of indirect regeneration through somatic embryogenesis in eggplant and they observed that, MS medium supplemented with 0.05 mg/l BAP and 2 mg/l NAA gave best result on somatic embryogenesis.

Prakash *et al*.(2008) studied on effect of growth regulators on shoot morphogenesis in hypocotyl and cotyledonary leaf explants, shoot elongation and root induction and reported that hypocotyl explants showed callus initiation and direct regeneration response after 3-5 and 13-15 days of culture initiation respectively. Cotyledonary leaf explants showed callus initiation and callus-mediated shoot regeneration response after 7-8 days and 3 weeks of culture initiation. Growth regulators did not affect callus initiation response but they markedly influenced regeneration response. Culture medium containing 10 µM BAP and 0.5 µM NAA induced highest 18.33% shoot regeneration in cotyledonary leaf explants. Either increase or decrease in BAP concentration than this resulted in reduction in shoot regeneration response. Addition of BAP to shoot elongation medium was found beneficial for shoot elongation and shoot regeneration medium was efficient in shoot elongation also. Addition of auxins in root induction medium has reduced number of days taken for root induction and increased in number of roots and root length.

Eli Zayova *et al.*(2008) investigated the effect of some phytohormones and their combinations on callus induction in eggplant tissue cultures. They observed that cotyledons and hypocotyls from 30-day-old seedling were the best explants for callus induction. Murashige and Skoog (MS) medium supplemented with naphthalene acetic acid (NAA) 2.0 mg/l and 6-benzylaminopurine (BAP) 0.5 mg/l proved to be very suitable for callus induction. Callus was obtained from 90.0% of cotyledon explants and from 63.3% of hypocotyl explants.

A. Ferdausi *et al.* (2009) reported that, Shoot tip is the best explant for callus induction and maximum callus can be obtained on

MS medium supplemented with 2mg/l NAA and cytokinin (0.5 mg/l BAP) shows highest percentage of shoot regeneration.

While studying on *in-vitro* plantlet regeneration through somatic embryogenesis in *Solanum melongena,* B. Swamynathan *et al*.(2010) observed that, cotyledon cultures were highly responsive for callus production on medium supplemented with NAA (10.6 mg/l) alone. However embryo explants cultures showed good responses for callus production and embryogenesis on MS medium supplemented with NAA (8.0mg/l) and kinetin (0.1mg/l). Callusing response for shoot culture was low. Embryos derived from cotyledon and shoot explants gives shooting on hormone free medium for germination.

Ray *et al.* (2010) studied on, plant regeneration from seedling derived explants through callus of eggplant (*Solanum melongena L.)* and observed that, the highest amount of callus (48.66%) was produced on MS medium containing 2.0 mg/l BAP and 0.5 mg/l NAA from stem after 8.2 days and the highest percentage of regeneration (23.28%) was recorded in MS media containing 2.0 mg/l BAP + 0.5 mg/l NAA from stem after 38.8 days.

Dhavala *et al*.(2010) reported that MS medium supplemented with 2 mg/l NAA+2.5 mg/l BAP was optimum for callus initiation, total callus quantity and embryogenic callus formation, and MS medium supplemented with 2.5 mg/l each BAP and kinetin were optimum for regeneration. Rooting of shoots occurred on half strength MS medium supplemented with 1 and 1.5 mg/l IBA respectively.

D.Sammaiah *et al*. reported that cotyledon explant gives maximum callus on MS Medium supplemented with 2 mg/l NAA and IAA + BAP (0.5+3.0 mg/1) was suitable for the multiple shoots and regeneration.

2.1.3 SOMATIC EMBRYOGENESIS

Somatic embryogenesis (SE) is the process by which somatic cells develop through the stages of embryogeny to give whole plants. The first observation of *in vitro* SE was made in carrot (Dacus carota)

(Reinert, 1958 and Steward *et al*., 1958). Ever since, many species of different genera have been tested and considerable information has been accumulated to establish the embryogenic potential of somatic plant cells. This aspect has been the subject of several reviews of different crop plants (Bannikova and Barabanova, 1990, Durzan and Gupta, 1988, Gray and Purohit, 1991, Tautorus *et al*., 1991, *Tisserat et al*., 1979 and Tomes, 1985). The high volume multiplication of embryogenic propagules is the most attractive application of *in vitro* SE, which can be utilized directly in various studies much as genetic transformation, somatic hybridization and somaclonal variation.

While studying temporal regulation of somatic embryogenesis by adjusting cellular polyamine content in eggplant, Jitender Singh Yadav and Manchikatla Venkat Rajam showed that spatial endogenous PA levels were associated with differential embryogenic ability (Sharma and Rajam, 1995; Yadav and Rajam, 1997). Furthermore, they demonstrated the association of elevated PUT levels and the importance of the ADC pathway in SE from eggplant leaves (Yadav and Rajam, 1997).

A. S. Kantharajah (2003) reported about the factors affecting on Somatic embryogenesis (Genotype, Source of explant, Growth regulators, Polyamines), induction, development and maturation of somatic embryos, Molecular aspects of somatic embryos etc.

Huda *et al*. (2007) reported that, MS Medium supplemented with 2mg/l NAA and 0.05 mg/l BAP gave best result for somatic embryogenesis. They could easily isolate the globular structures of somatic embryos from parent tissue as these were floated freely in the water.

B. Swamynathan *et al*.(2010) studied on *in-vitro* plantlet regeneration through somatic embryogenesis in *Solanum melongena* and observed that the medium supplemented with NAA (4.0 mg/l) and kinetin (0.5mg/l) induced 85% embryogenic callus in that 70% of embryoids were isolated and high concentration of NAA alone (10.6mg/l.) showed 40% of embryogenic callus out of which 60% of embryoids were isolated which is derived from embryo explants. In

cotyledon explants the medium supplemented with high concentration of NAA (0.6mg/l) was good for the embryoid initiation. This medium contributed 60% of embryogenic callus and in large size out of which 80% of embryoid was isolated and the low concentration of NAA (4.0mg/l.) also shows 40% of embryogenic callus out which 45% of embryoid was isolated. The various combination and concentration of NAA, TDZ, BAP and 2-4D did not respond to convert the callus to embryogenic callus.

2.1.4 ROOTING

The root formation in Brinjal was reported in half-stength MS medium (Taha and Tizan; Sarker *et al.,*), ½ MS medium supplemented with 0.6 mm IAA (Magioli *et al.*,1998), ¼ MS medium (Dobariya and Kachhadiya,) and MS medium containing 1.0 mg/l 3-indole butyric acid (Borgato *et al 2007*).

P. Sharma *et al*.(1997) studied on Induction of laterals in root cultures of eggplant (*Solanum melongena* L.) in hormone-free liquid medium: A novel system to study the role of polyamines and reported that polyamine spermidine (Spd) dramatically increased both root length and number of laterals at 0.5 and 1 mM.

C. Magioli *et al*. (1998) reported rooting was induced on half-strength MS medium supplemented with 0.6 mM IAA.

Sarker *et al*. (2006) investigated that regenerated shoots produced healthy roots when they were cultured on MS medium without hormonal supplements.

According to Carmina Gisbert *et al*. (2006) MS medium is better for rooting. M Rahman *et al*. (2006) reported that for root induction, MS + 3.0 mg/l IBA was proved to be better treatment for average number (14-15) and mean length (12 cm) of roots than those of other treatments.

Sharmin *et al*. (2008) reported that for root development from meristem derived shoots, 1.0 mg/l IBA was found most responsive.

Ferdausi *et al*. (2009) reported that, MS basal medium was proved to be better treatment for average number (12-15) of roots.

Shivaraj G (2010) reported that indole butyric acid (IBA) at 3.0 mg/l best rooting medium. While studying on effect of genotype, explant and hormonal concentration on *in vitro* response of eggplant. Dhavala et al. (2010) observed that rooting of shoots occurs on half strength MS medium supplemented with 1 and 1.5 mg/l IBA.

2.1.5 HARDENING AND ESTABLISHMENT OF THE REGENERATED PLANTS

Shivaraj G. (2010) reported that, rooted plantlets can hardened on MS basal liquid medium and subsequently transferred to polycups containg miculate:soil:sand (1:2:2). Plantlets, thus developed were successfully established and finally transferred to a greenhouse the plantlets showed high survival rate (80%) in the soil.

Dhavala *et al*. (2010) transferred plantlets at five leaves stage to sterilized soil + vermiculate mix (1:1) in growth chamber for two weeks and further they were transferred into the pots and experimental garden.

M Rahman *et al*. (2006) *reported that* acclimatized of *in-vitro* regenerated Eggplants can be achieved on sterile sand, soil and compost (1:2:1) with highest survival rate.

Mohinder Kaur *et al*. (2011) reported that hardening of rooted plantlets in wet cotton resulted in softening and killing of plants. That may be due to the excess of water supplied by wet cotton during hardening. Hardening of rooted plantlets on wet filter paper increased the survival. They also reported that the addition of 0.2% bavistin to the tap water further enhanced the survival efficiency of plantlets.

Table 2.1: Somatic embryogenesis in eggplant

Species	Explant	Basal medium	Growth regulator	Reference
S.melongena	Hypocotyls	MS	4-30 mg/l NAA	Matsuoka(1983)
S.melongena	Leaf	MS	10 mg/l NAA	Gleddie et al.(1983)
S.melongena	Protoplasts isolated from young leaves	MS	2,4-D	Gleddie (1986)
S.melongena v.Dourga	Leaf	MS	10 mg/l NAA	Rotino et al. (1987)
S.melongena cv.Imperical black beauty	Cotyledon	MS	5-10 mg/l NAA	Fobert and Webb(1988)
S.melongena, S.melongena var. insanum, F1 of *S.melongena x S.melongena* var. insanum	Hypocotyl	MS	0.5-2 mg/l 2,4-D	Ali et al. (1991)
F1 of *S.melongena var. suphal*	Leaf	MS	8mg/l NAA+ 0.1mg/l kin	Rao and singh(1991)
S.melongena cv.Giulietta	Seedling explant	MS	10 mg/l NAA	Mariani (1992)
S.melongena cv. Nagate shinkuro	Cotyledon	MS	50µM 2,4-D	Saito and Nishimura (1994)
S.melongena cv.Pusa purple long, white cluster, pusa kranti, pusa purple cluster	Hypocotyl, Cotyledon, Leaf	MS	5.7-53.7µM NAA	Sharma and Rajam (1995)

Table 2.2 : *In vitro* regeneration studies of eggplant

Mechanism	Explant	Growth regulators (µM)	Reference
Organogenesis	Hypocotyl	5.7 IAA + 4.4 BAP	Kamat & Rao 1978
	Hypocoty	1 BAP alone or with 0.09 NAA	Matsuoka & Hinata 1979
	Hypocotyl, cotyledon and leaf	1.8 2.4-D	Alicchio *et al.* 1982
	Leaf	44.4 BAP	Gleddie *et al.* 1983
	Leaf	9.3 KIN	Mukherjee *et al.* 1991
	Hypocotyl, cotyledon and leaf	11.1 BAP + 2.9 2.4-D	Sharma & Rajam 1995
	Epicotyl, hypocotyl, cotyledon, leaf and node	0.2 TDZ	Magioli *et al.* 1998
	Root	0.5 TDZ + 13.3 BAP	Franklin & Sita 2003
Embryogenesis	Zygotic embryos	5.4 NAA	Yamada *et al.* 1967
	Hypocotyl	43 NAA	Matsuoka & Hinata 1979
	Cell suspension culture and leaf	54 NAA	Gleddie *et al.* 1983
	Cotyledon	27 NAA	Fobert & Webb 1988
	Leaf and cotyledon	5.4 NAA	Fillippone & Lurquin 1989
	Hypocotyl	2.7 – 10.8 NAA	Ali *et al.* 1991
	Leaf	43 NAA + 0.5 KIN	Rao & Singh 1991
	Leaf and cotyledon	50 2.4-D	Saito & Nishimura 1994
	Hypocotyl, cotyledon and leaf	5.7 - 54 NAA	Sharma & Rajam 1995a
	Leaf and cotyledon	54 NAA	Magioli *et al.* 2001

2.2 TRANSFORMATION STUDIES

Plant transformation studies in many ways have been quite common after Avery *et al.* (1944) showed that the DNA is the

transforming principle in Bacteria and Chilton *et al.* (1977) elucidated the molecular events leading to the development of crown gall in dicotyledonous plants. Among the plant transformation methods now available, direct exposure of appropriate cells, tissues, organs and protoplasts to DNA and those that involve *Agrobacterium* with Ti plasmid vector are widely practiced (Vancannet *et al.*, 1990).

2.2.1 DIRECT TRANSFORMATION

Direct transformation is applicable to both stable and transient gene expression studies. Physical as well as chemical methods have been developed to facilitate DNA delivery across the plasma membrane. Although the frequency of stable transformation by these methods is low, direct DNA uptake is applicable to those plants which are not amenable to *Agrobacterium tumefaciens* mediated transformation, particularly monocotyledons (Siregar and Sundarsono, 1997 and Rohini and Sankaro Rao, 2001). The first report on direct delivery of DNA molecules into plant protoplasts has been well documented by Davey *et al.* (1989).

Electroporation has been used to transform not only protoplasts but also walled plant cells, either growing in suspension or as part of intact tissues (Bechtold *et al.*, 2000).

In eggplant biolistics approach to direct gene transfer has been tested by Singh A.K, *et al*. (2010). They developed a method for plastid transformation in eggplant. Plastid transformation in eggplant was achieved by bambardment of green stem segments with pPRV111A plastid expression vector carrying the aadA gene encoding aminoglycoside 300-adenylyltransferase. Biolistic delivery of the pPRV111A plasmid yielded transplastomic plants at a frequency of two per 21 bambarded plates containing 25 stem explants each. Integration of the aadA gene in the plastome was verified by PCR analysis and also by southern blotting using 16S rDNA (targeting sequence) and the aadA gene as a probe. Transplastomic expression of the aadA gene was verified by RT-PCR.

2.2.2 *AGROBACTERIUM*-MEDIATED DNA TRANSFORMATION

Genetic transformation of eggplant via *Agrobacterium* was first reported by Guri & Sink (1988b), using leaf explants and a cointegrate vector, although no success was achieved with a binary vector. Later, Fillipone & Lurquin (1989) reported the transformation of leaf and cotyledon explants using the wild supervirulent strain A281. Transgenic plants were obtained by Rotino & Gleddie (1990) and Fari *et al.*(1995), using organogenic regeneration systems. An optimization of factors that influence transformation efficiency, including length of pre and post-coculture periods, explant type, and genotype was performed using a TDZ-based organogenic system (Magioli *et al.*2000). The efficiency of transformation protocols based on organogenesis may be influenced by the antibiotic used to eliminate *A. tumefaciens.* For example, augmentin can cause enhanced shoot proliferation induced by TDZ (Billings *et al.* 1997).

