# GENETIC TRANSFORMATION OF CHILLI (*Capsicum annuum* L.) WITH *OSMOTIN* GENE

By

**RESMY HENRY T.**



**THESIS**

*submitted in partial fulfilment of the requirement for the degree of*

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# DECLARATION

I hereby declare that this thesis entitled **"Genetic transformation of chilli (*Capsicum annuum* L.) with *osmotin* gene"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, fellowship or other similar title, of any other University or Society.

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# CERTIFICATE

Certified that this thesis, entitled **"Genetic transformation of chilli (*Capsicum annuum* L.) with *osmotin* gene"** is a record of research work done independently by **Ms. Resmy Henry T.** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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Dedicated to My
Loving Parents
&
Sister

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## ABBREVIATIONS

| | |
|---|---|
| BA | Benzyl Adenine |
| % | Per cent |
| *A.tumefaciens* | *Agrobacterium tumefaciens* |
| AC | Activated charcoal |
| bp | Base pair |
| *C. annuum* | *Capsicum annuum* |
| CaMV | Cauliflower mosaic virus |
| $CH_3COOK$ | Potassium acetate |
| $CH_3COONH_4$ | Ammonium acetate |
| cm | Centi metre |
| DNA | Deoxyribo Nucleic Acid |
| dNTP | Deoxyribo Nucleoside Triphosphate |
| EDTA | Ethylene Diamine Tetra Acetic acid |
| $g\ l^{-1}$ | Grams per litre |
| GA | Gibberelic acid |
| GUS | Glucuronidase |
| h | Hour(s) |
| HCl | Hydrochloric acid |
| IAA | Indole Acetic Acid |
| IBA | Indole Butyric Acid |
| kD | Kilo Dalton |
| M | Molar |
| $mg\ l^{-1}$ | Milligrams per litre |
| min | Minute(s) |
| ml | Milli litre |
| mm | Milli metre |
| MS | Murashige and Skoog's medium |
| NAA | Naphthalein Acetic Acid |

| | |
|---|---|
| NaOH | Sodium hydroxide |
| nm | Nano metre |
| nos | Nopaline synthase |
| npt | Neomycin phospho transferase |
| °c | Degree celcius |
| OD | Optical Density |
| PCR | Polymerase Chain Reaction |
| $p^H$ | Hydrogen ion concentration |
| PR | Pathogenesis related protein |
| psi | Pounds per square inch |
| RNA | Ribo Nucleic Acid |
| rpm | Revolutions per minute |
| SDS | Sodium Dodecyl Sulphate |
| SH | Schenk and Hilderbrandt's medium |
| TAE | Tris acetate EDTA buffer |
| T-DNA | Transfer DNA |
| TE | Tris EDTA buffer |
| U | Unit |
| UV | Ultra violet |
| X-gluc | 5-bromo-4-chloro-3-indolyl glucuronide |
| YEM | Yeast Extract Mannitol medium |
| β | Beta |
| µg | Micro grams |
| µl | Micro litre |
| µM | Micro molar |

# *Introduction*

# 1. INTRODUCTION

*Capsicum annuum* L., commonly known as chilli is an important vegetable and spice crop of India having high export potential. Chilli, besides imparting pungency and red colour to the dishes is a rich source of vitamin A, C and E and also assists in digestion.

In India, the export of chilli was increased from one per cent of total production (1980) to 13 per cent (2004) (Spices Board, 2005). However, growing chilli in 21$^{st}$ century will be still adversely affected by biotic and abiotic stresses apart from the considerable loss due to post harvest damages. Chilli is highly susceptible to pathogens like *Ralstonia solanacearum, Colletotrichum capsici* and viruses. Spraying of fungicides and pesticides can control the diseases and pests to some extent, however effective resistance against several destructive pathogens is still not possible. It would be a boon to chilli growers if tolerance of the crop to these abiotic and biotic stresses can be improved.

Molecular biology has proved to be a strong tool and many gene products have been identified from various organisms which confer to them the resistance and /or tolerance against various biotic and abiotic stresses. These genes provide an opportunity for the biotechnologists to manipulate and explore the possibilities to improve the crop plants so that they can be profitably grown on the marginal lands.

Genetic transformation facilitates the introduction of specifically desirable genes without co-transfer of any undesirable genes from donor species, which normally occurs by conventional breeding methods. *Agrobacterium* mediated gene transfer is now a routine procedure for introducing foreign genes into many plant species including vegetable crops such as tomato (Fischhoff *et al.*, 1987), brinjal (Fari *et al.*, 1995), *Brassica* (Metz *et al.*, 1995) etc. The two most important pre-requisites for the success of this method are the availability of a

plant regeneration system from the explants and a suitable method for transformation. Therefore, the integration of biotechnology into chilli improvement programs requires efficient plant regeneration systems.

Pathogenesis related proteins (PR proteins) are naturally produced by plants in response to environmental stress signals, such as pathogen invasion, drought and wounding. Osmotin is a basic, pathogenesis related protein produced by plants when challenged by biotic and abiotic stresses. It is classified under PR 5 group. Barthakur *et al.* (2001) gave evidence that over expression of osmotin induces proline accumulation and confers tolerance to osmotic stress in transgenic tobacco. Singh *et al.* (1987) reported that osmotin protein has antifungal activity against a variety of fungi. So, transformation of chilli with *osmotin* gene is expected to impart tolerance to drought, salinity and fungal pathogens like *Colletotrichum*. Ujwala being a market preferred variety of chilli, such sincere attempts of crop improvement is the need of the hour.

Hence, the present study was undertaken with the following objectives:

1) To standardize the *in vitro* regeneration in chilli (*Capsicum annuum* L.) through organogenesis
2) To standardize the *Agrobacterium* mediated genetic transformation in chilli
3) To genetically modify chilli with *osmotin* gene using *Agrobacterium* mediated transformation.

# Review of Literature

# 2. REVIEW OF LITERATURE

Chilli (*Capsicum annuum* L.) is an economically important vegetable and spice crop. Conventional breeding programmes in this crop have been based on mass and individual selection, pedigree breeding, back crossing and hybrid breeding. More over, *in vitro* culture techniques have been adopted for micro propagation of elite plants, selection of somaclonal variants and the production of microspore or pollen derived haploid and dihaploid plants. Breeding for resistance to biotic and abiotic stresses is of crucial importance because they significantly limit the productivity and yield of chilli. Genetic transformation offers the possibility to introduce the desired trait into the elite germplasm without changing any other attribute to the transformed genotype.

Almost 20 years ago, several solanaceous species, such as tobacco, tomato and petunia became model systems for the development of the genetic transformation technology (Horsch *et al.*, 1985; Mc-Cormick *et al.*, 1986). Transgenic cultivars of these solanaceous crops have been released to the market, or are at different stages of field trials. By contrast, chilli (*Capsicum annuum* L.) lags behind, and is only at the entrance to the era of advanced biotechnology and of transgenic breeding.

For successful plant transformation, there are three essential requirements viz, a) a foreign gene imparting desired character, b) a well developed and efficient regeneration protocol for the crop under consideration and c) an efficient gene delivery system.

## 2.1 APPLICATION OF *IN VITRO* CULTURE TECHNIQUES IN TRANSFORMATION

Development of an efficient and reproducible regeneration system is a pre-requisite for genetic transformation of plants. Direct organogenesis, indirect

organogenesis and somatic embryogenesis have been effectively utilized in production of genetically transformed plants.

## 2.2 *IN VITRO* CULTURE STUDIES IN CAPSICUM

Effective in vitro regeneration systems in chilli could serve three main purposes: a) micro propagation of special value elite plants (e.g. male sterile plants or F1 plants displaying heterosis) (Gupta *et al.*, 1998), b) production of transgenic plants, c) generation of microspore or pollen derived dihaploid plants.

### 2.2.1 Regeneration via organogenesis

The ratio of auxins to cytokinins influences the balance between root and shoot organogenesis from cultured tissues. Generally, a higher auxin relative to cytokinin favoured root formation and the reverse situation favoured shoot formation (Warren, 1991).

*In vitro* regeneration of red chilli (*C. annuum*) was first reported by Gunay and Rao in 1978. They found that IAA and BA are the best for shoot regeneration in chilli whereas 2,4-dichlorophenoxy acetic acid (2,4-D) induced only callusing. Since then, almost all the laboratories in chilli growing countries are intensively involved in *in vitro* culture studies of several cultivars of chilli with different explants, different media and with different additives. In almost all reports regeneration was induced and accomplished by exogenous growth regulator supplements to the medium. In rare cases, spontaneous shoot regeneration from explants cultured on a medium devoid of growth regulators has been observed (Ezura *et al.*, 1993; Binzel *et al.*, 1996b). The explants used were: seed explants (Binzel *et al.*, 1996b), cotyledons (Arroyo and Revilla, 1991; Mathew, 2002), hypocotyls and decapitated hypocotyls (Szasz *et al.*, 1995; Ramirez-Malagon and Ochoa-Alejo, 1996; Ramage and Leung, 1996), shoot tips (Fari and Tury, 1990; Christopher and Rajam, 1994), leaf tissue (Christopher and Rajam, 1996; Zhu *et al.*, 1996) or protoplasts derived from leaves taken from *in vitro* shoots (Prakash *et al.*, 1997). Explants were placed onto an agar solidified shoot inoculation medium supplemented with a cytokinin usually benzyl adenine (BA), kinetin, zeatin or

thidiazuron (TDZ)) and often also an auxin (IAA, IBA or NAA). Subsequently, shoot elongation may, in some cases take place after transplantation of small shoots *ex vitro* (Ediba and Hu, 1993). However, in most instances shoot or shoot bud clusters were transferred to a stem elongation medium *in vitro*, because shoot elongation has repeatedly been found as an obstacle in obtaining normal chilli plants (Liu *et al.*, 1990; Arroyo and Revilla, 1991). Arroyo and Revilla (1991) obtained adventitious shoot buds from the acropetal section of hypocotyls, but the shoot buds grew into rosettes with numerous well-developed leaves and did not elongate. They made several attempts to elongate the rosettes, such as culture in hormone free medium, reduction of cytokinin concentration, addition of gibberellic acid or dark and cold treatments. But none of the treatments were found successful.