Following the establishment of basic protocols, successful introduction of agronomic traits into eggplant was achieved. Resistance to Colorado Potato Beetle (*Leptinotarsa decemlineata* Say) (CPB), a pest that has developed resistance to synthetic insecticides and became a serious problem for agriculture in Europe and America (Arpaia *et al*. 1997), has been pursued by a number of groups. Chen *et al.*(1995) have produced transgenic eggplant lines with the introduction of *Bacillus thuringiensis* (*Bt*) genes, but resistance to CPB was not observed. Later, different groups obtained lines resistant to CPB by using mutagenized versions of *cryIIB* (Arpaia *et al.* 1997; Iannacone *et al.* 1997) and a synthetic version of *cryIIIA Bt* genes (Jelencovic *et al.* 1998). Field trials demonstrated high levels of resistance in transgenic plants produced after the introduction of a mutagenized *Bt cryIIIB* gene, without detrimental effects on nontarget arthropods (Acciarri *et al.* 2000). These resistant transgenic eggplants can potentially be used for the development of new varieties.

Besides CPB resistance, there are other examples of genetic improvement of eggplant via *A. tumefaciens.* Resistance to *Leucinodes orbanalis* was obtained in transgenic plants harboring the *Bt* (*Cry1Ab*) gene. Tolerance against osmotic stress induced by salt, drought and chilling stress was achieved in transformants expressing the bacterial mannitol-1-phosphodehydrogenase (*mtlD*) gene (Prabhavathi *et al.* 2002) and transgenic hybrids harboring the parthenocarpic gene *DefH9-iaaM* presented a significant yield increment that resulted in a 10% reduction in cultivation costs (Donzella *et al.* 2000).

Ellialtıoglu S *et al*. (2001) reported that the GV2260 (Containing GUS gene) was more effective than EHA105 (Containing GUS gene) in Eggplant transformation.

Nazzareno Acciarri *et al*.(2002) developed Genetically modified parthenocarpic eggplants by using the *DefH9-iaaM* gene. The *iaaM* gene codes for tryptophan monoxygenase and confers auxin synthesis, while the *DefH9* controlling regions drive expression of the gene specifically in the ovules and placenta. And they observed genetically engineered (GM) parthenocarpic eggplants demonstrated a significant increase (an average of 33% increase) in fruit production concomitant with a reduction in cultivation costs.

G. Franklin and G. Lakshmi Sita (2003) developed an efficient variety-independent method for producing transgenic eggplant (*Solanum melongena* L.) *via Agrobacterium tumefaciens*-mediated genetic transformation. They transformed root explants by co-cultivation with *Agrobacterium tumefaciens* strain LBA4404 harbouring a binary vector pBAL2 carrying the reporter gene β-glucuronidase intron (GUS-INT) and the marker gene neomycin phosphotransferase (NPTII). Transgenic calli were induced in media containing 0.1 mg/l thidiazuron (TDZ), 3.0mg/l N6-benzylaminopurine, 100 mg/l kanamycin and 500 mg/l cefotaxime. The putative transgenic shoot buds elongated on basal selection medium and rooted efficiently on Soilrite irrigated with water containing 100 mg/l kanamycin sulphate. Transgenic plants were raised in pots and seeds subsequently collected from mature fruits. Histochemical GUS assay and polymerase

chain reaction analysis of field-established transgenic plants and their offsprings confirmed the presence of the GUS and NPTII genes, respectively. Integration of T-DNA into the genome of putative transgenics was further confirmed by Southern blot analysis. Progeny analysis of these plants showed a pattern of classical Mendelian inheritance for both the NPTII and GUS genes.

V. Prabhavathi, M.V. Rajam (2007) reported transgenics expressing mtlD gene with mannitol accumulation exhibit increased resistance against three fungal wilts caused by *Fusarium oxysporum*, *Verticillium dahliae* and *Rhizoctonia solani* under both *in vitro* and in vivo growth conditions. Mannitol levels could not be detected in the wild-type plants, but the presence of mannitol in the transgenics could be positively correlated with the disease resistance. These results and the previous data suggest that mtlD gene can be used for engineering crop plants for both biotic and abiotic stress tolerance.

Phan Dinh Phap *et al.* (2010) a synthetic *cryIAb* was transferred into brinjal using cotyledonary leaves by *Agrobacterium*-mediated transformation method. They observed that when the inoculated cotyledonary leaves were kept on regeneration media supplemented with 70 mg/l kanamycin, regeneration efficiency ranged from 1.11% to 9.33% and the highest transformation efficiency was 4.0%. However, transformation efficiency was 7.5% on selection medium (50-100 mg/l kanamycin) using cotyledonary leaves. It was 20.8% on selection media (50 mg/l kanamycin) and 7% on selection media containing 100 mg/l kanamycin using leaf explants.

Dharmendra Pratap *et al*.(2011) attempted genetic transformation in Eggplant using cotyledon explants and *Agrobacterium tumefaciens* (strain LBA 4404) carrying cucumber mosaic virus (CMV) coat protein (CP) gene construct under the control of CaMV35S promoter in pROK2 binary vector. Out of 110 co-cultivated explants 67 putative transgenic plants were established in greenhouse. Integration of CMV-CP transgene in the genome of transgenic plants was confirmed by PCR using CMV-CP specific primers. They also used CMV-CP specific probes for confirmation of

single or double copy no. of transgene in their genome, which successfully transcribed and translated 26 KDa protein. To generation transgenic plants showing significant degree of resistance against CMV were observed when screened by challenge inoculation.

E. Filippone and Paul F.Lurquin (1999) studied on use of hypervirulant *Agrobacterium tumefaciens* carrying a binary vector to transform Eggplant cotyledonary leaf discs and reported that strain A281 carrying plasmid pGA472 is capable of successful transfer of NPTII gene.

The production of transgenic eggplant through somatic embryogenesis either fails to occur (Fillipone & Lurquin 1989) or is achieved with very low efficiency (Fari *et al.* 1995). It has been demonstrated that both co-cultivation with *Agrobacterium* and the presence of bactericidal antibiotics used in transformation protocols cause a reduction of 80-99% in the number of embryo/explant. The inhibitory effect on somatic embryos development may result from the interaction between the physiological alterations caused by these treatments and the delicate processes of gene regulation that are induced in early culture stages (Magioli *et al.* 2001a).

Fari *et al. (*1995) studied on *Agrobacterium* mediated genetic transformation and plant regeneration via organogenesis and somatic embryogenesis from cotyledon leaves in eggplant. After co-cultivation with *Agrobacterium* vectors harboring neomycin phosphotransferase *(nptll)* as selectable marker, transgenic plantlets were regenerated on selective media containing 100 mg/l kanamycin. Transformants were recovered from embryogenic calli induced by 4mg/l α-naphthaleneacetic acid (NAA), and from organogenic calli induced by the addition of 2 mg/l zeatin plus 0.01 mg/l NAA. Nineteen independent transgenic lines were grown to maturity. The structural integrity, expression and sexual transmission of the introduced genes for neomycin phosphotransferase and L-glucuronidase *(gus)* were investigated.

Wang Feng-hua *et al*.(2000) studied genetic transformation of eggplant callus mediated by *Agrobacterium tumefaciens* they observed

that the transformation ratio was the highest when pre-culture for nine days in advance, invading for 20 min and co-culture for one day. The factors affecting transformation rate was as follows: the pre-culture time was the most important, next was invading time, and co-culture time was the last. The molecular determination indicated that seven plantlets among eleven positive plantlets were amplified bar gene, which indicated that these seven plantlets has been successfully transformed. The rate of transgenic plantlet to positive plantlets was 63.6%.

Table 2.3 : Genetic transformation of eggplant via *Agrobacterium.*

Explant	Gene	Observations	Reference
Leaf	*NptII*	Success with a binary vector, no information about transformation efficiency	Guri & Sink 1988
Cotyledon / Leaf	*NptII*	Stable transformation using the wild supervirulent strain A281	Filippone & Lurquin 1989
Leaf	*NptII* and *CAT*	Transformation efficiency of 7.6%	Rotino & Gleddie 1990
Cotyledon	*NptII* and *GUS*	10% of transgenic organogenic calli regenerated plants	Fari *et al.* 1995
Hypocotyl	*Bt (cry IIIB)*	Resistance to CPB was not observed	Chen *et al.* 1995
Hypocotyl	*Bt (cry IIIB)*	Resistance to CPB using a mutagenized version of the *Bt cryIIIB* gene	Arpaia *et al.* 1997
Cotyledon	*Bt (cry IIIB)*	Resistance to CPB using a mutagenized version of the *Bt cryIIIB* gene	Iannacone *et al.* 1997
Leaf	*Bt (cry IIIB) NptII* and *GUS*	Influence of growth regulators and antibiotics on transformation efficiency	Billings *et al.* 1997
Leaf	*Bt (cry IIIA) and GUS*	Resistance to CPB using a synthetic version of the *Bt cryIIIA* gene	Jelenkovic *et al.* 1998
Cotyledon	*Bt (Cry 1Ab)*	Resistance to *Leucinodes orbonalis* using a synthetic *cry1Ab* gene	Kumar *et al.* 1998
Leaf	*Luc*	Evaluation of the stability of luciferase gene expression	Hanyu *et al.* 1999
Cotyledon	*pAtgtrp-5::GUS* and *NptII*	Optimization of factors which influence transformation efficiency	Magioli *et al.* 2000
Cotyledon	*DefH9-iaaM*	Parthenocarpic transgenic plants	Donzella *et al.* 2000
Cotyledon	*MtlD*	Tolerance against osmotic stress	Prabhavathi *et al.* 2002
Root	*NptII* and *GUS-INT*	Stable transformation using root explants	Franklin & Sita 2003

NptII - neomycin phosphotransferase II, *CAT - chloramphenicol acetyltransferase*, *Bt (cry IIIB) - Bacillus thuringiensis cry IIIB*; *Bt (cry IIIA) - Bacillus thuringiensis cry IIIA*; *Bt (Cry 1Ab) - Bacillus thuringiensis cry 1Ab*; *GUS - α-glucuronidase*; *Luc - luciferase*; *pAtgrp-5* - regulatory region of the *Arabidopsis thaliana* glycine rich protein 5; *DefH9-iaaM* - regulatory region of the *DEFICIENS 9* gene from snapdragon and the auxin-synthesizing gene coding region *(IaaM)* from *Pseudomonas syringae* pv *savastanoi*; *MtlD* – bacterial *mannitol-1-phosphodehydrogenase* gene.

2.3 EGGPLANT AS A MODEL PLANT

In addition to providing tools for selection of valuable agronomic traits, *in vitro* culture systems can be used as models to study molecular signals in plant physiology and development. The establishment of efficient *in vitro* regeneration systems is also the first step for developing genetic transformation protocols in order to introduce novel traits or to study regulation of gene expression in plants. The availability of efficient protocols for *in vitro* regeneration, both via organogenesis and embryogenesis, as well as for genetic transformation of eggplant, offers an excellent model system to investigate plant physiology *in vitro*. Accordingly, reports published in the last few years provided several examples for the use of eggplant as a model plant and will be discussed herein.

Different plant *in vitro* morphogenic systems have been used to study the role of polyamines (PA), which are proposed as a new class of growth regulators involved in differentiation, reproduction, disease resistance and stress (Scoccianti *et al.* 2000). Eggplant tissues may favour a clearer picture of these molecular signals in differentiation processes, considering their ability to regenerate plants *in vitro* through different morphogenetic processes. The effect of polyamines in the process of *in vitro* morphogenesis has been studied in eggplant by analyzing cellular levels of free and conjugated polyamines and the activity of enzymes involved in biosynthesis and oxidation of polyamines or by treating explants with exogenous polyamines and inhibitors of synthesis of polyamines.

The effect of polyamines, polyamine precursors and biosynthetic inhibitors during embryogenesis was reported by Fobert & Webb (1988). A relationship between the spatial distribution of free and conjugated endogenous polyamine and the differential morphogenetic potential within explants have been observed during embryogenesis (Yadav & Rajam 1997) and organogenesis (Scoccianti *et al.* 2000). Putrescine has been positively correlated to the efficiency of somatic embryogenesis and shown to occur in different levels during the

process, while spermine and spermidine showed no significant effect (Sharma & Rajam 1995; Yadav & Rajam 1997;1998). In hypocotyl segments, high levels of conjugated spermidine along with high levels of total PA could be correlated with the formation of somatic embryos (Sharma & Rajam 1995). Studies using root cultures of eggplant demonstrated that PAs, particularly spermidine, are intricately involved in root growth and differentiation of lateral roots (Sharma *et al.* 1997).

The accumulation of BiP (Binding Protein), an endoplasmic reticulum resident, stress-related protein of the Heat shock protein (Hsp) 70 family, was demonstrated in hyperhidric plants originated from an organogenic system of eggplant. This finding supports the assumption that the monitoration of BiP synthesis can be used to detect intracellular stress in plants (Picoli *et al.* 2001). Eggplant was also used as model plant for the study the expression and the incorporation of the luciferase gene (*luc*) and LUC activity in a longterm period. The expression of the *luc* gene was found to be stable and its specific activity was shown to fluctuate in response to environmental conditions (Hanyu *et al.* 1999).

Somatic embryogenesis systems in eggplant are also good models to study the earliest stages of embryo development. Specific alterations in gene expression during early stages of somatic embryogenesis induced by 2,4-D have been described through differential display of RNA species by Momiyama *et al.* (1995) and Afele *et al.* (1996), with the identification of three classes of genes that were newly expressed or showed enhanced expression during the first 10 days of culture. Differential cultivar responses to the induction of somatic embryogenesis were correlated to differences in gene expression patterns (Afele *et al.* 1996). In addition, an antioncogen homolog and the activation of retrotransposon were described during the early phase of somatic embryogenesis (Momiyama *et al.* 1996). Differential display and Restriction Fragment Length Polymorphism (RFLP) analyses were used to study alterations in DNA methylation and gene expression in cell suspension cultures which produced either somatic embryos or shoots, resulting in the identification of one

organogenesis and two somatic embryogenesis related transcripts (Bucherna *et al.* 2001).

The expression pattern of *Atgrp-5* gene (glycin rich protein isolated from *Arabidopsis thaliana*) in transgenic eggplants harboring a construct containing an *Atgrp-5* promoter-GUS fusion showed to be highly regulated during developmental processes and to have preferential expression in epidermis and stem phloem (Magioli *et al.* 2000), confirming the observations on tobacco and *Arabidopsis* (Sachetto-Martins *et al.*1995). One important aspect of this work was that embryogenesis in eggplant could be efficiently induced from leaves of *in vitro* transgenic plants. In contrast, induction of somatic embryogenesis in *Arabidopsis* is based on the use of immature zygotic embryos, involving difficult and time-consuming procedures.