Franck-Duchenne *et al.* (1998) attempted increasing stimulation of stem elongation by planting shoot buds on medium including 2, 4 epi-brassinolide. Although, this growth regulator did improve the rate of plantlet recovery, it did not provide a general alleviation of stem elongation difficulties. Adventitious shoots root spontaneously on a hormone free medium or consequently planting shoots onto a medium supplemented with a low auxin concentration. Girija *et al.* (2004) reported that pulse treatment with IBA enhanced early rooting (4 days) when compared to the untreated one (9.5days).

The number of plants regenerated per explant stands usually in the range of 1-10. In some cases even lower regeneration rates have been indicated (Steinitz *et al.*, 1999).

In chilli, the regeneration is highly genotype specific (Christopher and Rajam, 1996) and there is no reproducibility to other cultivars. Wolf *et al.* (1998) screened genotypes for regeneration capabilities. Explants regenerated from young seedling organs were cultured according to numerous protocols published. Out of more than 40 genotypes tested, only 4 genotypes were finally identified to have consistent repeatable regeneration capabilities. Other genotypes had either lower regeneration rates or did not display any normal regeneration.

Conclusions emerge from the studies on *in vitro* regeneration of *Capsicum annuum* by different laboratories are:

a) The hormonal composition and the sequence of application of growth regulators have to be adapted to the genotype and to explant type.

b) Cytokinin, whether applied alone or in combination with an auxin, is the critical regulator for adventitious shoot induction and they are not unique to chilli species

c) Invariably of chilli genotype or explant type, profuse bud formation is visible within 2-3 weeks from culture initiation. Unfortunately a majority of these buds develop leafy structures or stunted and otherwise aberrant shoots. Defects in shoot meristem differentiation or primordial organization cause the recurrent low incidence of normal plant recovery. Conditions that permit abundant normal shoot development have generally not been detected.

### 2.2.2 Regeneration via somatic embryogenesis

Direct somatic embryogenesis was first described in chilli pepper by Harini and Sita (1993) and in sweet pepper by Binzel *et al.* (1996a). In both studies, immature zygotic embryos were inoculated on a medium containing 2,4-D, kinetin or TDZ, coconut water and 6-10% sucrose. Somatic embryos formed directly on the immature zygotic embryo without formation of an intermediate phase of embryonic callus. The entire process, from embryogenesis inoculation to somatic embryo maturation, was accomplished on the initial medium without subculture. Noteworthy, somatic embryogenesis occurred on 10-85% (Binzel *et al.*, 1996a) or even on all (100%) zygotic embryos explanted (Harini and Sita, 1993)

Somatic embryos were obtained also from mature zygotic embryos, however through an intermediate stage of embryogenic callus. The callus was regenerated on a medium with 2, 4-D and then transferred through a sequence of subcultures in different media (Buyukalaca and Mavituana, 1996). More over a recurrent somatic embryogenesis process was developed: all stages of embryogenesis, from growth of the embryogenic suspension cultures to embryo

maturation, were performed in a bioreactor as a series of drain and fill batches, keeping the embryos in the bioreactor all the time (Mavituana and Buyukalaca, 1996). The multiplication rates obtained by recurrent somatic embryogenesis in a bioreactor were significantly higher than multiplication rates reported for caulogenesis. The production of artificial seeds, consisting of somatic *Capsicum annuum* embryos encapsulated in calcium alginate gel beads, has also been achieved (Buyukalaca *et al.*, 1995). Although, it is conceivable that some details of the culture conditions will require genotype dependent adaptations, propagation in automated computer controlled bioreactors could become the way to profitable large-scale micro propagation of elite material. However, variations amongst culture-derived plants were detected (Shen *et al.*, 1994). So the future research and development of mass propagation technologies should include genetic stability and a follow up in horticultural fidelity of the propagules (Steinitz *et al.*, 1999).

## 2.3 *AGROBACTERIUM TUMEFACIENS* AS A VECTOR FOR PLANT GENETIC ENGINEERING

The phytopathogenic bacteria *Agrobacterium tumefaciens* (Smith and Townsend, 1907) and *A. rhizogenes* (Riker, 1930) are the causative agents of the wide spread plant diseases "crown gall" and "hairy root" respectively. *Agrobacterium* are gram-negative rods that belong to the bacterial family Rhizobiaceae. These are found near soil level at the junction of plant stem and root.

Virulent strains of *Agrobacterium tumefaciens* are characterized by the presence of a large (200-250kb) plasmid known as Ti plasmid (Scott *et al.*, 1988). These bacteria induce neoplastic growth on most dicots and gymnosperms (Decleene and De-Leney, 1976) but also on a few monocots (Bytebier *et al.*, 1987; Eady *et al.*, 2000) where it results in so called crown gall tumours. *In vivo*, the infection requires wounding of the plant tissue (Kahl, 1982). After attachment to cell walls of wound activated plant cells, *Agrobacterium* transfer part of their Ti plasmid (the T-region) into the nucleus of the host plant, where it becomes stably integrated (T-DNA). Several gene loci on the bacterial chromosome (Thomashow

*et al.*, 1987) and a set of virulence (*vir*) gene located on the Ti plasmid (Stachel and Nester, 1986) code for functions involved in plant cell recognition and attachment as well as for the excision, transfer and probably the integration of T-DNA into the plant genome. The products of *vir* genes A and G recognize signal molecules derived from wounded plant cells (e.g.acetosyringone) and trigger the activation of the other *vir* genes B, C, D and E (Engstrom *et al.*, 1987). The *vir* D locus encodes a site specific endonuclease (Yanofsky *et al.*, 1986) which specifically cuts within the conserved 25 bp right terminus of T-DNA (Veluthambi *et al.*, 1987). The 25 bp repeat occurs at both ends of T-DNA, but only the right terminus has been shown to be essential for T-DNA transfer (Wang *et al.*, 1984). After cutting, a single stranded T-strand is generated unidirectionally from the right border and is transported into the plant cell by an as yet unknown mechanism, in which circular intermediates are probably involved (Koukolíkova-Nicola *et al.*, 1985). The whole process of *Agrobacterium* mediated T-DNA transfer probably represents a specialized case of bacterial conjugation applied to plant cells (Lichtenstein, 1987).

Once transferred to the nucleus, the T-DNA is covalently integrated into the plant genome in one to several copies (Lemmers *et al.*, 1980). The integration sites are randomly distributed throughout the plant genome. Multiple insertions as well as aberrant integration patterns may occur (De-Frammond *et al.*, 1986) including tandemerization in direct (Zambryski *et al.*, 1980) and inverted repeats (Jones *et al.*, 1987), rearrangements and truncation of T-DNA and of plant target sequences (Weising *et al.*, 1988). After its integration, T-DNA adopts eukaryotic features of chromatin organization and DNase I /(hyper) sensitivity (Coates *et al.*, 1987).

By taking advantage of the *Agrobacterium* Ti plasmid system, it was possible as early as 1980, to transfer foreign DNA sequences to plant cells either by transposon mutagenesis or by site specific insertion of genes into T-DNA, followed by recombination with a wild type Ti plasmid (Matzke and Chilton, 1981). However, these early attempts that relied on a double cross over event were

time consuming and cumbersome and resulted in low transformation frequencies. More efficient vector systems have subsequently been developed to facilitate bacterial genetic manipulations and to allow selection and regeneration of transformants.

Among the first such improvement were cointegrate vectors, in which new genes are introduced via homologous recombination into an artificial T-DNA already present on the Ti plasmid (Zambryski *et al.*, 1983). Binary systems in which new genes are cloned into plasmids containing an artificial T-DNA, which are subsequently introduced into an *Agrobacterium* strain harbouring a Ti plasmid with an intact vir region, but lacking the T region (Bevan, 1984).

### 2.3.1 Chimeric gene vectors

The nuclear plant gene consists of different regions, each involved in different functions of transcription and translation of mRNA. Starting with 5' end, there is a promoter region that is involved in the initiation of transcription, together with enhancer/silencer regions that confer regulation of expression, a transcriptional start or cap site, and the so called CAAT and TATA boxes, which help in binding RNA polymerase. One or more untranslated or intron regions are present within the transcribed region. The end of the translation region is determined by a stop codon and followed by a terminator at the 3' end polyadenylation signal (Chawla, 2002).

Plants are usually transformed with relatively simple constructs in which the gene of interest is coupled with an appropriate promoter, 5' leader and 3' terminator sequences to ensure efficient transcription, stability and translation of mRNA. The promoter can be of plant, viral or bacterial origin. Some promoters confer constitutive expression, whereas others may be selected to permit tissue specific expression or environmentally inducible expression.

Plant viruses which are dependent on plant transcription and translation factors have been used as sources of regulatory elements. Most commonly used are

the promoters of the 35S RNA of the cauliflower mosaic virus (*CaMV*). It directs high levels of expression in most tissues. Others such as maize ubiquitin 1 promoter, *rbcs* (ribulose biphosphate carboxylase small sub unit), *Adh* 1 (alcohol dehydrogenase), *nos* (nopaline synthase) and the rice actin promoter / intron sequences are often preferred for expression in monocots.

The promoter derived from maize alcohol dehydrogenase 1 (*Adh* 1) 5' flanking sequence showed the level of expression in monocot cells equivalent to or higher than *CaMV* 35S promoters.The presence of the *Adh* 1 intron 1 between promoter and coding sequence has also been shown to increase expression of the *npt*II gene I maize (Callis *et al.*, 1987). When this intron was used with the *CaMV* 35S promoter, high levels of expression were found than were obtained without the intron region. Similar results were obtained with first intron of the maize shrunken-1 locus (Vasil *et al.*, 1989).

The control of gene expression plays an important role in plant development and it is obvious that consideration has to be given to the regulation of gene introduced artificially. As the DNA constructs become more sophisticated, they will contain complex chimeric nucleotide sequences that are a combination of enhancer and silencer sequences, transcription promoters and terminators, protein-coding open reading frames possibly with organelle targeting or signal sequences, selectable markers, reporter genes and vector sequences. Thus, it is the chimeric gene constructs that are used for expression of transferred genes

### 2.3.2 Gene fusion markers in transgenic plants (Reporter genes)

A reporter gene is a test gene whose expression results in a quantifiable phenotype. A reporter system is useful in the analysis of plant gene expression and standardization of parameters for successful gene transfer in a particular technique.

Gene expression can be controlled at the initiation of transcription or translation and or during the process of transport, degradation of mRNA or protein. The use of these gene fusions can simplify analysis of these complex processes and

delineate the contribution of transcriptional control by eliminating the specific signals for post-transcriptional controls and replacing them with sequences from a readily assayed reporter gene. By using gene fusions to individual members of such families and individual genes separate from the background of other members of the gene family (Jefferson *et al.*, 1987).

Gene fusions using the *Escherichia coli* β-galactosidase (Helmer *et al.*, 1984) proved difficult to assay because of high endogenous β galactosidase activity in plants. Use of *Agrobacterium tumefaciens* Ti plasmid encoded genes nopaline synthase (Bevan *et al.*, 1983a) and octopine synthase (De-Greve *et al.*, 1982) promised to overcome problems associated with endogenous activity, because the opines produced by these genes are not found in normal plant cells. However, these reporter genes are not widely used because the assays are cumbersome and difficult to quantify, they cannot be used to demonstrate enzyme action (Otten and Schilperoort, 1978) and octopine synthase cannot tolerate amino terminal fusions (Jones *et al*, 1985).

The bacterial genes chloramphenicol acetyl transferase (CAT) and neomycin phospho transferase (*npt*II) that encodes enzymes with specificities not normally found in plant tissues (Fraley *et al.*, 1983; Herrera-Estrella *et al.*, 1983; Bevan *et al.*, 1983b) have been most commonly useful reporter genes. In addition, *npt*II can tolerate amino terminal fusions and remain enzymatically active, making it useful for studying organelle transport in plants (Broeck *et al.*, 1985).

Neomycin phosphotransferase II (*npt*II) gene from transposon Tn5, detoxify neomycin, kanamycin and G418 by phosphorylation. It is widely used in dicotyledon systems, including tobacco, potato and tomato (An *et al.*, 1986), legumes such as clover (White and Greenwood, 1987) and pea (Puonti-Kaerlas *et al.*, 1989) and woody species such as *Pseudotuga menziesii* (Ellis *et al.*, 1989). But this marker gene proved unsuitable for some dicotyledonous species, such as *Arabidopsis*, in which large number of non-transformed cells survive and for many

monocotyledonous species whose growth is not significantly inhibited by the antibiotic (Potrykus *et al.*, 1985; Dekeyser *et al.*, 1989).

Hygromycin phosphotransferase (*hpt*) gene was originally derived from *E. coli*. This inactivates the antibiotic hygromycin. This gene was successfully used as a selectable marker in strawberry (Nehra *et al.*, 1990) and *Solanum* sp. (Kumar, 1995).

The firefly luciferase gene has been used as a marker in transgenic plants (Ow *et al.*, 1986), but the enzyme is labile and difficult to assay with accuracy. This reaction is complex and there is little, if any, potential for routine histochemical analysis or fusion genetics.

It was earnestly felt at this juncture to develop new gene fusion systems that are easy to quantitate, highly sensitive, and moreover allowing analysis of genes whose products are of moderate and low abundance in contingent on a complete absence of any intrinsic reporter activity in plants. Activity of the reported enzyme should be maintained when fused to other proteins at its amino terminus to allow the study of translation and processing events involved in protein transport. The reporter enzyme should be detectable with sensitive histochemical assays to localize gene activity in particular cell types. Finally, the reaction catalyzed by the reporter enzyme should be sufficiently specific to minimize interference with normal cellular metabolism and general enough to allow the use of a variety of novel substrates to maximize the potential for fusion genetics and *in vitro* analysis (Jefferson *et al.*, 1987).

Jefferson *et al.* (1987) developed the *E. coli* β-glucuronidase gene as a reporter gene system for transformation of plants. They showed that glucuronidase is easily, sensitively and cheaply assayed reporter gene system. It can be assayed histochemically to localize GUS activity in cells and tissues. Many plants assayed to date lack detectable glucuronidase activity, providing null background in which to assay chimeric gene expression.

β-glucuronidase, which is is encoded by the uid A locus (Novel and Novel,1973), is a hydrolase that catalyses the cleavage of a wide variety of β-glucuronides, many of which are available commercially as spectrophotometric, fluorometric and histochemical substrates. The β-glucuronidase gene has been cloned and sequenced. It encodes a stable enzyme that has desirable properties for the construction and analysis of gene fusions (Jefferson *et al.*, 1987).

Striekema *et al.* (1988) used GUS reporter gene for transforming potato plant. Battraw and Hall (1990) studied the *CaMV* 35S promoter-β-glucuronidase gene expression in transgenic rice plants. To determine the tissue and cell types of cereal plants that the promoter function in, transgenic rice plants containing a *CaMV* 35S promoter /GUS chimeric gene were localized for GUS activity. Histochemical localization of GUS activity confirmed that the *CaMV* 35S promoter functions in cells of the leaf epidermis, mesophyll and vascular bundle. Eapen and George (1994) in their *Agrobacterium* mediated gene transfer in peanut found that the expression of GUS activity in the regenerated shoots was not directly correlated with kanamycin resistance. The lack of expression of the *gusA* gene in kanamycin resistant shoots may be due the alteration or loss of *gusA* gene. The copy number and localization of insertion and subsequent rearrangements can significantly affect expression level of the gene. Methylation of GUS reporter gene is also known to alter gene expression in potato (Ottaviani *et al.*, 1993).

Genes conferring resistance to herbicide are also used as selectable marker. The Bar gene isolated from *Streptomyces hygroscopicus* confers resistance to the herbicide phosphinothricin (PPT), bialophose and gluphosinate. The bar gene codes for the enzyme phosphinothricin acetyl transferase (PAT), which converts PPT /bialophose into non-herbicidal acetylated form. This gene has been inserted and expressed in tomato, tobacco and potato (De-Block *et al.*, 1989).

Modified version of EPSP isolated from *E. coli* confers resistance to glyphosate in transformed plants. Such transformants have been produced in

tomato (Fillatti, 1987), tobacco (Comai *et al.*, 1985) and soyabean (Hinchee *et al.*, 1988).

### 2.3.3 *Agrobacterium* mediated plant genetic transformation

The *Agrobacterium* system was historically the first successful plant transformation system, making the breakthrough in plant genetic engineering in 1983 (Fraley *et al.*, 1983; Herrera-Esterella *et al.*, 1983). Since then this gene delivery system has been used widely in a number of crops and transgenic plants of commercial importance were produced (Lindsey, 1992).

#### *2.3.3.1 Preculture*

One of the critical factors in achieving high frequencies of transformation is the preculture of explants on the inoculation medium prior to co-cultivation.

Venketachalam *et al.* (2000) pre cultured the cotyledonary explants of groundnut for 0, 1, 2, 3, 4 and 5 days and transformation frequency was calculated by their embryo forming ability in the selection medium. When cotyledon explants were precultured on the regeneration medium for 2 days, the hypersensitivity response was reduced compared to co-cultivation without preculture. Preculture of explants in the regeneration medium for 2 days prior to co-cultivation was found to be optimum for efficient transformation

Li *et al.* (2003) studied the effect of the length of preculture on the differentiation rate of cotyledon explants of *Capsicum annuum* and found that the explants preconditioned for a relatively long time cannot be infected well by *A. tumefaciens*. In their study the 2-day period gave the best result with differentiation rate of 85 per cent.

#### *2.3.3.2 Inoculum density*

Concentration of bacterial cells in the inoculation medium is an important factor to be considered for efficient transformation. Very low density of bacterial

population could lead to ineffective transformation, whereas very high density may lead to necrosis and death of the explant.

Iannamico *et al.* (1993) evaluated the effect of bacterial densities on the transformation frequency of *Solanum sodomeum*. The O.D. (550nm) of 0.01 and 0.05 permitted to obtain a higher number of kanamycin resistant calli and a higher shoot differentiation.

Fillatti *et al.* (1987) tried five concentrations of bacteria for their transformation works in tomato. Eighty percent of the cotyledons co-cultivated with $5x10^8$ bacteria produced shoots on selection medium; however, when the concentration of bacteria increased or decreased five fold the rate of transformation was reduced by at least 20%.

Clercq *et al.* (2002) carried out experiments in which a higher density of bacterial inoculum (1.6-2.4 $O.D._{600nm}$) and a longer co-cultivation period was used to transform *Phaseolus acutifolius*. These circumstances prevented proper killing of the bacteria after co-cultivation and were detrimental for the calli, resulting a drastically decreased survival rate.

#### *2.3.3.3 Inoculation time and media*

The explant, in their most receptive stage is exposed to the *Agrobacterium* culture in the inoculation medium at an optimum bacterial density. Both the composition of the inoculation media and time of inoculation may have a role in the efficiency of transformation.