Considering that epidermis and stem phloem are sites for pathogen penetration and diffusion, *Atgrp* promotor has potential biotechnological interest and may be used in programs aiming at the genetic improvement of eggplant by introducing genes which confer resistance to diseases or pests. In addition, the analysis of GUS expression during the early stages of somatic embryogenesis using the embryogenic system established by Magioli *et al.* (1998) demonstrated that this promoter is activated simultaneously with the first anatomical events leading to embryo development, indicating that *Atgrp-5* may participate in the early cellular events necessary for entering an embryogenic program (Magioli *et al.* 2001b).

In conclusion, eggplant provides a unique system to study morphogenesis and somaclonal variation, taking into account that *in vitro* regeneration can be induced from different explants, by distinct growth regulators and morphogenetic pathways. In addition, the availability of efficient transformation protocols favors gene regulation studies, especially those related to embryogenesis, with advantages over other species. From this perspective, eggplant can be considered as an alternative model plant to study different aspects of plant biology.

2.4 rd29A::DREB1A GENE FOR ABIOTIC STRESS TOLERANCE

Drought, Salt-loading and freezing conditions are stresses that have adverse effect on the growth of plants and on crop yields. The physiological response to these stresses arises from changes in the cellular gene expression profile, and a number of genes are induced by exposure to such conditions (Thomashow *et al*.1994; Shinozaki and Yamaguchi- Shinozaki 1996). The products of these genes can be classified into two groups: those that protect against environmental stresses directly and those that regulate gene expression and signal transduction during the stress response (Shinozaki and Yamaguchi-Shinozaki 1997). The first group includes proteins that are most likely to function by protecting cell from dehydration, including enzymes required for the biosynthesis of various osmoprotectants, late-embryogenesis-abundant (LEA) proteins, antifreeze proteins, chaperons, and detoxification enzymes (Ingram and Bartels 1996; Bray 1997; Shinozaki and Yamaguchi- Shinozaki 1997).

In nature when plants are exposed to conditions of stress, they can develop tolerance, but this is a slow process. To overcome this time lag, many genes related to different stress responses have recently been transferred to various plants to improve stress tolerance (Pilon-Smits *et al*. 1995; Goddijn *et al*.1997; Kasuga *et al*.1999; Huang *et al*. 2002). Transgenic methods involving several different gene transfer approaches have been used to improve the stress tolerance of plants. To investigate the possibility of simultaneously enhancing tolerance towards multiple abiotic stresses using *Agrobacterium*-mediated gene transfer, Kasuga *et al*. (1999) used this system to test a stress-inducible transcription factor that regulate many genes involved in stress tolerance in *Arabidopsis thaliana*. A cis-acting elements has been identified in the promoter region of rd29A gene and is responsible for both dehydration and cold-induced expression (Yamaguchi- Shinozaki 1994). This sequence (TACCGACAT), called the dehydration responsive elements (DRE), is essential for regulating dehydration responsive gene expression (Yamaguchi- Shinozaki and

Shinozaki 1994) and is found in the promoter region of other genes induced by dehydration and cold stresses (Yamaguchi- Shinozaki and Shinozaki 1994; Wang *et al*.1995).

The c-DNAs encoding the DRE-Binding proteins DREB1A and DREB2A have been isolated using Yeast one-hybrid screening (Lui *et al*.1998), and both proteins specifically bind to and activate transcription of genes containing the DRE sequence in *Arabidopsis.* The overexpression of the DREB1A (CBF3) c-DNA, under the control of CaMV 35S Promoter, was subsequently shown to result in the strong expression of the strong expression of target genes that are stress-inducible following conditions of drought, high salt are freezing temperatures (Lui *et al*.1998; Kasuga *et al*.1999; Gilmour *et al*.2000). Therefore, the stress- inducible rd29A promoter was used to drive the expression of DREB1A, with the aim of minimizing the negative effects on plant growth experienced with the use of the 35S CaMV promoter. Improvements in the stress tolerance responses of the transgenic plants and much improved growth under normal conditions were observed with this construct (Kasuga *et al*.1999).

Novillo *et al.* (2004) reported that CBF2/DREB1C is a negative regulator of CBF1/DREB1B and CBF3/DREB1A expression and plays a central role in stress tolerance in *Arabidopsis* and CBF/DREB1(C-repeat-binding factor/dehydration responsive element-binding factor 1) genes encode a small family of transcriptional activators that have been described as playing an important role in freezing tolerance and cold acclimation in Arabidopsis.

Yamaguchi-Shinozaki *et al*.(2000) reported that there is a common signal transduction pathway for dehydration and cold stress involving the DRE/CRT cis-acting element. Thus, tolerance affected to one stress response might provide cross tolerance to another. These genes can be divided into two groups; genes involved in cell protection during drought stress and genes involved in regulation of other genes involved in drought response.

Mie Kasuga *et al.*(2004) have reported that a combination of the *Arabidopsis* DREB1A gene and stress-inducible *rd29A* promoter

improved drought- and low-temperature stress tolerance in tobacco by gene transfer and concluded that, overexpression of *DREB1A* improved drought- and low-temperature stress tolerance in tobacco. The stress-inducible *rd29A* promoter minimized the negative effects on the plant growth in tobacco. Furthermore, they detected overexpression of stress-inducible target genes of DREB1A in tobacco. These results indicate that a combination of the *rd29A* promoter and DREB1A is useful for improvement of various kinds of transgenic plants that are tolerant to environmental stress.

Oh *et al.* (2005) reported that, *Arabidopsis* CBF3/DREB1A and ABF3 in transgenic rice has increased tolerance to abiotic stress without stunting growth.

Bobak Behman *et al*. (2006) studied on the *Arabidopsis* DREB1A gene driven by stress-inducible rd29A promoter increases salt-stress tolerance in proportion to its copy number in tetrasomic tetraploid potato and reported that, the gene transfer of rd29A::DREB1A could be used to increase the salt, dehydration, freezing tolerance of important agricultural crops such as, tetrasomic polyploidy potatoes, as occurs in diploid model species such as, *Arabidopsis.*

Table 2.4 : Characteristics *of two* desiccation-responsive genes, rd29B and rd29A. from *A. thaliana*

Organism	Arabidopsis thaliana, Columbia ecotype
Location	Multigene family of unknown location
Function	Unknown, drought-inducible hydrophilic protein
Techniques	lsolation of RD29 cDNA clone was described by Yamaguchi-Shinozaki et al. (1992). Cenomic DNA library from Clontech (Palo Alto, CA) was screened with RD29 cDNA clone (Yamaguchi-Shinozaki and Shinozaki, 1992).
Expression Characteristics	The rd29A gene was induced within 20 min after desiccation began, but rd29B mRNA did not accumulate to a detectable level until 3 h after desiccation (Yamaguchi-Shinozaki et al.,1992; Yamaguchi-Shinozaki and Shinozaki, 1992). Both genes were induced by exogenous ABA 3 h after ABA was applied to plants (Yamaguchi-Shinozaki and Shinozaki, 1992).
Features of Genes	The nucleotide sequence of 8048 bp includes the two coding regions for rd29B (from nucleotide 1786 to nucleotide 3870) and rd29A (from nucleotide 5512 to nucleotide 7909) in this order, 1785-bp 5' flanking region of rd29B, 1641-bp spacer region between rd29B and rd29A, and 139-bp 3' flanking region of rd29A. The rd29A gene has three introns of 85, 96,and 84 bp. The rd29B gene contains three introns of 81, 113,and 76 bp at the same positions as those of the rd29A Introns

2.5 BIOCHEMICAL AND PHYSIOLOGICAL STUDIES OF TRANSFORMANTS

Development of transgenic plants using biotechnological tools has become important in plant-stress biology. The various abiotic stresses cause changes in plant processes at all levels of organization i.e. morphological, physiological, biochemical and molecular. Also affects the productivity and quality of crops. Abiotic stress is the primary cause of crop loss worldwide, reducing average yields for most major crop plants by >50% (Mayra Rodriguez *et al*., 2005). Drought, salinity, extreme temperatures and oxidative stress are interconnected and affect the water relations of a plant on the cellular as well as whole plant level causing specific as well as unspecific reactions (Beck *et al.* 2007). This leads to a series of morphological, physiological, biochemical and molecular changes that adversely affect plant growth and productivity (Wang *et al*. 2001). In plant, primary and secondary damage and stresses have been differentiated. The secondary stress and damage is caused by reactive oxygen species (ROS) (Randy

1995) generated from imbalances of electron transport rate and also from the impacts of cold or excess of light energy, which are primary stresses (Huner *et al*., 1998).

As tolerance to stress is multifactorial syndrome rather than result of a single reaction or gene (Mittler 2006;Shinozaki & Yamaguchi-Shinozaki; 2000), tackling of the primary stress reactions by gene transfer can also alleviate the secondary stress and generate plant with higher stress tolerance (Beck *et al.* 2007). During drought stress, protoplast volume shrinkage by water loss leads to loss of turgor, osmotic stress and a potential change of membrane potentials. Upon severe loss of water from the cells, membrane disintegration and abolition of metabolic processes occur (Mahajan and Tuteja 2006).

Osmotin is a protein that plays a role in conferring tolerance to drought and salt stress in plants. Over expression of *osmotin* induces proline accumulation and confers tolerance to osmotic stress in transgenic plants. Amino acid proline is enhanced due to accumulation of osmotin as reported earlier (Kavi Kishor *et al*. 1995). Osmotin accumulate vacuolar inclusion bodies of cells which adapted to NaCl. It is also localized in the cytoplasm to a much lower level and is loosely associated with isolated plasma membrane and tonoplast vesicles (Singh *et al*. 1987).

During the evaluation of T3 Osmotin-transgenic tomato, Danswrang Goyary (2009) observed lesser leakage of electrolyte and increased concentration of proline in transgenics as compared to the control under stress. Mahmoud M. Saker *et al*. (2011) studied on comparative analysis of transformed potato microtubers and its non-transformed counterpart using some biochemical analysis along with inter simple sequence repeat (ISSR) marker and this study concludes that essentially nutritional equivalence was found between transgenic and nontransgenic potato lines. It was further added that, there were no significant differences between the transformed and non-transformed lines in the total amount of amino acids, some minerals, total phenolic, total flavonoid and β-carotene contents. These preliminarily results thus confirm that the nutritional quality of

transgenic potato line was mostly substantially equivalent to that of the nontransgenic counterpart. No detrimental changes in the nutritional composition of the transgenic potato were observed as a result of the insertion of CP-PVY gene.

Mohamed Salah Beltagi (2008) studied on Molecular responses of Bt transgenic corn (*Zea mays* L.) plans to salt (NaCl) stress and observed lower chlorophyll a/b ratio in non-transformed corn plants, the high ratio of chlorophyll a/b as well as the stability of chlorophyll b content in Bt transgenic corn plants which can be ranged as an index of salt toleranceand concluded this might produce higher photosynthetic rate and consequently high yield (Raja Babo *et al*., 2005). Moreover, the chlorophyll stability index (CSI) is an important index for screening plant tolerance to abiotic stresses (Michael Gomaz and Rangasamy, 2002; Yagameena, 2004). In this investigation, the high values of chlorophyll satiability index (CSI%) verified the tolerance of Bt transgenic corn plants to salinity stress, results were supported by the findings of Raja Babo *et al*. (2005).

V. Prabhavathi *et al*.(2001) studied on Abiotic stress tolerance in transgenic eggplant (*Solanum melongena* L.) by introduction of bacterial mannitol phosphodehydrogenase gene and reported that the morphology of the transgenic plants was normal as controls, but the chlorophyll content was higher in some of the lines.

CHAPTER-III

Materials & Methods

This highlighted the "spark plugs" to ignite the material and methods for final result. This is said to be the plan of work for the entire research work.

III. MATERIAL AND METHODS

The present study on transgenic research in brinjal was carried out at the Department of Agricultural Biotechnology, College of Agriculture, Orissa University of Agriculture And Technology, Bhubaneswar-751003 (Orissa). The details of materials used and the experimental techniques adopted during the course of investigation are presented in this chapter.

3.1 MATERIALS

3.1.1 Genotypes

The popular high yielding genotype Utkal Anushree adapted to different agroclimatic situations in Orissa was chosen for the study. The seeds of brinjal cultivar Utkal Anushree were collected from the vegetable Breeder, Dean of Research, Orissa University of Agriculture and Technology, Bhubaneswar and were stored in desiccators to be used for the purpose.

Plate 1: Seeds of *Solanum melongena* cv. Utkal Anushree

Table 3.1: Characteristic Features Of Eggplant cultivar Utkal Anushree

Variety	Utkal Anushree
Duration	80-85 days
Grain Yield (kg/ha) Potential average	3849
Special Features	Medium height, oblong, small, clustered, and green fruits. Tolerant to bacterial wilt.

3.1.2 Explant source

Four different types of explants from 25 days' old sterile seedling of cultivar Utkal Anushree were used for *in vitro* regeneration and transformation studies. They are abbreviated as mentioned below.

• Cotyledonary leaves (E1)

• Hypocotyl (E2)

• Cotyledonary nodal region/shoot tip (E3)

• Root (E4)

3.1.3 Plant growth regulators (Source- Merck)

The following plant growth regulators were used in different experiments at different concentrations.

Auxins

- Indole Acetic Acid (IAA)
- Naphthalene Acetic Acid (NAA)
- 2, 4-Dichlorophenoxy Acetic Acid (2, 4-D)

Cytokinins

- Benzylaminopurine (BAP)
- Kinetin (KIN)

3.1.4 Plant nutrient medium

Murashige and Skoog (1962) basal salts (Source- Merck) (Appendix I) were used.

3.1.5 Antibiotics (Source- Merck)

The following antibiotics were used in different experiments at different concentrations.

- Kanamycin
- Rifampicin

- Gentamycin
- Cefotaxime
- Carbenicillin

3.1.6 *Agrobacterium* strain and plasmid vector

The disarmed *Agrobacterium* strain GV3107 harboring pCAMBIA2300 was used for *in vitro* transformation and it was received from IARI, New Delhi. pCAMBIA2300 contains *rd29A::DREB1A* gene and *nptII* marker, linked to the CaMV35S promoter and nos terminator (Fig. 1).