For inoculation, regeneration medium of the explant that can support bacterial growth like Murashige and Skoog medium is used. It can also be the bacterial culture medium. The pH of the medium usually maintained range from 5.5 to 5.7.For infection the explants are mostly immersed in the inoculation medium for a time varying 30 sec to 1 hour.

Murashige and Skoog salts and vitamins were used for preparation of inoculation medium by Nagaraju *et al.* (1998) during their experiments with Gerbera hybrid. They immersed the explants in inoculation medium for 5 minutes and got successful transformation rate.

In an attempt to standardize the *Agrobacterium* mediated genetic transformation in *Capsicum*, Fatima *et al.* (2005) studied the effect of different infection times on survival rate of explants. Even though there was a positive GUS expression in all the infection times tested, the survival rate of transformed tissues was highest (85%) in 10 minutes when compared to 5 and 15 minutes with 50 percent and 45 percent rate of survival respectively. The low survival rate in an inoculation time of 5 minutes may probably due to low rate of transformation and in 15 minutes it was attributed to bacterial overgrowth.

Archiletti *et al.* (1995) tried various inoculation times ranging from 10 minutes to 180 min. during their transformation studies in almond. Using MS medium for inoculation they found that 30 minutes were sufficient for inoculation to occur, while longer times did not increase the transformation frequency and caused problems in eliminating bacteria.

Le *et al.* (1996) reported inoculation time of 1 hour for successful transformation of *Casuarina glauca*. Transformation works in *Pinus pinea* L. revealed that the inoculation time of 5 minutes gave better transformation frequency compared to 30 minutes of inoculation time (Humara *et al.*, 1999).

***2.3.3.4 Co-cultivation***

The initial studies on transfer of foreign genes to plants involved the co cultivation of plant protoplasts with *Agrobacterium* (Fraley *et al.*, 1983; Herrera-Estrella *et al.*, 1983). The co cultivation method involves dipping explants into a culture of modified *Agrobacterium* on sterile filter paper and culturing on callusing or regeneration media. Co-cultivation period plays an important role in the success of the transformation. It is during this period that the *vir* genes are activated and T-

DNA transferred into plant cell. However increasing the co-cultivation period might lead to the necrosis and death of the explant due to hypersensitive reaction of the tissue. Sometimes it might also lead to uncontrollable bacterial over growth. Hence the length of co-cultivation period should always be the shortest interval necessary to obtain the maximum frequency of transformation in terms of the number of transgenic plants recovered following co- cultivation.

Fillatti *et al.* (1987) during their transformation experiments in tomato, tried different co-cultivation period of 24, 48 and 72h to get the best transformation efficiency. An average of 60 per cent of the cotyledons co-cultivated for 48h produced kanamycin resistant shoots, while the average transformation rate observed after cotyledons were co-cultivated for 24 and 72h was less.

In Gerbera hybrida, Nagaraju (1998) found 48h to be the optimum co-cultivation period and a period beyond that resulted in bacterial overgrowth and inhibition of callus formation. In lettuce the percentage of explants producing transformed calli were similar at 58, 72, 96 and 120h of co-cultivation. The co-cultivation period of 48h produced the maximum transgenic shoots followed by 24 and 72h of co-cultivation. Co-cultivation of 96 and 120h had an inhibitory effect on shoot formation giving no shoots at all (Torres *et al.*, 1993).

In pear and citrus, a prolonged co-cultivation period of more than 30 days was used to develop transgenic plants (Mourgues *et al.*, 1996; Cervera *et al.*, 1998).

Wu *et al.* (2003) cultured immature embryos (IEs) of wheat on co-cultivation media for 1-5 days and a co-cultivation period of 3 days was found optimum. The longer co-cultivation times reduced the capacity of IEs to form embryogenic callus and regenerate.

Jayashree *et al.* (2003) evaluated the effect of co-cultivation period on transformation frequency of rubber tree by culturing *Agrobacterium* infected calli

for 1, 2, 3, 4 and 5 days in the co-cultivation medium. The highest transformation frequency (4%) was observed following a 3 day co-cultivation. No transformation was observed when the callus was transferred to selection medium immediately after infection with *Agrobacterium* (without co-cultivation).

Kim *et al.* (2004) investigated the factors affecting the genetic transformation of perilla with *Agrobacterium* harbouring *gus* gene.The duration of co-cultivation with *Agrobacterium* is one of the factors affecting transformation per cent and the optimal duration has reported to be more than 4 days (Tang *et al.*, 2000).

After co-cultivation, the explants are transferred to media containing bacteriostatic agent to kill the bacteria. Complete elimination of the bacteria from the explant after co-cultivation is very essential otherwise it will interfere with the growth and organogenesis of the explant. Elimination of the bacteria from the explant is done by use of antibiotics. The most commonly used antibiotics for this purpose are carbenicillin and cefotaxime.

***2.3.3.5 Screening of transformed cells***

Selection of transformed cells is an important aspect of transformation works. The explants are transferred to a selection medium containing appropriate selection agent (antibiotics, herbicides, etc.) depending on the plant selectable marker employed for transformation. Resistant/tolerant tissues are continuously grown and selected on selection agent followed by regeneration.

***2.3.3.6 Analyses of transformed plant cells***

Selection and growth of plant cells on selective media provide initial phenotypic evidence for transformation. However, spontaneous variants with increased resistance to many chemicals can be readily selected in plant tissue culture. This includes resistance to kanamycin (Owens, 1981), the most commonly used selection agent for plant transformation. Therefore biochemical and molecular evidence is essential to confirm expression and integration of transferred genes.

The presence of a selectable marker allows selection of transgenic cells but does not guarantee 100 per cent co-transmission and/or expression of the other genes on the same section of T-DNA. Independently selected transgenic plants often show varying degrees of gene expression. Several factors other than the absolute effects of the regulatory sequences can influence the magnitude of gene expression. These include copy number, position effects resulting from the site of insertion and methylation of the transferred genes.

The integration of the foreign genes into the plant nuclear genome can be determined by via Southern analyses and the use of the Polymerase Chain Reaction (PCR). Southern analyses allow the number of copies and nature of the integration of the integration of specific genes or DNA regions to be determined. PCR is a new and powerful technique for confirming DNA insertion in transgenic plants (Lassner *et al.*, 1989). Primers can be designed which simultaneously amplify specific genes or T-DNA regions on the T-DNA that are expected to be integtrated into the genome of plants. Advantages include the rapid manner in which large collection of transgenic plants can be analysed and the very small amount of plant tissue required.

## 2.4 STATUS OF TRANSFORMATION STUDIES IN *CAPSICUM*

Genetic transformation in *Capsicum* has been attempted by several groups of research workers. An *in vitro* regeneration and transformation was defined using 6 cultivars of bell pepper. Transformed shoot buds and leaf like structure were obtained showing β-glucuronidase activity, but no functional transgenic plants could be secured (Liu *et al.*, 1990). Wang *et al.* (1991) reported successful transformation of *Capsicum frutescens*. *Agrobacterium tumefaciens* transformed plantlets were obtained using strains C58 and GV 3111 and Horsch's leaf disc method. Co-culturing of plantlets regenerated from hypocotyl, cotyledon and leaf explants of bell pepper with *Agrobacterium* carrying construct with *gus* gene yielded transgenic plants (Christopher and Rajam, 1995)

Szasz *et al.* (1997) carried out genetic transformation experiments on cotyledon and rooted hypocotyl explants of *C. annuum* using a disarmed *Agrobacterium.* The key step in the *Agrobacterium* mediated genetic transformation of chilli pepper using cotyledons as explants was the elongation of the shoots surviving the first kanamycin selection. It was observed that cefotaxime used to eliminate the bacteria has a phytohormone effect detrimental to the regeneration step.

Mouse Adenine Deaminase (ADA) gene was transformed into hot pepper (Tae *et al.*, 1999). *Agrobacterium tumefaciens* and the cotyledon explants were co-cultivated for 48 hours. Transformed plants were selected on medium containing kanamycin and carbenicillin. Integration and expression of ADA was confirmed by PCR and spectrophotometric analysis. The bar (herbicide resistance) gene was also introduced into hot pepper, and progenies of the transgenic plants were obtained.

Cucumber Mosaic Virus (CMV) satellite RNA was transformed into hot pepper plants through *Agrobacterium.* The level of protection against CMV in the progeny of transgenic plants that express CMV satellite RNA was investigated. Polymerase Chain Reaction and RNA gel blot analysis showed that the introduced gene was stably transmitted and expressed in the progeny. Partial attenuation of symptoms and a decrease of virus titer were observed (Kim *et al.*, 1997).

*Capsicum annuum* plants containing CMV (cucumber mosaic cucumovirus) satellite cDNA were obtained by *A. tumefaciens* mediated transformation. Transformants were regenerated on selection medium and some flowered and set fruit (Dong *et al.*, 1992).

Fertile transgenic sweet pepper (*C. annuum* var.grossum) plants were regenerated from explants that were co-cultivated with *A. tumefaciens* strain GV 3111 SE harbouring a plasmid that contains the cucumber mosaic cucumovirus coat protein (CMV-CP) gene (Zhu *et al.*, 1996). Southern analysis of DNA isolated

from transgenic plants revealed the presence of the transformed gene. Expression of the gene was confirmed by Western Blot analysis.

Venkataiah *et al.* (2001) performed *A. tumefaciens* mediated transformation of 3 explants-hypocotyls, cotyledon and leaf explants of four capsicum species viz. *C. annuum, C. baccatum, C. frutescens and C. praetemissum.* They found that the transformation efficiency certainly depends upon regeneration efficiency of explant tissue and species as well as the vector system. This observation shows that explants and *Agrobacterium* compatibility plays a very important role in the transformation methods in chilli pepper. Siregar and Sudarsono (1997) also made similar observations in *Capsicum.*

Kim *et al.* (2001) obtained only two transgenic plants from 255 seed explants that were infected with *Agrobacterium* strain LBA 4404. One of them showed morphological change, such as dwarfism and early flowering by the constitutive expression of the rice *OsMADS1* gene. The transformation efficiency was 0.8%.

Nianiou *et al.* (2002) established a regeneration and transformation protocol for the sweet red pepper type Florinis and for two pepper hybrids using hypocotyl explants. In order to achieve the transformation of pepper they applied two different methods, using *Agrobacterium* and particle gun. Following the first method fertile transgenic plants were regenerated from hypocotyl explants that were co-cultivated with *A. tumefaciens* strain LBA4404, but the percentage of transformed plants obtained using this method was rather small. As an alternative they have used biolistic method. The number of kanamycin resistant plants that were produced through particle gun seems to be quite large.

Cotyledonary explants were transformed with *A. tumefaciens*, shoot buds were elongated and rooted in presence of kanamycin. Transgenic plants were transferred to half MS for root hardening (Manoharan *et al.*, 1998). For transformation, around 200 cotyledonary leaves were co-cultivated with

*Agrobacterium* for 48h. The shoot bud induction was observed after 15 days of inoculation on the selection medium. Of the ten explants with shoot buds, only four elongated and shoots with roots were obtained.

Jayapadma *et al.* (2005) in their transformation studies in chilli observed that the major obstacle to chilli pepper plant transformation and regeneration was the lack of elongation of newly formed buds. The frequency of bud elongation reported was generally too low to permit production of respectable amount of transformants. The key elements involved in successful transformation of chilli depend on the right choice of explant and media.

Shivegowda *et al.* (2002) transformed cotyledonary explants of Pusa Jwala with *A. tumefaciens* strain C58 containing binary vector pGV1040 harbouring the two reporter genes *npt* II and GUS. The presence of transgene was confirmed through histochemical staining of GUS, PCR and Southern hybridization analysis of *npt* II gene. *Agrobacterium* mediated genetic transformation was standardized by Fatima *et al.* (2005). They studied the effect of different infection times and dilutions of bacterial suspension on the survival rate of explants.

## 2.4 PATHOGENESIS RELATED (PR) PROTEINS

Pathogenesis related (PR) proteins are naturally produced by plants in response to environmental stress signals such as pathogen invasion, drought and wounding. Pathogenesis related (PR) proteins were first discovered as polypeptides that accumulate in genotypes of tobacco that respond hyper sensitively to infection with tobacco mosaic virus (TMV) (Van Loon *et al.*, 1970). Since then, many PR proteins have been described as occurring in a wide variety of plant species. The PR proteins and genes that encode them have now been categorized into five major groups. Increasing evidence has mounted showing that many members of the PR super family have antifungal activity in *in vitro* assays (Bol *et al.*, 1990).

Osmotin is a small basic pathogenesis related protein produced by plants when challenged by biotic and abiotic stresses. Singh *et al.* (1985) studied the

protein in tobacco var. Wisconsin 38 and gave the name to a basic 24 Kda protein that accumulates in cells on osmotic stress adaptation. On the basis of similarities in amino acid sequence and expression pattern, osmotin has been classified as a member of the PR-5 proteins of tobacco (Brederobe *et al.*, 1991; Linthorst, 1991). Further studies have shown that the synthesis and accumulation of osmotin mRNA are developmentally regulated by at least six hormonal or environmental signals including abscisic acid (ABA), ethylene, tobacco mosaic virus infection, salinity, dessication and wounding in both cultured cells and whole plants of tobacco (La-Rosa *et al.*, 1985; Singh *et al.*, 1987; La-Rosa *et al.*, 1987). However, substantial accumulation of osmotin promoter occurs only in response to osmotic stress and ethylene in tobacco cultivar, W 38, which shows no hypersensitive response (La-Rosa *et al.*, 1992). Woloshuk *et al.* (1991) demonstrated *in vitro* that osmotin has antifungal activity against a variety of fungi including *Phytophthora infestans, Candida albicons, Neurospora crassa* and *Trichoderma reesi*. So, these proteins have a dual function: response to plant pathogenesis and to osmotic stress (Kononowicz *et al.*, 1992; Raghothama *et al.*, 1993).

Barthakur *et al.* (2001) gave evidence that over expression of osmotin induces proline accumulation and confers tolerance to osmotic stress in transgenic tobacco. Osmotin induces proline biosynthesis by activating the $\Delta^1$ pyrroline-5-carboxylate synthetase (P5CS) enzyme that catalyses the rate limiting step in proline synthesis, by repressing the proline catabolic pathway, or by repressing feed back inhibition of proline biosynthesis. Further it may be pointed out that osmotin is a proline rich protein and its degradation could also possibly lead to accumulation of proline, at least under conditions when the protein is over produced.

Osmotin, a 24 KD protein isolated from Nicotiana tabacum 'Xanthi nc' was very toxic to *Phytophthora infestans in vitro* assay. Li *et al.* (1999) produced two osmotin mutants by PCR mutagenesis. Both mutant genes driven by CaMV 35S promoter was introduced in potato by *Agrobacterium* mediated genetic

transformation. Disease resistance test using *P. infestans* complex race showed that expression of both osmotin mutants in transgenic potato inhibited the development of fungal disease in inoculated leaves.

Sarad *et al.* (2004) developed transgenic tomatoes with osmotin gene by *Agrobacterium* mediated transformation. Proline content was higher in transformed plants under normal and stress conditions. Preliminary tests have revealed transgenics with the osmotin gene are more tolerant of cold than wild types. Abdin (2005) mobilized tobacco osmotin gene via *A. tumefaciens* mediated genetic transformation in chicory (*Cichorium intybus* L.). The results confirmed the integration of osmotin gene in the chicory genome and its role as osmoprotectant has been proved. To validate the performance of these putative transgenics, physiological and biochemical assessments were made employing leaf disc assay on salt solution. The leaf discs from transgenic chicory plants showed less senescence over the marked period and retained chlorophyll than those from non-transformed plants. Also proline, the biochemical marker of osmotolerance in plants accumulated upto 90 per cent higher in transgenic plants as compared to wild type, at 250mM NaCl. These results confirmed the over-expression of the osmotin gene in transgenic plants and their improved tolerance when exposed to salt stress.

Pathogenesis related protein, osmotin, when introgressed into *Brassica juncea* plants, provided tolerance to fungal attack (Taj *et al.*, 2004). Osmotin is thought to influence the signal transduction pathway. Possible influence of osmotin gene transfer on the cell cycle and cell pathways was investigated using transformed *B. juncea* calli as model systems. It was observed that partially purified *Alternaria* toxin inhibited the growth of non transformed calli; whereas, transformed calli resisted the effect of toxin, which appears to counteract the inhibitory effects of phytotoxin.

# *Materials and Methods*

# 3. MATERIALS AND METHODS

The study entitled 'Genetic transformation of chilli (*Capsicum annuum* L.) with *osmotin* gene' was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara, from December 2003 to September 2005. Details regarding the experimental materials used and the methodologies adopted for various experiments under are described below.

## 3.1 SOURCE OF EXPLANT

Seedlings of *Capsicum annuum* L. var. Ujwala raised under *in vitro* conditions on Murashige and Skoog (MS) medium containing 2 per cent sucrose served as the source of explant.

## 3.2 CULTURE MEDIUM

### 3.2.1 Chemicals

The major and minor elements required for the preparation of media were of analytical grade and procured from M/s Sisco Research Laboratories (SRL), British Drug House (BDH) and M/s Merck India Ltd. The amino acids, vitamins and chemicals used for molecular biology work were obtained from M/s Merck India Ltd.,SRL and Sigma Chemicals, USA. The antibiotics used for the transformation study was obtained from Himedia.

### 3.2.2 Glass wares and plastic wares

Borosilicate glasswares of Corning/Borosil were used for the experiment. The glasswares were cleaned initially by soaking in potassium dichromate solution for 12h followed by thorough washing with jets of tap water in order to remove completely all traces of potassium dichromate solution. They were further cleaned with 0.1 per cent teepol detergent solution and were washed thoroughly with water and rinsed twice with double distilled water. These were

then air dried in hot air oven at $105^0$c for 24h and later stored in cupboards free of dust till further use.

The plastic wares used for the experiment were procured from Tarsons India Ltd. and Axygen. The plastic wares were autoclaved at 121°C by applying 15 psi pressure for 20 min and stored in cupboards free of dust till use.

### 3.2.3 Composition of media

Basal MS medium (Murashige and Skoog, 1962), B5 medium (Gamborg *et al.*, 1968), SH medium (Schenk and Hilderbrandt, 1972) supplemented with different plant growth regulators were used for plant tissue culture in the present study. Yeast Extract Mannitol (YEM) medium was used for culturing *Agrobacterium tumefaciens* strains during the study. The basal compositions of these media are given in Appendix-I.

### 3.2.4 Preparation of tissue culture medium

Standard procedures (Gamborg and Shyluk, 1981) were followed for the preparation of plant tissue culture media. Stock solutions of major and minor elements were prepared and stored in pre-cleaned amber coloured bottles in refrigerated conditions.

A clean steel vessel, rinsed with distilled water was used to prepare the medium. Aliquots from all stock solutions were pipetted in proportionate volumes in the vessel. A little amount of distilled water was added to it and later on, required quantities of sucrose and inositol were added and dissolved in it. The desired volume was made up by adding distilled water. The pH of the medium was adjusted between 5.5 to 5.8 using 0.1N NaOH or HCl.