3.1.7 Primers for PCR analysis

DREB1A gene specific primers
Forward primer : 5'TGATTATATTCCGACGCTTG3'
Reverse primer : 5' TTCATGATTATGATTCCACT3'

3.1.8 Chemicals for biochemical analysis

3.1.8.1 Photosynthetic pigment estimation

- 80% Acetone
- Ethyl alcohol (absolute)

3.1.8.2 Proline estimation

- Ninhydrin reagent (1.25 gm Ninhydrin + 30 ml Glacial acetic acid + 20 ml 6M Phosphoric acid)
- L-Proline
- 3% sulfosalicylic acid

Fig. 1: Restriction map of *rd29A:: DREB1A* gene construct

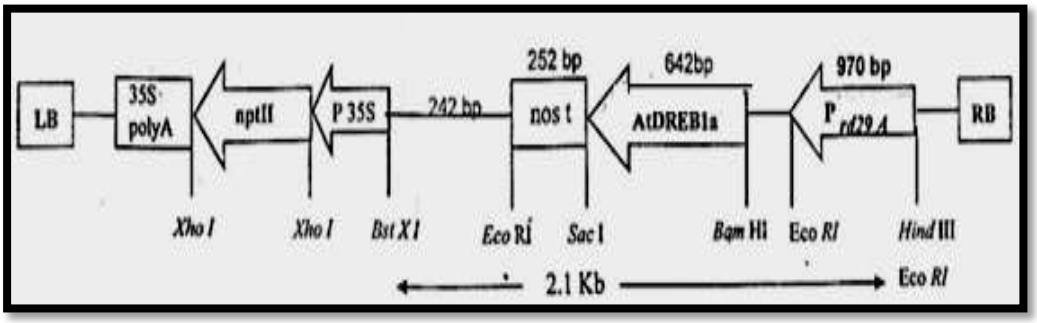
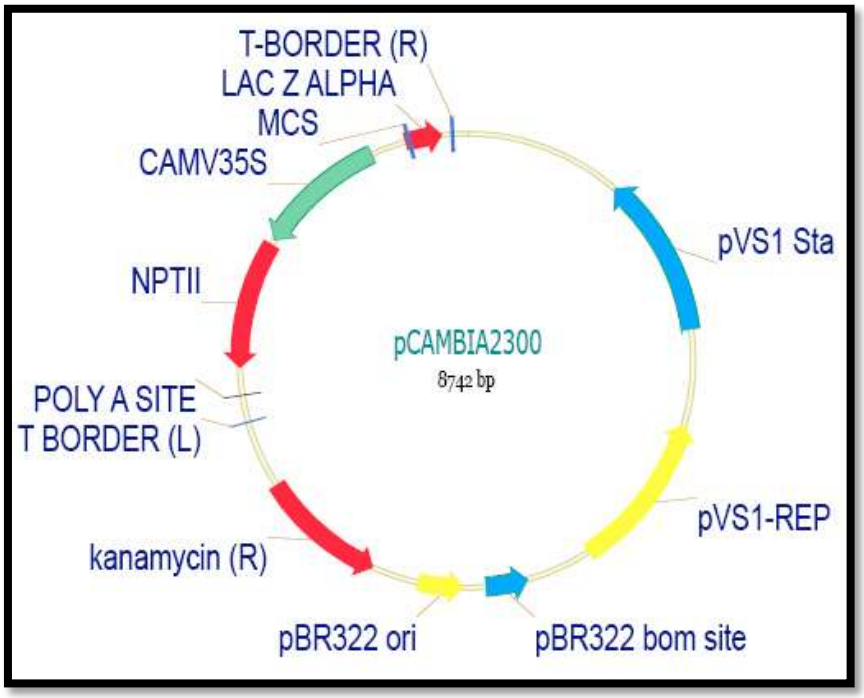


Fig. 2: Schematic map of vector pCAMBIA2300



3.2 METHODOLOGY

3.2.1 Preparation of explants

Seeds were surface sterilized with 0.1 per cent mercuric chloride ($HgCl_2$) for 3 minutes, washed thoroughly with sterile water 4 to 5 times to remove the traces of $HgCl_2$ and then blot dried. Sterilized seeds were used for raising seedling *in vitro* on ½ MS medium.

The following explants were taken from 25 day old seedlings under sterile conditions using scalpel blade.

- Cotyledonary leaves (E1)
- Hypocotyl (4 mm) (E2)
- Shoot tip along with cotyledonary nodal region (E3)
- Root (E4)

3.2.2 Preparation of media

MS basal salts supplemented with different growth regulators at different concentrations were used depending on the purpose of the individual experiment. Separate stock solutions of macronutrients, micronutrients, iron source, and organic supplements (except myoinositol) were prepared (Appendix I). The medium was prepared by adding appropriate quantities of the stock solutions as mentioned below and correct volume was made up with the distilled water. Sucrose 30 g/l and myoinositol 100 mg/l were added fresh. pH was adjusted to 5.6 to 5.8 by using 0.1N HCl or 0.1N NaOH. Then agar (8 g/l) was added and melted. About 12.5 ml of medium was poured into each test tube (150 × 25 mm) before it melts, and plugged with cotton plugs. The media was autoclaved at 121°C for 15 minutes and kept for inoculation.

Amount of stock solution added to the media

Sl.no.	Stock solution	Strength	Amount to be added (ml/l)
1	Macronutrient	20X	50
2	Micronutrient	200X	5
3	Iron source	200X	5
4	Organic supplements	200X	5

Myoinositol was weighed separately @ 100 mg/l and added during media preparation.

3.2.3 Physical factors for *in vitro* culture

All the plant tissue culture experiments were conducted under defined conditions of the culture room maintained at 25 ± 2°C, uniform light (1000 lux) provided by fluorescent tubes over a photoperiod of 16/8 hours. The *in vitro* culture work was carried out aseptically in a laminar airflow chamber. Borosilicate culture tubes (150 × 25 m), glasswares and analytical grade chemicals were used.

3.2.4 Preparation of antibiotic stocks

Kanamycin and gentamycin stocks of 100 mg/ml were prepared in sterile double distilled water. Rifampicin stock of 100 mg/ml was prepared in DMSO. Cefotaxime and carbenicillin stock of 100 mg/ml were prepared in double distilled sterile water and all the stocks were filter sterilized and stored at 4°C for future work.

3.2.5 Direct regeneration

Multiple shoot induction

Different explants such as cotyledonary leaves, hypocotyls and cotyledonary nodal region/shoot tip explants were cultured on MS medium supplemented with different levels of BAP (1.0, 1.5, 2.0 mg/l) and kinetin (1.0, 1.5, 2.0mg/l) to obtain multiple shoots. The observations were recorded from 5th day of inoculation at 2 days interval. The experiment was repeated for four times.

Elongation of shoots

The shoots obtained in the above experiments were subcultured on the same medium for their elongation and then number of shoots showing elongation, number of elongated shoots per culture and days taken for elongation were recorded.

3.2.6 Callus mediated regeneration

3.2.6.1 Callus induction

Three explants *viz.,* cotyledonary leaf, hypocotyl and root explants were cultured on MS medium supplemented with different growth regulators and incubated in dark for callus induction. Observations were recorded on days to callus initiation, number of explants responding/treatment and quantity of callus induced, type of callus induced (Friable, cottony, watery, compact), colour of callus, visual callus quality. MS medium with following combinations of growth regulators were used for callus induction.

	BAP			
2, 4-D	mg/l	0	0.5	1.0	1.5
	1.0	T1	T4	T7	T10
	1.5	T2	T5	T8	T11
	2.0	T3	T6	T9	T12

3.2.6.2 Regeneration through callus

Calli induced on the different media combinations were subcultured for regeneration on graded doses of BAP (1.0, 1.5 & 2.0 mg/l) and Kinetin (1.0, 1.5 & 2.0 mg/l). Type of response (callus growth, regeneration), days to shoot bud initiation, number of shoots regenerated from callus were recorded by taking observation from 5th day of inoculation at 2 days interval.

3.2.7 Rooting and hardening

Well elongated shoots were transferred to MS medium, ½ strength MS medium and MS medium supplemented with NAA (0.01, 0.05, 0.1 mg/l) to optimize protocol for root formation. Number of shoots producing roots and type of roots (profuse, scanty) produced were recorded after 8 days of culture.

Rooted shoots were transferred to pots containing soil:sand:FYM (2:1:0.5) mixture as substrate for establishment.

3.3 OPIMIZATION OF METHODS FOR EFFICIENT GENETIC TRANSFORMATION IN BRINJAL

In this study, *Agrobacterium* mediated *in vitro* transformation method was tried for gene transfer.

3.3.1 Maintenance and growing of *Agrobacterium*

The *Agrobacterium* strain carrying plasmid pCAMBIA2300 containing rd29A*:: DREB1A* gene construct was maintained on the solid yeast extract mannitol agar (YEMA) (Appendix II) medium containing 50 mg/l kanamycin, 10 mg/l rifampicin and 10 mg/l gentamycin. Sub-culturing was done every month on fresh medium. For transformation experiment, single colony of *Agrobacterium* was taken from the plate and inoculated in 100 ml YEM broth containing selective antibiotic and

was incubated on shaker for 48 hrs at room temperature. The culture that read 0.8 OD at 600 nm λ was chosen for bacterial infection of the totipotent plant tissue.

3.3.2 Co-cultivation

Shoot tip explants were taken from 25 days old *in vitro* grown seedlings and co-cultivated with *Agrobacterium*. The overnight grown culture was centrifuged at 10,000 rpm for 5 minutes at 22ºC, the supernatant was discarded and the bacterial pellet was suspended in liquid AB medium (AppendixIII). Shoot tips were suspended in *Agrobacterium* suspension and kept for 5 minutes with gentle shaking. Excess bacteria were blot dried and explants were placed in petriplate lined with blotting paper which was wet by liquid plant growth medium (LPGM) (Appendix IV). Plate was wrapped with aluminium foil to avoid the drying of blotting paper due to evaporation of LPGM medium and kept for 2 days in culture room for co-cultivation. Then the explants were washed with sterile Distil water having cefotaxime (500 mg/l), blot dried and inoculated on the MS+ 2.0mg/l BAP + 250mg/l cefotaxime + 100 mg/l kanamycin medium.

3.3.3 Cefotaxime/Carbenicillin sensitivity test

To find out the suitable concentration of cefotaxime/carbenicillin to avoid bacterial contamination during morphogenesis and to know minimal level of cefotaxime/carbenicillin which wouldl completely eliminate the excess bacteria after co-cultivation, this test was conducted at 100, 200, 300, 400 and 500 mg/l cefotaxime/carbenicillin along with control.

3.3.4 Kanamycin sensitivity

Initially, kanamycin sensitivity test was carried out to find out the minimum concentration of kanamycin required to inhibit the growth of normal, untransformed explants to design the medium for selection of transformed plants. This was done by culturing the explants on optimized MS medium for regeneration of plants with the following levels of kanamycin added to it (0, 20, 40, 60, 80, 100,125, 150, and 200 mg/l).

3.3.5 Isolation of plasmid DNA from *Agrobacterium* (Alkali lysis method- Sambrook and Russel, 2001)

- A single *Agrobacterium* colony was picked up aseptically using a sterile inoculation needle and was grown overnight in 10 ml YEMA (Appendix II) medium containing kanamycin (50 mg/l), gentamycin (10 mg/l) and rifampicin (10 mg/l) in a sterile conical flask.
- Overnight grown culture was transferred to centrifuge tube and centrifuged for 15 min at 10,000 rpm.
- The supernatant was removed and cell pellet was dried.
- 200 µl ice cold suspension buffer (Appendix V), was added in pellet and pellet was dissolved by vortexing.
- 200 µl of freshly prepared lysis buffer (Appendix V) was added, and stored in ice for 5 min.
- 200 µl of 1.5 M K-acetate (Appendix V) was added and mixed well (not by vortexing) and stored in ice for 10 minutes.
- Lysate was centrifuged for 15 min at 12,000 rpm. Supernatant was transferred to next tube.
- Equal volume of phenol-chloroform was added, vortexed and centrifuged for 10 min at 10,000 rpm. Aqueous upper layer was transferred to a fresh tube.

- DNA was precipitated by adding 600 µl of isopropanol and kept at -20°c temperature for overnight.
- Suspension was centrifuged at 12,000 rpm for 15 min and DNA pellet was dried.
- Pellet was washed with 1 ml of 70 percent ethanol and pellet was dried completely.
- The pellet was dissolved in 30 µl of $T_{10}E_1$ buffer (Appendix VI).
- 2 µl of RNAse was added and incubated at 37°c for 1 hr.
- Purified sample was stored at -20°c temperature for further use.

3.3.6 Isolation of Plant genomic DNA (CTAB Method- Edwards *et al.*, 1991)

- Plant tissue was collected using 1.5 ml eppendorf's tube lid to ensure uniform size.
- Collected tissue was macerated in pestle and mortar at room temperature without buffer for 15 sec.
- Extraction buffer (0.4 ml) (Appendix VII) was added and sample was vortexed for 5 sec (can be kept in room temperature for more than 1hr).
- The solution was centrifuged at 13,000 rpm for 1 minute and 300 µl supernatant was transferred to fresh eppendorf's tube.
- Supernatant was mixed with 300 µl isopropanol and incubated at room temperature for 2 minutes and centrifuged at 13,000 rpm for 2 min.
- Pellet was dried and suspended in 100 µl 1X $T_{10}E_1$.
- 100 µl RNase (1 mg/ml) was added to the DNA and incubated at 37ºC in water bath for half an hour.
- DNA was precipitated using $1/10^{th}$ volume of 3M Na-acetate and ethanol and incubated over night at 4^0C.
- The solution was centrifuged at 13,000 rpm for 2 min and pellet was dried again.
- Pellet was suspended in 50 µl 1X $T_{10}E_1$.

3.3.7 PCR amplification for confirmation of transformants

The chromosomal DNA of plants obtained from co-cultivated explants, control plants and plasmid DNA (positive control) were used as template for PCR confirmation of the targeted DREB1A gene with primers specific to this gene. Nucleotide sequence of DREB1A specific primer is as follows;

Forward : 5'TGATTATATTCCGACGCTTG3'

Reverse : 5' TTCATGATTATGATTCCACT3'

PCR reaction mix used was;

Template DNA	-	50 ng
Primer	-	20 ng
dNTPs	-	0.1 mM
Taq DNA polymerase	-	0.33 µl (1 unit)
Taq assay buffer	-	1x

PCR amplification conditions were as follows.

Stage	Temperature (ºC)	Duration (min)	Cycles
Denaturation	92	5	1
Denaturation	92	2	35
Annealing	51	1	
Primer extension	72	2	
Primer extension	72	10	1
Soak	4	1	-

After completion the samples were stored at 4ºC and contents were loaded on the agarose gel for electrophoresis.