For solid medium, agar was added at 0.75 per cent (w/v) concentration, after adjusting the pH. The medium was stirred and heated to melt the agar, and was poured when hot into culture vessels and were plugged with non-absorbent cotton. For solid media, test tubes (15 cm x 2.5 cm) were used whereas for liquid

media conical flasks (100, 250 and 500 ml) were used as culture vessels. Fifteen ml medium was poured in each test tube, 50 ml medium in 100 ml conical flask, 100 ml in 250 ml conical flask and 250 ml in 500 ml conical flask. Vessels containing media were sterilized in an autoclave at 121°C by applying 15 psi pressure for 20 min. The medium was allowed to cool to room temperature and stored in culture room until used.

### 3.2.5 Preparation of YEM medium for *Agrobacterium*

Clean steel vessels, rinsed with distilled water were used to prepare the media. The ingredients were weighed on electronic balance and were added into the vessels. Little amount of distilled water was added to it and the ingredients were dissolved. The desired volume was made up by adding distilled water. The pH of the media was adjusted to 7.0 using a standard pH meter by adding either 0.1N NaOH or HCl.

For solid media agar was added at the rate of 20 g $l^{-1}$. The media were stirred and heated to melt the agar and were poured when hot into conical flasks (100 ml). Fifty ml medium was poured in each conical flask and the conical flasks were plugged with non-absorbent cotton. Autoclaving was done at 121°C at 15 psi for 20 min to sterilize the medium. The sterilized flasks were kept in the culture room until used.

## 3.3 TRANSFER AREA AND ASEPTIC MANIPULATIONS

All the aseptic manipulations were carried out under the hood of a laminar air flow cabinet fitted with UV lamp for extra protection.

## 3.4 CULTURE CONDITIONS

The cultures were incubated at 26 ± $2^0$c in an air conditioned culture room with 16 h photoperiod (1000 lux) supplied by fluorescent tubes. Humidity in the culture room varied from 60 to 80 per cent according to the climate prevailing.

## 3.5 EXPLANTS

Various explants viz. cotyledons, hypocotyls and leaf segments were used for the study.

### 3.5.1 Establishment of aseptic plants

Seeds of *Capsicum annuum* L. var. Ujwala obtained from the Department of Olericulture, College of Horticulture, Vellanikkara were used for the study. The seeds were washed in water containing few drops of teepol and rinsed with running tap water to remove teepol. The seeds were taken to the laminar flow and surface sterilized with $HgCl_2$ (0.1 per cent) for one minute. It was then washed free of $HgCl_2$ by rinsing with three changes of sterile water. The seeds were drained on sterile blotting paper. The surface sterilized seeds were incubated in basal MS medium containing 2 per cent sucrose. The cultures were incubated in the culture room.

### 3.5.2 Preparation of explants

The *in vitro* raised seedlings were taken out of the culture tubes in a laminar flow cabinet on pre-sterilized steel plates. The seedlings were cut with sterile blade to separate cotyledon and hypocotyl. The hypocotyls were cut approximately 2-2.5 cm below the embryo axis, separated from the roots. The leaf segments were cut into 1 $cm^2$ (approximately) with a sterile blade.

## 3.6 STANDARDIZATION OF *IN VITRO* REGENERATION

### 3.6.1 Standardization of explants

Cotyledons, leaf segments and hypocotyls taken from *in vitro* raised seedlings were cultured in MS media supplemented with different combinations of auxins and cytokinins. The hypocotyl segments (2-2.5 cm long) were placed upside down in the culture medium. The cultures were incubated in the culture room. Observations regarding the response of different explants were recorded.

### 3.6.2 Standardization of basal media

The best explant from the previous experiment was cultured in different basal media viz.full strength, MS, SH and B5 supplemented with different combinations of auxins and cytokinins. The cultures were incubated in the culture room. Observations regarding the response of explants were recorded at weekly intervals.

### 3.6.3 Standardization of growth regulators

The explants were cultured in MS medium supplemented with different concentrations of BA (1.0, 2.0, 3.0, 5.0 and 7.0 mg $l^{-1}$) and IAA (0.2, 0.3, 0.5, 0.7 and 1.0 mg $l^{-1}$). The cultures were incubated in the culture room. Observations on the response of explants were recorded at weekly intervals.

### 3.6.4 Elongation of regenerated buds

The regenerated buds were cut into smaller pieces using sterile scalpel blade and cultured in MS medium supplemented with different combinations of growth regulators, for shoot elongation (Table 1). The cultures were maintained in the culture room. Observations regarding the response of explants to different media combinations were recorded at weekly intervals.

Table 1. Different media combinations used for elongation of regenerated buds in *Capsicum annuum* L. var. Ujwala

| Sl. No. | Media combinations |
|---|---|
| 1 | MS + 3.0 per cent sucrose |
| 2 | MS + 2.0 % sucrose |
| 3 | Half MS |
| 4 | MS + 3% sucrose + 1.0 mg $l^{-1}$ IAA |
| 5 | MS + 3% sucrose + 0.5 mg $l^{-1}$ IAA |
| 6 | MS + 3% sucrose + 1.0 $mgl^{-1}$ GA |
| 7 | MS + 3% sucrose + 0.5 $mgl^{-1}$ GA |
| 8 | MS + 3% sucrose + 0.05 % AC |
| 9 | MS + 3% sucrose + 0.025% AC |

### 3.6.5 Rooting

Elongated buds were excised using a sterile blade and dipped in IBA (1000 mg $l^{-1}$) for one minute. These were inoculated in full MS medium containing 2 per cent and 3 per cent sucrose. The cultures were maintained in the culture room. Observations regarding the percentage of rooting were recorded at weekly intervals.

### 3.6.6 Hardening

Rooted plantlets were taken out of the culture tubes and washed in running tap water to remove the adhering media. These plants were planted in pots containing sterile potting mixture. These TC regenerants were kept in net house (temperature 28-30$^0$c, relative humidity-80%) for 15 days and then planted out.

### 3.6.7 Planting out

The hardened plants were transferred to pots (capacity-2kg) containing sterile potting mixture in net house. Regular watering of the plants was done. Observations regarding the percentage of plants established were recorded.

## 3.7 *AGROBACTERIUM* MEDIATED GENETIC TRANSFORMATION

### 3.7.1 Evaluation of the sensitivity of explants to various antibiotics

Both hypocotyls and cotyledonary explants were tested for their sensitivity to various antibiotics, in order to select a suitable marker for *Agrobacterium* mediated genetic transformation. The antibiotics used for testing sensitivity were kanamycin, rifampicin, carbencillin and cefotaxime.

The standardized regeneration medium was supplemented with 50 mg $l^{-1}$, 100 mg $l^{-1}$, 200 mg $l^{-1}$, 300 mg $l^{-1}$ and 400 mg $l^{-1}$ of each antibiotic, separately. Cotyledonary leaf segments of 1 $cm^2$ (approximately) and hypocotyl segments of 2-2.5 cm length were inoculated into the media. A control having no antibiotic was also maintained. Observations regarding the response of explants were recorded at weekly intervals.

### 3.7.2 *Agrobacterium tumefaciens* strains

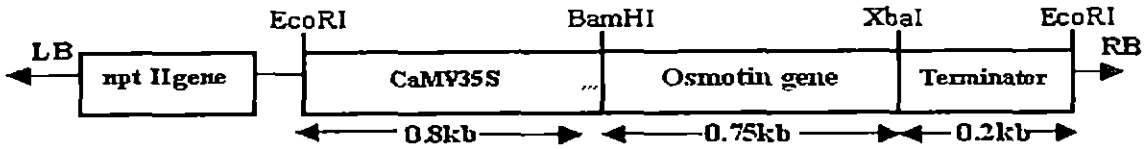
*Agrobacterium tumefaciens* strains EHA105 and GV 2260 were used for the study. The EHA 105 contains the plasmid p35SGUSINT. This plasmid contains *gusA* gene fused to the *CAMV 35S* promoter and the *npt* II gene controlled by the nos promoter. The *gusA* gene has an intron preventing its expression in *Agrobacterium*. The GV 2260 contains the plasmid pGV 2260. This plasmid contains *osmotin* gene and *npt* II gene tagged to the *CaMV 35S* promoter. Details of gene constructs are given in Table 2 and Fig. 1.

Table 2. Details of constructs used for transformation studies in chilli var. Ujwala

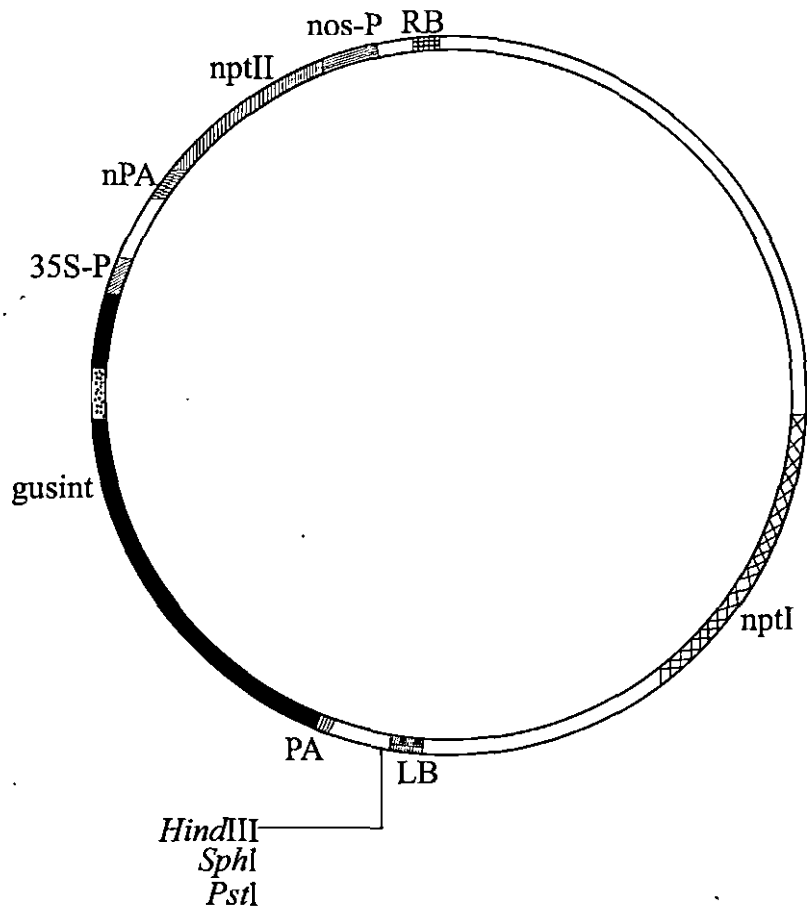
| Sl. No. | Name of construct | Strain of *Agrobacterium* | Plant selection marker | Reporter gene | Useful gene | Source of construct |
|---|---|---|---|---|---|---|
| 1 | p35SGUSINT | EHA105 | Kanamycin | GUS | - | NRCPB, IARI, New Delhi |
| 2 | pGV 2260 | GV2260 | Kanamycin | - | Osmotin | NRCPB, IARI, New Delhi |

### 3.7.3 Culturing of *Agrobacterium tumefaciens* strains

The bacterial cultures were maintained in YEM medium containing suitable antibiotics. The *Agrobacterium* strain EHA105 was cultured on YEM medium containing 50 mg $l^{-1}$ kanamycin and 20 mg $l^{-1}$ rifampicin. The *Agrobacterium* strain GV2260 was cultured on YEM medium containing 100 mg $l^{-1}$ carbenicillin, 50 mg $l^{-1}$ kanamycin and 75 mg $l^{-1}$ rifampicin.




pGV 2260 with *osmotin* gene and *npt* ll as selection marker




p35SGUSINT with GUS intron as reporter and *npt* II as selection marker

Fig. 1. Details of gene constructs used for transformation in chilli var. Ujwala

The YEM medium was prepared in required volume in conical flask and kept in culture room until use. On the day of use, the media was melted and then cooled to 40 to 50°C. Inside a laminar flow cabinet, the antibiotics were added to the medium and mixed thoroughly by vigorous shaking. The medium was then distributed in sterile petriplates and allowed to solidify and attain room temperature. Using sterile bacterial loop a single colony was scooped from the previous bacterial culture plate and streaked on to the freshly prepared solidified medium. The newly streaked bacterial plate was sealed with parafilm and incubated in inverted position at room temperature (28-30°C). The bacterial strains were subcultured once in a month. The morphological and cultural characteristics of the bacteria were noted.

### 3.7.4 Maintenance of strains

Stabs of the YEM medium for each strain were prepared and maintained for further use. Glycerol stocks of the *Agrobacterium* strains were also prepared and stored at $-20^0$c.

#### *3.7.4.1 Preparation of stabs*

The YEM medium containing the marker antibiotic for each strain was poured in sterilized culture tubes and allowed to solidify. The transfer loop was flamed, cooled and plunged in a single cell bacterial colony. The loop loaded with bacteria was used to stab the solid medium in the culture tube. Similarly, stabs for the two bacterial strains were prepared and allowed to stand in the culture room on racks for growth of the bacteria in the medium. The stabs showing good growth of bacteria were further stored in refrigerator at 4-6°C till further use.

#### 3.7.4.2 Preparation of glycerol stock

Liquid YEM medium was prepared in required volume in conical flask and kept in culture room until use. On the day of use the required antibiotics were added to the medium and shaken well in a laminar flow. Using a sterile bacterial loop, a single colony of the bacteria from the culture was taken and inoculated into the YEM liquid medium and shaken well to form a uniform bacterial suspension.

The conical flask was plugged and incubated in shaker (120 rpm) at 28°C for 18 to 24 hours to bring the bacterial density to 1.0 $OD_{600}$ nm

Inside the laminar air flow, 800 µl of the bacterial suspension was added to a sterile eppendorf tube. To this 200 µl autoclaved glycerol was added and stored at $-20^0$c.

### 3.7.5 Screening of *A. tumefaciens* strains for antibiotic sensitivity

The *A. tumefaciens* strains used for the study were tested for their sensitivity to various to antibiotics. The antibiotics used for testing sensitivity were kanamycin, rifampicin, carbenicillin and cefotaxime.

The YEM medium was supplemented with 50 mg $l^{-1}$, 100 mg $l^{-1}$, 200mg $l^{-1}$, 300 mg $l^{-1}$, 400 mg $l^{-1}$ of each antibiotics, separately. The bacteria from a single cell colony were spotted gently on the antibiotic medium in petriplates. A control having no antibiotic was also maintained. The observations regarding the growth of bacteria were documented.

### 3.7.6 Plasmid isolation from *Agrobacterium*

Binary vector constructs were isolated from the bacterial strains to confirm their presence using alkali lysis method (Birnhoim and Doly, 1979). Composition of various reagents used for plasmid isolation is given in Appendix II.

#### *3.7.6.1 Procedure*

Five ml of the YEM media containing antibiotics was taken in test tubes. To these, about 10 bacterial colonies were inoculated and incubated in a shaker (120 rpm) for 36-48 hours at 28°C. 1.5 ml aliquot of the broth was transferred into an eppendorf tube and centrifuged at 5000 rpm for 3 min at 4°C. The pellet was saved. To this 500 µl of ice cold solution I was added and incubated on ice for 30 min. 200 µl of freshly prepared solution II was added and mixed gently by inverting the tube 5 times. 150 µl of ice-cold solution III was

added, mixed gently and incubated on ice for 1 hour. This was centrifuged at 14,000 rpm for 10 min at 4°C. The supernatant was collected promptly, added 1 ml 100 per cent ethanol, incubated on dry ice (-20°C) for 30 minutes and centrifuged at 14,000 rpm for 10 min at 4°C. The pellet was air dried and resuspended in 200 µl solution IV and incubated on ice for 30 min. 100 µl of cold 100 per cent ethanol was added, incubated on dry ice for 15 min, centrifuged at 14,000 rpm for 10 min at 4°C. The pellet was dissolved in 200 µl TE buffer. The resuspended pellet was extracted once with 200 µl phenol: chloroform:isoamyl alcohol (25:24:1), centrifuged at 14,000 rpm for 10 min at 4°C. Collected the aqueous phase and pelleted the DNA by addition of 0.5 volume of solution V and 2.5 volume of 100 per cent ethanol for 1 hr at 20°C. The pellet was washed with 70 per cent alcohol and dissolved in TE buffer (pH - 8.0) and checked on 0.7 per cent agarose gel.

### 3.7.7 Standardization of *Agrobacterium* mediated transformation

*Agrobacterium* mediated genetic transformation of *Capsicum annuum* L. var. Ujwala was standardized with construct having *gus* reporter gene. The best explant and the best media from the previous experiment were used for the study.

#### *3.7.7.1 Preparation of Agrobacterium culture*

Liquid YEM medium was prepared in required volume in conical flask and kept in culture room until use. On the day of use kanamycin (50 mg $l^{-1}$) and rifampicin (20 mg $l^{-1}$) were added to the medium and shaken well in a laminar flow. Using a sterile bacterial loop, a single colony of the bacteria from the culture was taken and inoculated into the YEM liquid medium and shaken well to form a uniform bacterial suspension. The conical flask was plugged and kept in shaker (120 rpm) at 28°C for 18 to 24 hours according to the required density of the bacteria. The bacterial density was measured in Spectronic Genesis Spectonic instrument, USA at a wavelength of 600 nm.

### *3.7.7.2 Culture establishment*

The axenic seedlings raised in test tubes were brought to the laminar flow cabinet. Hypocotyls of approximately 2-2.5 cm were cut and separated from the seedling. The hypocotyls were then placed upside down in regeneration media in sterile petriplates. The petriplates were sealed with parafilm and incubated in culture room for 2 days.

### *3.7.7.3 Preparation of inoculation media*

The liquid MS media was used for the preparation of inoculation media. The *Agrobacterium* suspension was centrifuged at 5000 rpm for 10 minutes at 4°C. The supernatant was discarded and the bacterial pellet was washed thrice with liquid MS. The bacterial pellet was resuspended in MS liquid.

### *3.7.7.4 Standardization of inoculum density*

Transformation was tried at different inoculum density (bacterial concentration) ranging from $OD_{600}$ nm= 0.1 to 1.0.

### *3.7.7.4 Standardization of infection time*

The inoculation medium was transferred into sterile petriplates and the hypocotyl explants were immersed in the media. The infection times tried in various experiments were 2, 5, 10 and 15 minutes. The explants were then blotted dry with sterile blotting paper.

### *3.7.7.5 Standardization of co-cultivation period*

The blot dried explants were transferred to regeneration medium (without antibiotics) in petri dishes.

The regeneration medium was prepared in required volume in conical flask and kept in culture room until use. On the day of use, the media was melted and then cooled to 40 to 50°C. Inside a laminar flow cabinet the medium was distributed in sterile petriplates and allowed to solidify and attain room

temperature. The blot dried hypocotyl explants were inoculated into the media in an upside down position. The cultures were maintained at 26 ± 2°C.

The period of co-cultivation tried ranged from one to four days.

### *3.7.7.6 Pre-selection*

After co-cultivation, the hypocotyl explants were washed with liquid MS containing 200 mg $l^{-1}$ cefotaxime. The explants were then blotted dry and inoculated in pre-selection medium containing bacteriostatic agent to kill the bacteria (regeneration medium + 200 mg $l^{-1}$ cefotaxime).