3.3.8 Separation of PCR amplified products by agarose gel electrophoresis

PCR amplified products (10 µl) mixed with 2 µl of bromophenol blue (Appendix VIII) were loaded on 1.2 % agarose gel. The electrophoresis was carried out at 60 V for 1 hr using 1X TAE buffer (pH 8.0) (Appendix IX). Molecular weight marker was run in a separate lane.

3.4. Morpho-physiological anslysis of transgene expression

3.4.1 Studies on growth parameters under stress

After 8 days' of moisture stress plant height, no. of flowers and no. of leaves of the control and transformed plants were recorded.

3.4.2 Relative water content (RWC)

After 8 days' of moisture stress relative water content of control and transformed plant was measured following the method described by, Barrs and Weatherley (1962).

Procedure:

- Leaf discs were collected from control and transformed plants in replicates
- Fresh weight of leaf discs was recorded (Fw) and they were drowned in distilled water in covered petriplates
- These petriplates were kept at room temperature for overnight to attain turgidity.
- Turgid weight (Tw) of the samples were recorded after the blot dry of leaves.
- The same leaf samples were then oven dried for 48 hrs at 80ºC in hot air oven.
- Dry weight (Dw) of the respective samples were recorded.
- RWC was determined using the following formula:

$$RWC\ (\%) = \frac{Fw - Tw}{Tw - Dw} \times 100$$

3.5 Biochemical analysis of transgene expression

3.5.1 Estimation of photosynthetic pigments

Photosynthetic pigments were estimated from transformed and control plants by using Acetone method (Arnon,1949) and carotenoid following Kirk and Allen (1968).

Procedure:

- 100 mg of leaf sample was taken and crushed with sterile sand in 2 ml of 80% acetone with the help of mortar and pestle.
- Extracted samples were collected in sterile centrifuge tube and centrifuged at 10,000 rpm for 10 min. at 4ºc in a refrigerated centrifuge.
- Supernatant was collected in fresh sterile centrifuge tube and the volume was made upto 2 ml with 80% acetone and kept in a dark.
- Absorbance was recorded at 663 nm, 645 nm and 470 nm spectrum of wave length using UV-VIS spectrophotometer.
- The amount of photosynthetic pigment was calculated by using following formulae:
  - Chla (µg/ml) = $(12.25 \times A_{663}) - (2.79 \times A_{645})$
  - Chlb (µg/ml) = $(21.5 \times A_{645}) - (5.1 \times A_{663})$
  - Chla+Chlb (µg/ml) = $(7.15 \times A_{663}) + (18.73 \times A_{645})$
  - Carotenoid (µg/ml) = $\dfrac{(1000 \times A470) - (1.82 \times \text{Chla (µg/ml)}) - (85.2 \times \text{Chla (µg/ml)})}{198}$

3.5.2 Estimation of Proline

Moisture stress for 8 days was given to transformed and control plant and then the proline was estimated from control(stressed and non-stressed) and transformed (stressed and non-stressed) following Bates *et al.* (1973) and Sadasivam and Manickam(1992).

Procedure:

- 100 mg of leaf sample was taken and homogenized in 3% sulfosalicylic acid
- The homogenate was filtered through whatmann no.1 filter paper
- 2 ml of filtrate was taken and 2 ml of ninhydrin solution was added and 2 ml of glacial acetic acid was added to make the final volume of 6 ml
- The mix. was incubated at 100ºc in heating waterbath for 1 hr.
- The reaction was stopped by putting on ice and 4 ml of toluene was added and the mix. was shaken vigorously for 20-30 sec.
- The aqueous toluene layer was separated
- The red colour was measured at 520 nm wavelength of light.
- The conc. of proline in each sample was calculated by plotting the absorbance against standard curve.
- The proline content was expressed on fresh weight basis(micromoles/gm tissue) as per the following formula:

$$\frac{\mu g \text{ Proline/ml} \times \text{ml of Toluene}}{115.5} \times \frac{5}{\text{gram of sample}}$$

Where, 115.5 = mol.wt. of Proline.

3.5.3 Estimation of Membrane stability Index

The membrane stability index of control and transformed plant both under stress for 8days and non-stress was estimated following Onwueme (1979).

Procedure:

- 100 mg of leaf samples were taken from all the above mentioned plants in replicate in the test tubes containing 10 ml of distilled water.
- One set of the test tubes containing plant samples was heated at 40ºC for 30 min. in water bath, and the electrical conductivity of the solution was measured in the conductivity meter. And the conductivity was marked as C1.
- Another set of test tubes was boiled at 100 ºC on boiling water bath for 10 min. and the electrical conductivity of the solution was measured in the conductivity meter. And the conductivity was marked as C2.
- Membrane stability index of each sample was calculated by using following formulae:
- Membrane stability index= {1- (C1/C2) x 100}

3.6 OBSERVATIONS

Various biometrical observations on the following parameters were recorded as per the details given below**.**

3.6.1 Assessment of percent shoot induction

Shoot induction at the end of 30 days of culture period was assessed by calculating number of explants responded for multiple shoot induction and was expressed in percentage.

$$\text{Percent shoot induction} = \frac{\text{No. of explants with multiple shoots}}{\text{Total no. of explants cultured}} \times 100$$

3.6.2 Assessment of percent callus initiation

Response of explants to callus initiation at the end of 10 days of culture was assessed by calculating number of explants responded for callus initiation and expressed in percentage.

$$\text{Percent callus initiation} = \frac{\text{No. of explants with callus initiation}}{\text{Total no. of explants cultured}} \times 100$$

3.6.3 Assessment of percent rooting

Response of regenerated shoots to rooting at the end of 10 days of culture was assessed by calculating number of shoots responded for rooting and expressed in percentage.

$$\text{Per cent rooting} = \frac{\text{No. of shoots with rooting}}{\text{Total no. of explants cultured}} \times 100$$

3.6.4 Assessment of percent kanamycin resistants (putative transformants)

All the co-cultivated explants were cultured on shooting medium containing 80mg/l kanamycin and then putative transformants were obtained by transferring the shoots obtained from shooting medium supplemented with 80mg/l kanamycin to shooting medium supplemented with 100mg/l kanamycin. And the percent kanamycin resistant (putative transformants) was calculated.

$$\text{Percent putative transformants} = \frac{\text{Total no.of explants survived on 100mg/l kanamycin} \times 100}{\text{Total no. of shoots obtained from co-cultivated explants}}$$

3.6.5 Transformation efficiency

After the PCR analysis of putative transformants the transformation efficiency was calculated by using following formula:

$$\text{Transformation efficiency} = \frac{\text{Total no. of PCR +ve plants} \times 100}{\text{Total no. of shoots obtained from co-cultivated explants}}$$

3.7 STATISTICAL ANALYSIS

Various biometrical observations taken on invitro studies were given due statistical treatment based on completely randomized design with four replications. The ‘F’ test was carried out after analysis of variance (ANOVA) and CD (p=0.5) and CV% values were calculated where the ‘F’ test was significant.

CHAPTER-IV

Results & Discussion

Back bone of any research this is the "bulk fruit" of the entire work of research. Interpretation and analysis gives it proper shape to churn out the summary & conclusion. In this segment of study the researcher tried to pen the obtained information in proper format and analyzing the trends. It is believed that, discussion of the observed result often help the future similar research studies.

4.1 EXPERIMENTAL RESULTS

The results of the experiments conducted in this study on *in vitro* regeneration and genetic transformation are presented in this chapter in the following heads.

4.1.1 *In-vitro* regeneration

4.1.1.1 Direct regeneration

4.1.1.2 Indirect regeneration (via callus)

4.1.1.3 Rooting and hardening

4.1.1.4 Kanamycin based selection system

4.1.2 Genetic transformation

4.1.3 Screening and confirmation of transformants

4.1.4 Morpho-physiological and biochemical analyses of transgene expression

4.1.1 *IN-VITRO* REGENERATION

A successful application of *in vitro* techniques for crop improvement rests upon a reproducible plant regeneration protocol. There are two methods: a direct and an indirect regeneration method with an intervening callus mediation tried for getting efficient and reproducible in vitro regeneration protocol in brinjal cv. U. Anushree for its genetic transformation.

4.1.1.1. DIRECT REGENERATION

Most often direct regeneration occurs through shoot proliferation from pre-existing meristems instead of *de novo* formation of a meristem. Direct somatic embryogenesis has better applicability in the improvement of crop since plant regeneration from callus cultures is often associated with genetic and cytological variations (Chaudhari, 2004).

It was designed to assess the direct regeneration response i*n-vitro* of different explants like, shoot-tip, hypocotyls, cotyledonary leaf in the

MS medium fortified with different concentrations of cytokinins (BAP, kinetin). The effect of cytokinins in MS medium on organogenesis and plantlet differentiation is presented in table- 4.1. Each experiment was carried out in four replications. Multiple shoots arose on MS medium supplemented with BAP (1.0-2.0 mg/l) with maximum production occurring at 2.0 mg/l BAP from both shoot-tip and hypocotyls explants (Plate 3). Multiple shoots also arose on MS medium supplemented with kinetin (1.0-2.0 mg/l) with maximum production occurring at 1.5 mg/l kinetin from shoot-tip explants and at 2.0 mg/l kinetin from hypocotyls explants. As compared to hypocotyl explants shoot-tip explants require minimum no. of days for the initiation of adventitious shoots. Both Shoot-tip and hypocotyl produces maximum no. of shoot buds (13 and 11.75) on MS medium supplemented with 2.0 mg/l BAP within 18 and 22.75 days respectively. Besides these treatments, lower(<1.0 mg/l) and higher(>2.0mg/l) concentrations of BAP and kinetin were used in the pilot experiments and it was found that, even in higher concentrations of BAP the avg. no. of shoot production was same as that of in 2mg/l BAP and very poor response with lower concentration of BAP(Data not presented). Along with shoot-tip and hypocotyl, cotyledonary leaf explant was also used but its response was nearly same as that of shoot-tip explant.

Table 4.1: Direct Organogenesis- Effect of Explant and PGRs

Treatment	Hormonal combination(mg/l)		Days to initiation of adventitious shoots		Avg. no. of shoots produced/tube	
	BAP	Kinetin	Shoot-tip	Hypocotyl	Shoot-tip	Hypocotyl
1	1.0	-	22.25	24.25	10.00	4.25
2	1.5	-	19.25	21.25	12.00	7.50
3	**2.0**	**-**	**18.00**	**22.75**	**13.00**	**11.75**
4	-	1.0	22.75	27.5	2.75	3.00
5	-	1.5	21.75	25.25	3.75	2.75
6	-	2.0	21.5	25.5	3.25	3.00
CD(0.05)			0.61	0.72	0.85	0.56
CV%			1.95	1.97	7.57	7.00

4.1.1.2. INDIRECT REGENERATION

4.1.1.2. A) CALLUS INDUCTION

Callus refers to the actively dividing unorganized mass of cells induced in culture. Generally, a higher auxin concentration in the growth medium induces callus formation (Skoog and Miller, 1957). Establishment of callus, which retains high morphogenetic potential is a preliminary step in tissue culture of any species. The quantity and quality of callus produced depends on a wide variety of conditions like explants, genotype, growth regulators and light/dark incubation etc. (Mathias and Boyd, 1986).

For induction of callus, cotyledonary leaf, hypocotyl and root explants were cultured on MS medium supplemented with different concentrations of 2,4-D(1.0-2.0 mg/l) in combination with BAP(0.5-1.5 mg/l). The effect of 2,4-D in combination with BAP in MS medium on callus induction is presented in table-4.2. Each experiment was carried out in four replications. Friable, compact, compact green, compact white, watery types of calli were observed irrespective of explants depending on graded doses of phytohormones. Calli obtained on MS medium supplemented with 2,4-D(2.0 mg/l) along with various concentrations of BAP (0.5-1.5 mg/l) were loose friable white and the similar result was obtained on MS medium supplemented with 2,4-D (2.0 mg/l) only within a short duration as compared to the former. The calli obtained from cotyledonary leaf, root and hypocotyl explant on MS medium supplemented with 2.0 mg/l 2, 4-D are represented in Plate 4. The quality of callus obtained from root explant was not good and it requires more than 30 days for the initiation of callus. Also it gave very less quantity of callus (data not presented- pilot experiments). All the three explants were also subjected to callus induction on MS supplemented with different concentrations of NAA (1.0-2.0mg/l) but instead of callus formation rooting was observed from explants (pilot experiments).

Table 4.2: Effect of Explant and PGRs on Callus Induction

Treatment	Hormonal combination (mg/l)		Percentage of responding explants		Mean Days of callus induction		Nature of callus	
	2,4-D	BAP	Cotyledonary leaf	Hypocotyl	Cotyledonary leaf	Hypocotyl	Cotyledonary leaf	Hypocotyl
1	1.0	0	80	90	12.75	12.5	watery green	Watery green white
2	1.5	0	70	80	10.25	11.25	Loose friable green white	Loose friable green white
3	**2.0**	**0**	**90**	**80**	**8.25**	**9.25**	**Loose friable white**	**Loose friable white**
4	1.0	0.5	80	70	12.25	11.75	Loose friable green	Loose friable green
5	1.5	0.5	90	70	11	11.5	Loose friable white green	Loose friable and white
6	2.0	0.5	90	90	9.5	9.25	Loose friable white	Loose friable white
7	1.0	1.0	80	70	10.25	11.5	Loose friable white green	Loose friable white green
8	1.5	1.0	90	60	10.5	11	Loose friable white green	Loose friable white green
9	2.0	1.0	90	80	8.75	9.25	Loose friable white	Loose friable white
10	1.0	1.5	80	70	11	11.25	Loose friable green	Loose friable green
11	1.5	1.5	80	60	10.25	10.5	Loose friable green	Loose friable green
12	2.0	1.5	80	70	10.75	10.25	Loose friable white green	Loose friable white green
CD(0.05)					0.67	0.68		
CV%					4.48	4.43		

4.1.1.2. B) INDIRECT ORGANOGENESIS

After 4 weeks of culture, the well proliferated calli were subcultured 2-3 times on the MS medium supplemented with low concentration of 2,4-D (0.5 mg/l) so as to reduce the effect of auxin. Afterward the calli were transferred to MS Medium supplemented with various concentrations of cytokinins (BAP and Kinetin) for induction of shoots. Both, the calli obtained from cotyledonary leaf and hypocotyl responded well for shoot initiation on MS medium supplemented with BAP (1.0-2.0 mg/l) but not responded well on MS medium supplemented with kinetin (1.0-2.0 mg/l). Days taken for initiation of adventitious shoots on MS Medium supplemented with BAP (2.0 mg/l) from cotyledonary leaf and hypocotyl explants were same (21) and no. of shoots obtained from both of the explants on the same medium were, 14.25 and 13.75 respectively. The effect of lower (<1.0mg/l) and higher(>2.0mg/l)

concentrations of BAP and kinetin is not reported (pilot experiments). The effect of BAP and kinetin on shooting from cotyledonary leaf and hypocotyl explant is presented in table-4.3. The shoots obtained from cotyledonary leaf and hypocotyl explant on MS Medium supplemented with 2.0 mg/l BAP are presented in Plate 5.

Table 4. 3 : Indirect Organogenesis- Effect of Explant and PGRs

Treatment	Hormonal combination (mg/l)		Days to initiation of adventitious shoots		Avg. no. of shoots produced/tube	
	BAP	Kinetin	Hypocotyl callus	Cotyledonary leaf callus	Hypocotyl callus	Cotyledonary leaf callus
1	1.0	-	23.75	23.75	6.75	9.25
2	1.5	-	22.25	22.25	11.25	12.25
3	**2.0**	**-**	**21.00**	**21.00**	**13.75**	**14.25**
4	-	1.0	33.00	33.00	3.50	3.25
5	-	1.5	29.25	29.25	3.75	3.25
6	-	2.0	27.25	27.25	3.50	2.50
CD(0.05)			0.88	0.88	0.79	0.86
CV%			2.25	2.25	7.44	7.70

4.1.1.3. RHIZOGENESIS AND ACLIMATIZATION

The healthy shoots obtained from both direct and indirect methods, were transferred to different media such as, MS, 1/4th MS and MS medium supplemented with various concentrations of NAA (0.01-0.1mg/l) for rhizogenesis. Within 8 days of transfer different types of roots were observed. After 15 days formation of healthy secondary roots were observed (Plate 6). As indicated from Table-4.4, 95% of shoots produced multiple, adventitious, long and thin roots in MS medium supplemented with 0.01 mg/l NAA. Before pre-hardening rooted plantlets were transferred into D.W and kept for 2 days so as to acclimatize them. Then these plants were transferred to polythene bag containing soil:sand:FYM (2:1:0.5) and kept in the culture room for 15 days. After 15 days these plants were transferred to pot containing

soil:sand:FYM (2:1:0.5) and kept in the poly-house for establishment (Plate 7)

Table 4.4 : Response of shoots on different rooting media

Response of shoots on different rooting media	No. of shoots producing roots	Percent response	Type of roots
MS control	8.50	85.00	Long and thin roots
1/4th MS	6.00	60.00	Short and thin roots
MS + NAA(0.01ppm)	9.50	95.00	**Long and thin roots (multiple, adventitious)**
MS + NAA (0.05ppm)	8.75	87.50	Short and thick roots(with callus at shoot cut)
MS + NAA(0.1ppm)	8.50	85.00	Short and thick roots(with callus at shoot cut)
CD(0.05)	0.68		
CV	5.42		

***** 10 shoots were cultured in each treatment under four replications

4.1.1.4. KANAMYCIN BASED SELECTION SYSTEM

The growth inhibiting dose of Kanamycin was determined by transferring the embryogenic calli to the MS Medium supplemented with 2mg/l BAP along with various concentrations of kanamycin (0-100 mg/l) (Table -4.5). It was observed that 100 mg/l is the lethal dose of Kanamycin for the control plant (Plate 8). 80 mg/l kanamycin was used for primary screening of putative transformed and the 100 mg/l kanamycin concentration was used for the final selection of putative transformants. The optimized kanamycin selection scheme eliminates the regeneration of non-transformed.

Table 4.5 : Kanamycin based selection system

***** 40 shoots were cultured in each treatment under four replications

4.1.2. GENETIC TRANSFORMATION

4.1.2.1 OPTIMIZATION OF FACTORS FOR EFFICIENT TRANSFORMATION

4.1.2.1 A) PRE CULTURE

The sterile shoot-tips were inoculated on MS medium supplemented with 2.0mg/l BAP for different periods prior to co-cultivation. There was no significant difference between explants pre-cultured for 24 (66.25%) and 48 hours (71.25%) with respect to their survival. However percent survival was reduced by 10 per cent on direct co-cultivation. Further incubation was carried out for 72 hours and observed that survival percentage is more (76.87%). Therefore, pre-culture period of 72 hrs.

Sl. No.	Kanamycin concentration (mg/l)	Explants survival after two weeks	Percent survival
1	Control	40.00	100
2	20	39.50	98.75
3	40	35.75	89.37
4	60	29.25	73.12
5	80	24.25	60.62
6	100	0	0
CD(0.05)		1.51	
CV		3.57	

is the best to obtain maximum survivability (Table 4.6).

4.1.2.1 B) DURATION OF CO-CULTIVATION

When 72 hrs pre-cultured explants were co-cultivated for 24 hrs, by dipping in *Agrobacterium* suspension in AB medium containing 200 mM Acetosyringone for 10 min, the survivability of explants was highest in carbenicillin washed explants (76.75%) and cefotaxime washed explants (73.75%)(Table 4.6). A co-cultivation period of 24 hrs is the best for 72 hrs pre-cultured explants to obtain maximum survivability.

4.1.2.1 C) INHIBITION OF GROWTH OF *Agrobacterium tumefaciens*

To study sensitivity of *Agrobacterium tumifaciens* to various levels of cefotaxime/ carbenicillin on the eggplant shoot-tip explants, MS medium containing 2.0 mg/l BAP was supplemented with various concentrations of cefotaxime/ carbenicillin (0-600 mg/l). Levels of carbenicillin/Cefotaxime differed significantly with respect to inhibition of *Agrobacterium* growth. Reappearance of *Agrobacterium tumefaciens* on explants was highest in cultures devoid of cefataxime/carbenicillin (100%) (Table 4.7). But this reappearance varied from 75.62-0% (In both cefotaxime and carbenicillin supplemented medium) when cultures were having 100-600 mg/l cefotaxime/carbenicillin respectively. So cefataxime/Carbenicillin at 500 mg/l was added to the culture medium routinely in the experiment (Plate 9).

Table 4.6: Effect of pre-culture and Co-cultivation period on survivability of brinjal

Sl. No	Effect of pre-culture period on survival of eggplant explants after co-cultivation			Effect of co-cultivation period on the explant survivability in eggplant						
	Duration (hrs) after preparation of explants	Avg. No. of explants alive after co-cultivation for 24 hrs	Percent survival	Duration of co-cultivation (hrs)	Response after fifteen days				*Agrobacterium* growth	
					after 4th Cefotaxime washing		after 4th Carbenicillin washing		In cefotaxime washed culture	In carbenicillin washed culture
					Alive	% survival	Alive	% survival		
1	Direct inoculation	22.5	56.25	**24**	**14.75**	**73.75**	**15.25**	**76.25**	**+**	**-**
2	24	26.5	66.25	48	10.5	52.5	14.75	73.75	+++	+
3	48	28.5	71.25	72	7.5	37.5	10.5	52.5	+++	+++
4	**72**	**30.75**	**76.87**							
CD(0.05)		1.07			0.50		1.04			
CV		2.48			2.64		4.45			

*40 explants were cultured under each treatment in four replications

\- : Complete inhibition of *Agrobacterium* growth

\+ : Slight appearance of *Agrobacterium* growth

+++ : Prominent growth of *Agrobacterium*

Table 4.7: Sensitivity of *Agrobacterium tumifaciens* to various levels of cefotaxime/ Carbenicillin on the eggplant shoot-tip explants

Sl. No.	MS + BAP(2.0PPM) + cefotaxime/ carbenicillin (mg/l)	Reapperaance of *Agrobacterium* in explant		% reappearance response		Growth of *Agrobacterium*	
		In Cefotaxime	In Carbenicillin	In Cefotaxime	In Carbenicillin	In Cefotaxime	In Carbenicillin
1	0	40	40	100	100	++	++
2	100	30.25	30.25	75.62	75.62	++	++
3	200	20.5	21.25	51.25	53.12	++	++
4	300	13	10	32.5	25	+	+
5	400	2.75	1.75	6.87	4.37	-	-
6	**500**	**0**	**0**	**0**	**0**	**-**	**-**
7	600	0	0	0	0	-	-
CD(0.05)		1.53	0.86				
CV		6.79	3.96				

*40 explants were cultured under each treatment in four replications

-- : Complete inhibition of *Agrobacterium* growth

+: Slight growth of *Agrobacterium*

++ : Prominent growth of *Agrobacterium*

4.1.3. SCREENING AND CONFIRMATION OF TRANSFORMANTS

4.1.3. A) KANAMYCIN BASED ANALYSIS AND MOLECULAR ANALYSIS OF TRANSGENE

Two hundred fifty-eight shoots were obtained after co-cultivation on MS supplemented with kanamycin 80mg/l (primary screening) after 45 days and these shoots were transferred to MS supplemented with kanamycin 100mg/l to obtain putative transformants. And after 20 days only 23 putative transformants were obtained. Then DNA from these putative transformants was isolated by using CTAB method and PCR analysis was carried out by using DREB1A specific forward and reverse primers. The PCR product was subjected to agarose gel electrophoresis (Plate11) to confirm the presence of transgene. Out of 23 plants 16 plants showed +ve result for presence of DREB1A gene. The transformation efficiency was calculated. On the basis on

kanamycin based selection system and on the basis of molecular analysis the transformation efficiency was found to be 9.20% and 6.40% respectively (Table 4.8).

4.1.3. B) MORPHOLOGICAL ASSESSMENT OF TRANSFORMANTS AFTER A MOISTURE STRESS

Morphological characters like plant height, no. of leaves, no. of flowers etc. were studied by providing 8 days' moisture stress to the transformed and non-transformed plants and data was presented in Table-4.9. The putative transformants were kept under a moisture stress for 15 days along with control plant. After 15 days it was observed that, leaves of control plants were completely dried and plant was near to die, but the putative transformants were bearing pale green leaves and were still alive (Plate 12).

4.1.4. PHYSIO- BIOCHEMICAL ANALYSIS OF TRANSGENE EXPRESSION

Product of DREB1A gene regulates gene expression and signal transduction during the stress response. This leads to biochemical changes in the transformed plant. Thus expression of DREB1A gene was studied by biochemical analysis.

4.1.4. A) ESTIMATION OF PHOTOSYNTHETIC PIGMENTS

Both the transformed and control plants were subjected to moisture stress for 8 days and then estimation of photosynthetic pigment was carried out and it was found that, Chl.a, Chl.b and carotenoid content was higher in transformed(under stress) plant as compared to others. (Table 4.9)

4.1.4. B) ESTIMATION OF PROLINE CONTENT

Both the transformed and control plants were subjected to moisture stress for 8 days and then proline content of non-stressed control, transformed plant and stressed control, transformed plant was estimated. It was observed that, proline content of non-stressed control and transformed plants was near about same (96 µg/mg and 95 µg/mg resp.). But the proline content of stressed control plant was less as compared to stressed transformed plant (Table 4.9)

4.1.4. C) ESTIMATION OF MEMBRANE STABILITY INDEX

Both the transformed and control plants were subjected to moisture stress for 8 days and then membrane stability index of non-stressed control, transformed plant and stressed control, transformed plant was estimated. It was observed that membrane stability index of non-stressed control and transformed plant was more (85 and 90 resp.) as compared to that of stressed control and transformed plant. Membrane stability index of transformed plant under stressed was more as compared to stressed control plant (Table 4.9)

4.1.4. D) Estimation of relative water content

Both the transformed and control plants were subjected to moisture stress for 8 days and then relative water content of non-stressed control, transformed plant and stressed control, transformed plant was estimated. It was found that, RWC of transformed plant under stress was high (90.89±1.64) as compared to others.

Table 4.8 *In vitro* transformation studies using rd29A::*DREB1A* gene

No. of shoots Obtained after co-cultivation with kan (80ppm) after 45 days	No. of Kan^r (100ppm) Shoots obtained 20 days after inoculation	No. of plantlets transferred to growth chamber	No. of plantlets survived in growth chamber	Transformation frequency (%) (based on kan selection system)	PCR +ve	Transformation frequency (%) Based on molecular analysis
250	23	23	17	9.20	16	6.40

Table 4.9: Morpho-physiological and biochemical analysis of transgene expression (After 8 days' of moisture stress)

Sl.no.	Morpho-physio-biochemical parameter	Control plant		Trasformed plant	
		Stressed	Unstressed	Stressed	Unstressed
1	Morphological				
	1. Plant height(cm)	88.29±1.67	88.31±1.69	88.33±1.74	88.32±2.01
	2. No. of leaves	11.20±1.30	14.00±1.50	13.8±1.48	14.02±1.09
	3. No. of flowers	6.80±1.16	10.60±1.01	7.4±0.08	10.40±0.80
2	Physiological				
	1. RWC (%)	83.98±0.99	81.41±0.24	90.89±1.64	81.52±0.20
3	Biochemical				
	1. Photosynthetic pigment				
	a) Chl.a (µg/gm)	138.11±0.82	141.02±1.1	155.13±4.3	141.03±0.91
	b) Chl.b (µg/gm)	33.19±0.62	35.15±1.23	46.37±0.53	35.32±0.34
	c) Chl.a+ Chl.b (µg/gm)	171.31±1.30	176.17±2.1	201.5±4.8	176.36±1.13
	d) Carotenoid	101.98±0.86	106.30±0.54	109.78±0.45	106.90±0.43
	2. Proline content (µg/mg)	107.56±1.49	96.60±2.18	110.83±1.61	96.38±1.84
	3. Membrane stability index	80.71±0.73	82.63±0.83	82.02±0.79	82.92±0.85

4.2 DISCUSSION

Brinjal is an important commercial crop of Indian subcontinent. Its fruits are rich sources of vitamins A, B and C and also it is famous for its various medicinal uses. India being the largest brinjal producer has vast potentiality to increase the production in order to promote export, besides meeting its domestic requirements (Anon., 1998). A number of limiting factors could be attributed to low productivity. A major bottle neck in the production of brinjal is damage caused by abiotic stresses like drought, salinity, extreme temperatures and oxidative stress.

Breeding for abiotic stress tolerance in brinjal received only limited attention in the past. Currently more emphasis is being placed on breeding brinjal crop tolerant to various abiotic stresses to minimize the productivity loss. There are several limitations with conventional breeding approaches which include linkage of resistant genes with the genes of inferior qualities and problem in recovery of desired gene in interspecific crosses (Chaudhari, 2004).

Genetic transformation of crop plants with desirable genes is the focus of many plant genetic engineering programs and several techniques to introduce foreign genes into plants have been developed.

A basic requirement for a successful gene transfer system for producing transgenic plants is the availability of a target tissue made up of a large number of regenerable cells that are accessible to the gene transfer treatment and that will retain the capacity for regeneration (Birch, 1997). There are several techniques for genetic manipulation, however, certain procedures are specific to some crop species. Stable integration and expression of foreign genes in plants is essential for crop improvement. Among the several methods used for transformation of plants, *Agrobacterium tumefaciens* mediated transformation is preferred in many cases because of several distinct

advantages over other methods. These include single copy integration, greater precision with excellent stability (Lee *et al.*, 1985).

Several studies in brinjal transformation are carried out very easily due to presence of reproducible regeneration system which is possible due to a high morphogenetic potential.

The protocol adopted in the current study uses transformation of competent cells in the shoot apex of germinating seedlings through *Agrobacterium* mediated transformation and for plant driven regeneration.

The present investigation was carried out to develop suitable regeneration and efficient transformation of local cultivar of Brinjal *viz.,* Utkal Anushree. The cultivar is most popular in parts of Orissa for its high productivity. Till now, no efforts have been made for genetic transformation in this cultivar for abiotic stress tolerance development. An attempt was made to generate the plants and transform with *rd29A::DREB1A* gene through *Agrobacterium* mediated transformation.

4.2.1 *IN VITRO* REGENERATION

Regeneration is the process by which a cell or group of cells differentiates to form organs. This is commonly inducted by manipulation of exogenous supply of phytohormones and occurs directly from explant tissues. For crop improvement through genetic transformation, standardization of plant regeneration protocol is the prerequisite. The nature and concentrations of a given growth regulator in association with specific genotype and explants can cause significant differences in morphogenetic response.

In brinjal shoot-tip, cotyledon and nodal segment could be used as primary explants, but cotyledonary leaf is the most commonly used explant for producing shoots (Mohinder Kaur *et al* 2011). In present study multiple shoot induction was observed in shoot-tip and

hypocotyls explants. The percent response was more in shoot-tip explant compared to other explants used. These results were in accordance with Sharmin *et al*. (2008), where he found among different explants percent response of shoot-tip explant is high.

Organ differentiation in plant is regulated by interplay of auxins and cytokinins (Skoog and Miller, 1957). This should not imply that for adventitious shoot formation both the hormones must be included. The medium in number of cases a cytokinin alone is enough for optimal shoot multiplication (Garland and Stolz, 1981). In the present study only cytokinins were used for shoot induction. Higher concentrations of BAP(2.0-2.5 mg/l) resulted in enhanced shoot induction. The highest frequency of shoot regeneration was obtained in response to BAP (2.0 mg/l) in all the explants used. However, kinetin gave very poor response for shoot induction. These results were validated with Mohinder Kaur *et al.* (2011) and Sharmin *et al*. (2008) where they obtained highest frequency of shoot-induction on MS medium supplemented with 2.0 mg/l BAP.

Virtually any part of the plant can be used to induce callus in brinjal *viz.,* cotyledon, hypocotyls, anther, midrib, petiole, stem, leaf, fruit and root segments (Rajam *et al.,* 2002). Among them cotyledonary leaf and hypocotyl are most widely used explants. In the present study, callus initiation was early in leaf explant (8.25 days) compared to hypocotyl explants similar to the findings of Eli Zayova *et al.*(2008) where they observed callus initiation in cotyledonary leaf within 8-9 days after inoculation. The calli produced on 2.0 mg/l 2, 4-D was friable in nature. As well as same type of calli were obtained when explants were cultured on MS medium supplemented with 2.0 mg/l 2,4-D and BAP(0.5-2.0 mg/l). So simple 2,4-D instead of the combination can be used for inducing callus for further study. Calli of both the cotyledonary leaf and hypocotyl explants gave maximum response for multiple shooting on MS medium supplemented with BAP but gave poor response on kinetin containing medium. The highest frequency of shoot regeneration was obtained when BAP was added @ 2.0 mg/l.

4.2.2 *IN VITRO* TRANSFORMATION

The *in vitro* transformants obtained in this study were from the explants pre-cultured for 72 hours before co-cultivation with *Agrobacterium tumefaciens*. The results indicated that the explants pre-cultured were more suitable for integration of transgenes as they would provide a higher probability for transformed cells to initiate division. Liu *et al.* (1990) and Rama (1997) also opined that presence of actively dividing cells in the shoot apex is important for *Agrobacterium*-mediated transformation.

Titer of bacterium is another factor that is known to affect transformation frequency. The titer of bacteria (0.5 × 108 cells/ml) is found sufficient for all the solanaceous crops (Koivu *et al.*, 1995). Bacterial culture with OD 0.8 at 600 nm was taken for co-cultivation.

Acetosyringone, a phenolic compound is known to increase virulence of *Agrobacterium*. The acetosyringone concentration of 200 mM was found optimum for solanaceous crops (Liu *et al.*, 1990). Hence, the concentration of 200 mM acetosyringone was used for induction of virulence. Although, cefotaxime is non-toxic to plant tissues, but at higher concentrations, it inhibits the growth of the plant. In this study, cefotaxime at 500 mg/l was found to control *Agrobacterium* effectively. This finding is in agreement with V Prabhavathi *et al*. (2002) and G Franklin *et al* (2003).

The antibiotic effect of kanamycin is normally attributed to its ability to inhibit translation in prokaryotes and in the plastids and mitochondria of eukaryotes, by binding to the ribosomal 30S subunit (Misumi *et al*., 1978). The gene construct being used here contains neomycin phosphotransferase (*npt*II) as a selectable marker which when expresses detoxifies the kanamycin. It was important to find out the toxic level of kanamycin which can completely inhibit the growth of the normal plant so that putative transformed plants can be isolated.

Prior to starting gene transfer experiments, shoot -tips were tested for *in vitro* kanamycin sensitivity. Isolated shoot-tips were inoculated on MS medium with different concentrations of kanamycin. The growth was partially inhibited at 80 mg/l kanamycin but when the concentration was increased to 100 mg/l the growth of shoot tip explants was completely checked as evidenced by browning and complete death of explants two weeks after incubation (Plate 8). This finding thus validates findings of V Prabhavathi *et al*. (2002) and G Franklin *et al* (2003).

In this investigation, out of 23 kanamycin resistant *in vitro* regenerated and well established plants, 16 were positive for PCR in T_0 generation (Plate 11). Presence of *DREB1A* specific band in the *in vitro* transformant, positive control and absence in the negative control and untransformed plant confirmed the presence of transgene in the tested plants. The transformation frequency was 6.40 percent. This low frequency of transformation was probably due to problems in sampling tissues from chimeric transformants obtained through organogenesis which involves more than one cell in the shoot initiation process. Kar *et al.* (1996) also opined that in an organogenetic system, there is a risk of losing events of transformation due to chimera. Repeated selection in rapidly multiplying shoots is thought to eliminate untransformed tissues.

Product of DREB1A gene regulates gene expression and signal transduction during the stress response. This leads to biochemical changes in the transformed plant. Thus expression of DREB1A gene was studied by physiological and biochemical analysis such as,

- Estimation of photosynthetic pigments
- Estimation of proline content
- Membrane stability index
- Estimation of relative water content

As compared to control plant, the photosynthetic pigments were present in less mount in transformed plants. It may be due to

kanamycin stress which was used for screening purpose. It was observed that although there was no morphological difference, the transgenic brinjal resists the moisture stress better than the non transgenic. Electrolyte leakage (Membrane stability Index) was also assessed in the T_0 generation plants by withdrawing moisture for 8 days and it was found that the leakage was lesser in transgenic in comparison to control after moisture stress. The biochemical analysis such as free proline concentration was quantified in leaves and it was observed that proline concentration was significantly increased in transgenic plants with respect to nontransgenic plants under stress condition. When both the group of plants were stressed further for the moisture, the control plant completely died whereas the growth of transformed plants was normal beyond 15 days of moisture stress (Plate 12).These findings suggest that the transgenic brinjal plant can be advanced for further generation and toxicology studies for clearance of biosafety issues for the release of transgenic brinjal.

4.3 FUTURE LINE OF WORK

Based on the results obtained in the present study, the following points need to be considered as thrust areas of research.

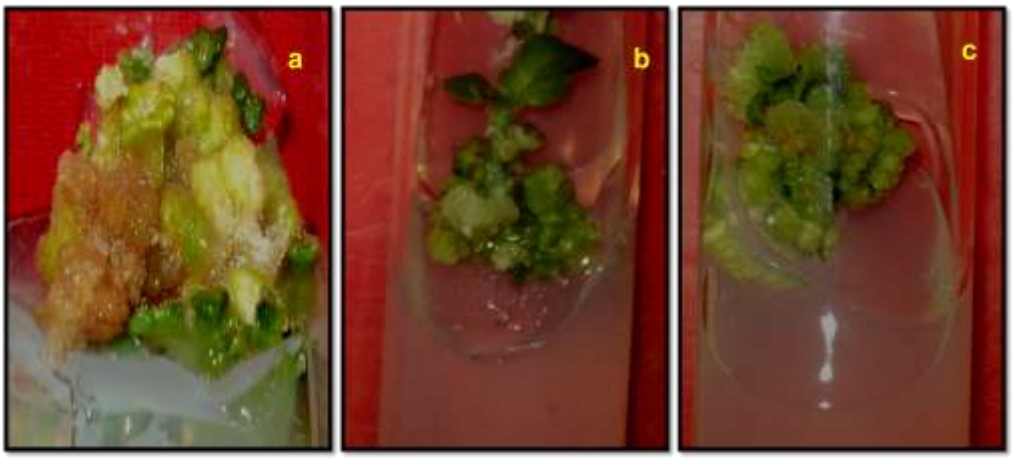
- Transformation *via* adventitious shoot was realized in present study. This method involves intense labour. Finding out effective screening technique for screening of transformant is essential. Kanamycin screening study is little erratic.
- Transformation via direct regeneration can also be made to use as an effective method, since gene integration is a random process, screening large number of independent transgenic event is essential.
- Further this research should be extended to find out the no and type of genes which are being activated and their levels of expression under stress condition.

PLATE 2

EXPLANT SOURCES

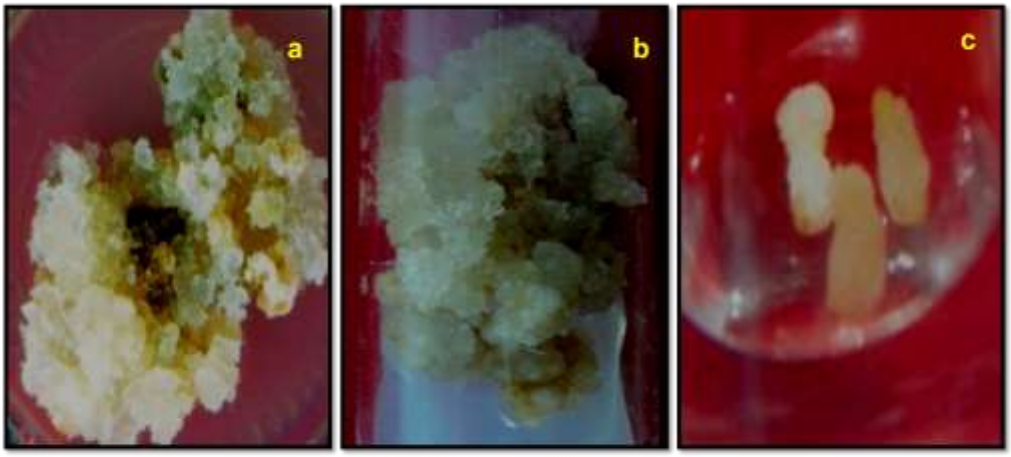
- Cotyledonary leaves (E1)
- Hypocotyl (E2)
- Shoot tip along with cotyledonary nodal region (E3)
- Root (E4)

PLATE 3



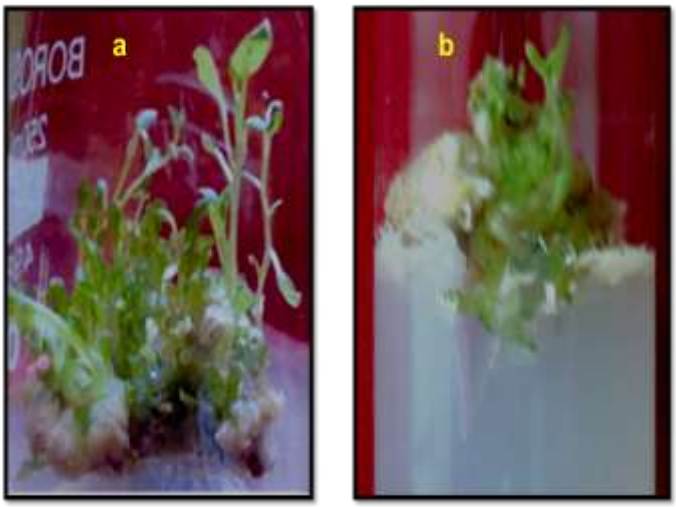
DIRECT ORGANOGENESIS: Initiation of adventitious shoots on MS+2 mg/l BAP from,
a) Shoot-tip
b) Cotyledonary leaf
c) Hypocotyl

PLATE 4



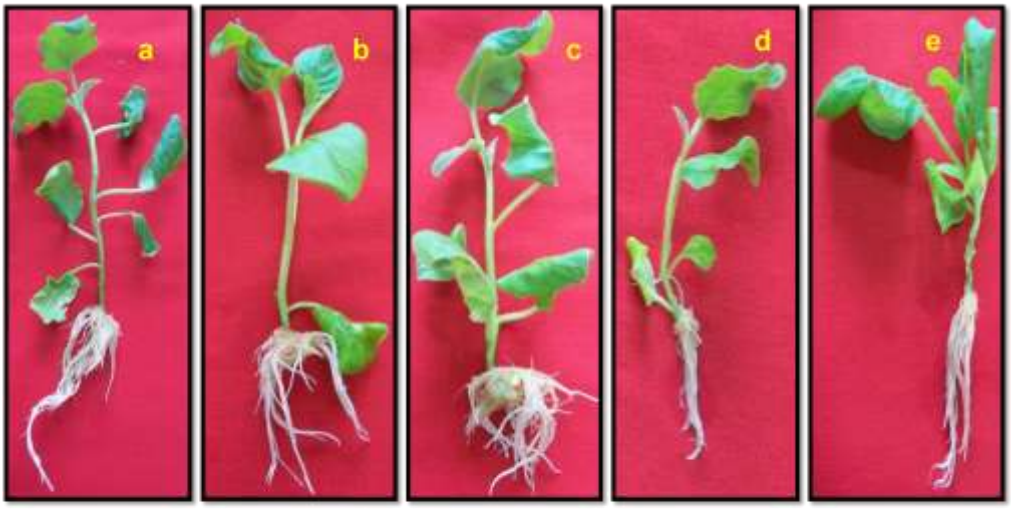
Callus induction on MS+ 2 mg/l 2,4-D from,
a) Hypocotyl
b) Cotyledonary leaf
c) Root

PLATE 5



INDIRECT ORGANOGENESIS: Initiation of adventitious shoots on MS+2 mg/l BAP from,
a) Cotylebonary leaf callus
b) Hypocotyl callus

PLATE 6



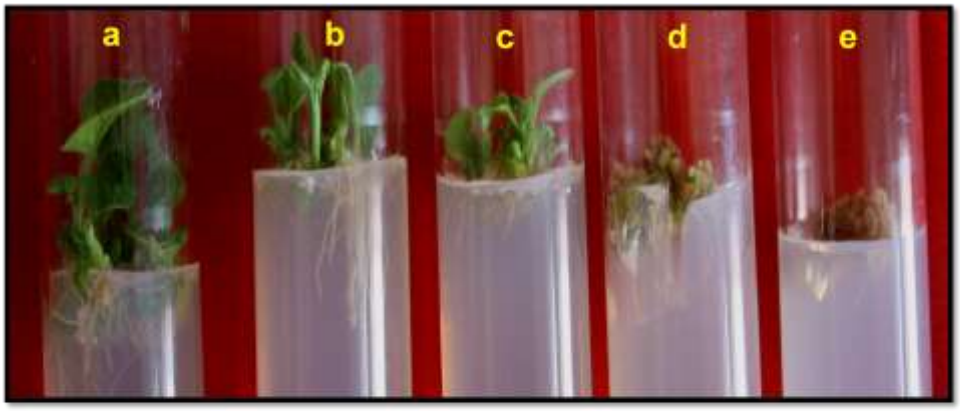
ROOTING ON DIFFERENT MEDIA

a) MS+0.01 mg/l NAA
b) MS+0.05 mg/l NAA
c) MS+0.1 mg/l NAA
d) 1/4th MS Media
e) MS Media

PLATE 7

HARDENING

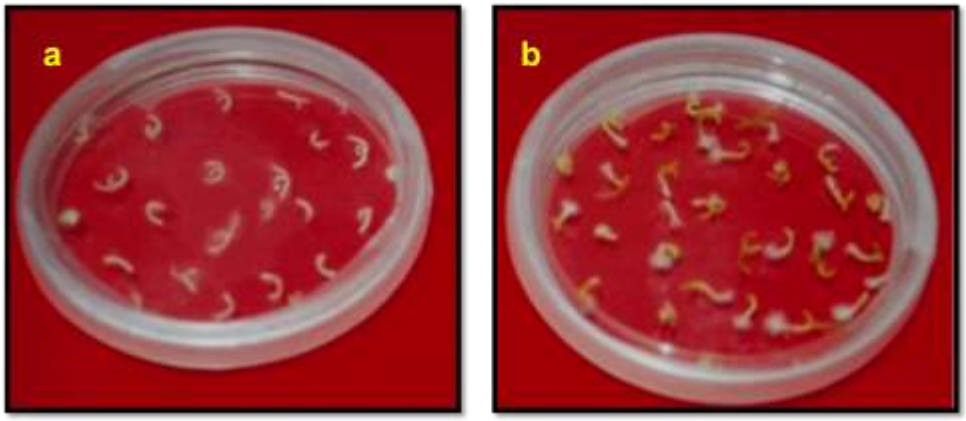
PLATE 8



KANAMYCIN BASED SELECTION SYSTEM
MS +2 mg/l BAP Supplimented with different concentrations of Kanamycin,

a) 0 mg/l (Control)
b) 40 mg/l
c) 60 mg/l
d) 80 mg/l
e) 100 mg/l

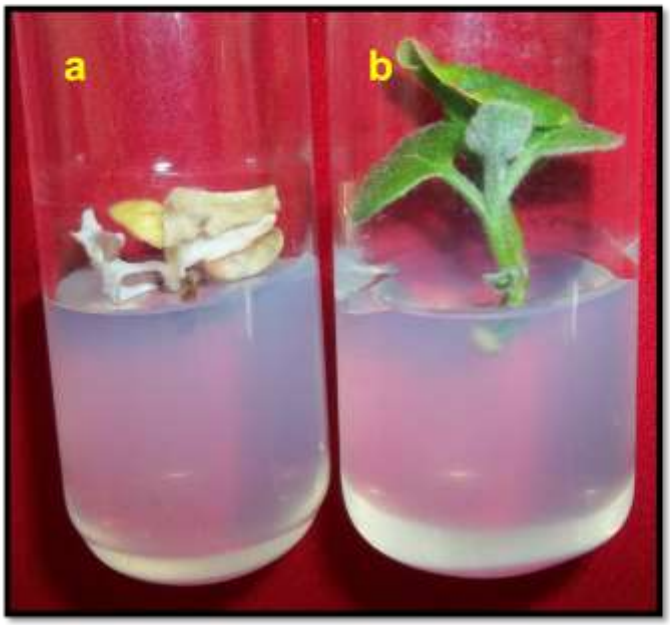
PLATE 9



LETHAL DOSE OF CEFOTAXIME

a) Shoot-tip explant on MS+Cefotaxime (500mg/l)
b) Shoot-tip explant on MS

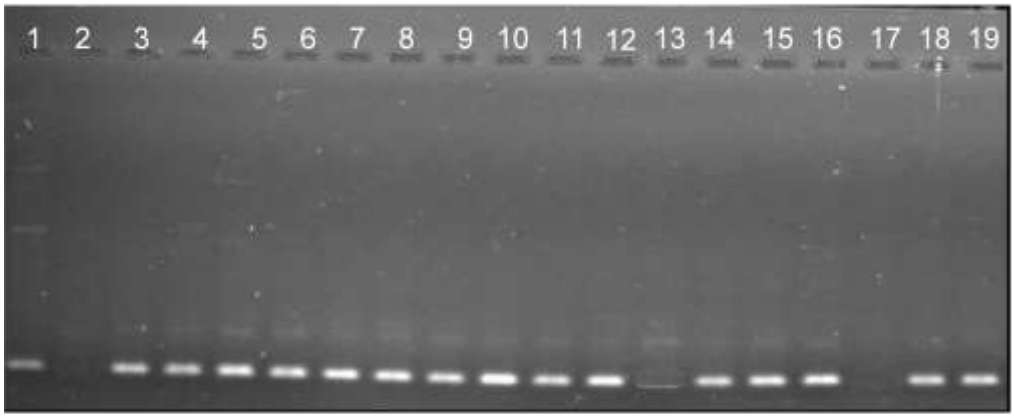
PLATE 10



SCREENING OF TRANSFORMED PLANTS ON MS+2 mg/l BAP+ 100 mg/l Kanamycin

a) Non-transformed
b) Transformed

PLATE 11



MOLECULAR ANALYSIS OF TRANSFORMED PLANT
Lane 1:- +ve control (plasmid DNA)
Lane 2:- -ve control
Lane 3-19 :- Putative transfomants DNA (T0 1-17 plants)

PLATE 12

SCREENING OF TRANSFORMED PLANTS AFTER 15 DAYS OF MOISTURE STRESS

CHAPTER-V

Summary & Conclusion

This is the ultimate butter from the entire research work. This is the "handy finding" which can be glanced through for assessing the result. Summary and conclusion are narrated to give the research work a final destination. Summary is the entire research result in a capsule form. Researcher had tried to concise the findings in an edited form. Further an attempt was made to suggest certain recommendations basing on the observed findings in form of conclusion for various stake holders in this field.

V. SUMMARY AND CONCLUSION

Eggplant (*Solanum melongena* L.) popularly known as brinjal is one of the important solanaceous vegetable crop cultivated worldwide. Its productivity is seriously affected by moisture stress. Genetic improvement can effectively be achieved through transgenic research for moistures stress tolerance by regulated expression of a large number of stress responsive genes. Over-expression of the transcription factor DREB1A/CBF3 in transgenic *Arabidopsis* plant has shown increased tolerance to freezing, drought and high salt concentration.The present study was carried out to develop moisture stress tolerance in a popular local cultivar of Brinjal cv. Utkal Anushree through *Agrobacterium*-mediated *in vitro* genetic transformation with rd29A:: DREB1A gene construct which required developing an efficient in vitro regeneration and genetic transformation protocol.

- The *Agrobacterium* strain GV3107 harboring the vector pCAMBIA 2300 containing gene construct DREB1A/CBF3 with a stress inducible promoter rd29A received from IARI and seed material of cv. Utkal Anushree obtained from Vegetable breeder, Dean Research, OUAT were used for the investigation.

- High frequency indirect somatic embryogenesis was achieved with cotyledonary leaf explants in basal MS salts supplemented with 2,4-D (2.0 mg/l) for callus induction and BAP (2.0mg/l) for shoot induction.

- Multiple shoot regeneration (20.0 shoot buds/explants) in vitro was also achieved with shoot-tip explants through direct regeneration in MS medium fortified with BAP (2.0mg/l).

- Rhizogenesis was achieved on MS-medium supplemented with 0.01 mg/l NAA.

- Various factors influencing transformation of brinjal shoot-tip such as the influence of explant pre-cultivation(72 hrs), co-

cultivation duration(24 hrs), bacterial inoculum density(0.8 OD at 600 nm wavelength), acetosyringone concentration (200 mM) and kanamycin sensitivity (100mg/l) for selection of putative transformants were optimized.

- Analysis of the putative transformants for the presence and expression of *DREB1A* gene through PCR amplification with gene specific primer revealed that the transformation efficiency was 6.40%.

- The morphological analysis for stress inducible expression of DREB1A in these transgenic plants did not indicate growth retardation or visible phenotypic alterations.

- Gene expression studies on the basis of physio-biochemical analysis like photosynthetic pigment count, membrane stability index, proline content, relative water content suggest that the transgenic brinjal plant over-expresses the stress inducible genes as regulated by rd29A::DREB1A gene construct and can be advanced for further generation and toxicology studies for clearance of biosafety issues for the release of transgenic brinjal.

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Appendices

Appendix I : Composition of Murashige and Skoog (1962) media

Components	Concentration in medium (mg/l)	Concentration in stock solution (mg/l)
MACRONUTRIENTS		20X
NH_4NO_3	1650.00	33000
KNO_3	1900.00	38000
$MgSO_4.7H_2O$	370.00	7400
KH_2PO_4	170.00	3400
$CaCl_2.2H_2O$	440.00	8800
MICRONUTRIENTS		200X
KI	0.83	166
H_3BO_3	6.20	1240
$MnSO_4.4H_2O$	22.30	4460
$ZnSO_4.7H_2O$	8.60	1720
$CuSO_4.5H_2O$	0.025	50
$CoCl_2.6H_2O$	0.025	5
$Na_2MoO_4.2H_2O$	0.25	5
IRON SOURCE		
$FeSO_4.7H_2O$	27.80	5560
$Na_2EDTA.H_2O$	37.30	7460
ORGANIC SUPPLIMENTS		200X
Thiamine HCl	0.5	100
Pyridoxine HCl	0.5	100
Nicotinic acid	0.5	100
Glycine	2.0	400
Myoinositol	Added freshly	100
Energy source - Sucrose(Added as solid) @30000.00mg/l		
Solidifying agent – Agar @8000.00 mg/l		

Iron-EDTA (200X) 500 ml Stock

5560 mg of $FeSO_4.7H_2O$ was dissolved in 500 ml of D.W. 7460 mg of Na_2 EDTA was added in 500 ml of D.W and boiled to dissolve it completely and it was then mixed with $FeSO_4.7H_2O$ solution.

Appendix II : YEMA medium (yeast extract mannitol salts medium)

- Mannitol - 10 g
- Yeast extract - 1 g
- KH2PO4 (2%) - 10 ml
- K2HPO4 (2%) - 10 ml
- MgSO4.7H2O (1 M) - 0.8 ml
- CaCl2.2H2O (1 M) - 0.4 ml
- Agar - 16 g
- Water - 1000 ml

Appendix III : AB MINIMAL MEDIUM

AB Buffer (20X) - 100 ml

K_2HPO_4 6.0 gm/l

NaH_2PO_4 2.0 gm/l Or

NaH_2PO_4 2.6 gm

Each salt was dissolved in 40 ml of D.W and then mixed together and final volume was made to 100 ml. p^H was adjusted to 7.0

AB Salt (20X) - 100ml

NH4Cl 2.0 gm

MgSO4.7H2O 0.6 gm

KCl 0.3 gm

CaCl2 0.3 gm Or

CaCl2.2H2O 0.31 gm

FeSO4.7H2O 0.005 gm

All the salts were dissolved in 80 ml of D.W and by making final volume 100 ml it was autoclaved and stored at R.T.

AB liquid Medium (100 ml)

500 mg of Glucose was dissolved in 90 ml of D.W and autoclaved then 5 ml of 20X AB buffer and 20X AB salt was added each.

Appendix IV: LPGM (Liquid Plant Growth Medium)

Liquid MS Medium + 2 mg/l BAP + 200 Mm Acetosyringone

Appendix V:

1) SUSPENSION BUFFER

Stocks :

- 1 M glucose (Stored at 40C)
- 0.5 M EDTA
- 1 M Tris-HCl (pH 8.0)

Working solutios :

5 ml of 1 M glucose + 2 ml of 0.5 M EDTA + 2.5 ml of 1 M Tri-HCL were combined and 5 mg/ml lysozyme was added to solution 1 buffer use.

2) LYSIS BUFFER

STOCKS :

- 10 N NaOH
- 10% SDS

Working solution :

0.8 ml 10 N NaOH + 4 ml 10% SDS + 35.2 ml of sterile distilled water

3) 1.5 M POTASSIUM ACETATE SOLUTION

Stock (stored at 4^0C):

5 M K-Acetate

Working solution :

60 ml of 5 M potassium acetate was mixed with 28.5 ml of glacial acetic acid and 11.5 ml of sterile distilled water. pH of the final solution was adjusted to 4.8 – 5.3 using glacial acetic acid

Appendix VI: TE BUFFER

- Tris-HCL - 10 mM
- EDTA - 1 mM

Appendix VII: EXTRACTION BUFFER

Extraction buffer (Edwards *et al.*, 1991)

- Tris HCl (pH 7.5) - 200 mM
- NaCl - 250 mM
- EDTA - 2.5 mM
- SDS - 0.5%

Appendix VIII : LOADING DYE

Bromophenol Blue

- 0.25% Bromophenol blue
- 40% (w/v) sucrose in water
- Stored at 4^0C

Appendix IX : TAE BUFFER

Tris base	- 242 g
Glacial acetic acid	- 37.1 ml
0.5 M EDTA (pH 8.0)	- 100 ml
Distilled water	- 1000 ml