The regeneration medium was prepared in required volume in conical flask and kept in culture room. On the day of use, the media was melted and cooled to 40-50°C. The antibiotics were added to the medium under aseptic conditions and mixed thoroughly by vigorous shaking. The medium was then distributed in sterile petriplates and allowed to solidify and attain room temperature. The co-cultivated explants were then transferred to the solidified media and cultured under 26 ± 2°C for a week.

### *3.7.7.7 Screening*

After pre-selection, the explants were transferred to screening media in petriplates or culture tubes. The screening media consisted of regeneration media containing antibiotics used as selecting agent and the bacteriocidal agent. Control explants (without co-cultivation) were also maintained in the screening medium.

The explants were then transferred to selection media. The cultures in the screening media were subcultured every 10 to 15 days. All the cultures were maintained in the culture room.

### *3.7.7.8 Transient expression of GUS reporter gene (histochemical assay)*

The histochemical GUS assay was carried out as described by Jefferson *et al.* (1987). The transient expression of GUS was monitored after 3 days of transfer to the pre-selection medium. The composition and preparation of X-Gluc stain is given in appendix III.

The explants were incubated in X-Gluc stain for 24-48 hrs at 37°C in dark. Both transformed and control explants were subjected to X-Gluc assay. After 24-48 h the explants were washed and kept in 100 per cent ethanol for preservation. Assayed tissues were observed under a microscope.

### 3.7.8 Transformation with *osmotin* gene

The *Agrobacterium* strain GV 2260 carrying the plasmid pGV2260 was used as vector system for transformation. The plasmid contains *osmotin* gene and *npt*II gene as plant selection marker.

The best parameters obtained from the previous experiments (explant, media, infection time, inoculum density and co-cultivation period) were used for transformation using *osmotin* gene.

#### *3.7.8.1 Transformation and regeneration*

The hypocotyl explants were pre-cultured in the regeneration medium for 2 days. Then the explants were infected for 5 min with *Agrobacterium* suspension, blotted dry with sterile blotting paper and returned to the regeneration medium for co-cultivation. Following the 2 days of co-cultivation, the explants were washed with liquid MS medium containing 250 mg $l^{-1}$ cefotaxime and transferred to pre-selection medium. Subsequently the explants were placed on selection medium, which is the regeneration medium complemented with 100 mg $l^{-1}$ kanamycin and 200 mg $l^{-1}$ cefotaxime. The explants were transferred to fresh selection medium after 15 days of culture. The explants with putative transgenic shoot buds were transferred to shoot elongation medium with 100 mg $l^{-1}$ kanamycin and 200 mg $l^{-1}$ cefotaxime.

#### *3.7.8.2 Confirmation of transformation*

The transformation was confirmed by Polymerase Chain Reaction (PCR) analysis using *npt*II primers.

*3.7.8.2.1 Plant DNA isolation*

For PCR analysis DNA was isolated from young leaves of transformed plantlets and control plants following Doyle and Doyle method (Doyle and Doyle, 1987). The composition of various reagents used for plant DNA isolation is given in Appendix II.

*3.7.8.2.1.1 Procedure*

Leaf samples weighing 0.5 g was ground with 6 ml of 1X extraction buffer, 50 µl β-mercapto ethanol and a pinch of sodium metabisulphate using an autoclaved mortar and pestle. To this ground material, 6 ml lysis buffer and 1 ml sarcosine were added and transferred into a 50 ml centrifuge tube. The tubes were kept in a water bath at 65° C for 10 minutes. The tubes were removed from the water bath and equal volume of chloroform:Isoamyl alcohol mixture (24:1 v/v) was added and mixed by gentle inversion. The tubes were centrifuged at 10,000 rpm for 10 min at 4°C. The clear aqueous phase was transferred to a new tube. 0.6 volume of ice-cold isopropanol was added and mixed gently and then kept in – 20°C deep freezer for 30 minutes for the complete precipitation of DNA. The tubes were centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant was discarded and the pellet was washed with 70 per cent ethanol by centrifuging at 10,000 rpm for 10 min at 4°C. The supernatant was discarded and the pellet was air-dried. The fresh pellet was dissolved in 100 µl of TE and stored at – 20°C.

*3.7.8.3 Agarose Gel Electrophoresis*

Agarose gel electrophoresis was performed based on the method described by Sambrook *et al.* (1989) to check the quality of DNA and also to separate the amplified products.

*3.7.8.3.1 Materials*

a) Agarose : 1.0 per cent (for genomic DNA)

Agarose : 0.7 per cent (for PCR samples)

b) 50x TAE buffer (pH 8.0)

| | |
|---|---|
| Tris base | - 242.0 g |
| Glacial acetic acid | - 57.1 ml |
| 0.5 M EDTA (pH 8.0) | - 100 ml |
| Distilled water | - 1000 ml |

The solution was prepared and stored at room temperature.

c) Electrophoresis unit, power pack, casting tray, comb.

d) 6x Loading/Tracking dye

| | |
|---|---|
| Bromophenol blue | - 0.25 per cent |
| Xylene cyanol | - 0.25 per cent |
| Glycerol | - 30 per cent |

The dye was prepared and kept in fridge at 4°C

e) Ethidium bromide solution

The dye was prepared as a stock solution of 10 mg $ml^{-1}$ in water and was stored at room temperature in a dark bottle.

f) UV transilluminator

g) Biorad gel documentation and analysis system.

*3.7.8.3.2* ***Procedure***

Four hundred ml of electrophoresis buffer (1x TAE) was prepared to fill the electrophoresis tank and to prepare the gel. The open ends of the gel-casting tray were sealed with a cellophane tape and placed on a perfectly horizontal leveled platform. Agarose (1.0 per cent for genomic DNA and 0.7 per cent for PCR) was added to 1x TAE, boiled till the agarose dissolved completely and then cooled to lukewarm temperature. Ethidium bromide was added to a final concentration of 0.5 $\mu g\ ml^{-1}$ as an intercalating agent of DNA, which will help in its visualization in UV rays. It was then poured into the gel mould and the comb was placed properly and allowed to solidify. After the gel was completely set (30-45 minutes at room temperature), the comb and cellophane tape were removed carefully. The casted gel was placed in the electrophoresis tank with the wells near the cathode and submerged with 1x TAE to a depth of 1 cm. A piece of cellophane tape was

pressed on a solid surface and 1 µl 6x loading buffer was dispensed in small quantity on the tape. A quantity of 3-5 µl of DNA was added to each dot (In the case of PCR products, 10.0-15.0 µl) mixed well by pipetting in and out for 2 to 3 times. Then the mixture was loaded into the wells, with the help of the micropipette. Lambda DNA (*Hind* III/*Eco*R I doble digest) was also added in one of the wells as a molecular weight marker. The cathode and anode were connected to power pack and the gel was run at a constant current of 60 volts. The power was turned off when the tracking dye reached at about 3 cm from the anode end.

### *3.7.8.4 PCR analysis of nptII gene*

Polymerase chain reaction was carried out using the primers designed for *npt*II gene. Two primers of *npt*II gene were used. The expected size of the fragment was 600 bp. Primers used for PCR amplification were:

Forward primer- 5' CAA TCG GCT GCT CTG ATG CCG 3'

Reverse primer-5' AGG CGA TAG AAG GCA ATG CGC 3'

Composition of the reaction mixture for PCR (25.0 µl)

a) Genomic DNA - 1µl (1:9 dilution)
b) 10X Taq assay buffer - 2.5µl
c) dNTP mix (1mM) - 1µl
d) Forward primer (2.5 pM) - 1µl
e) Reverse primer (2.5 pM) - 1µl
f) Taq polymerase (0.6U) - 2µl
g) Autoclaved distilled water - 16.5µl

The reaction mixture was given a momentary spin for thorough mixing of the cocktail components and15 µl of mineral oil was added to each tube to prevent the evaporation during the thermal cycling. Then the PCR tubes were loaded in a thermal cycler (PTC-200™ Programmable Thermocycler MJ Research).

Thermal cycler program

Profile 1 : 94 °C for 2 min - Initial denaturation

Profile 2 : 94 °C for 45 sec - Denaturation
Profile 3 : 54 °C for 1 min - Annealing
Profile 4 : 72 °C for 2 min – Extension
} 30 cycles

Profile 5 : 72 °C for 10 min - Final extension

Profile 6 : 4 °C for 15 min to hold the sample.

Amplified DNA fragments were electrophoresed on 0.7 per cent agarose ethidium bromide gel, observed under ultra-violet and documented using the gel documentation system (Biorad Imager Gel Doc XR).

# Results

# 4. RESULTS

The results of the investigations on the 'genetic transformation of chilli (*Capsicum annuum* L.) with *osmotin* gene,' carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara, from December 2003 to September 2005 are presented in this chapter.

## 4.1 *IN VITRO* REGENERATION

### 4.1.1 Standardization of explants

Based on the earlier reports on *in vitro* regeneration of *Capsicum*, MS medium containing BA (5.0 mg $l^{-1}$) and IAA (0.5 mg $l^{-1}$) was selected for the study. Response of hypocotyl, cotyledon and leaf explants in MS medium supplemented with BA (5.0 mg $l^{-1}$) and IAA (0.5 mg $l^{-1}$) are given in Table 3.

In the case of hypocotyl explants, regeneration started 13 days after inoculation. The average number of shoot buds per explant was 8.9 (Plate 1.a). The first sign of shoot bud initiation was swelling of basal part of the hypocotyl, followed by the appearance of a dark green ring like lump of tissue around the circumference of the cut ends. Hypocotyl explants produced roots on the middle and basal region. When the hypocotyl explants were placed horizontally, only the upper part of the hypocotyl showed the ability to differentiate shoot buds, whereas as roots were produced from the lower and middle regions (Plate.1.d). When the hypocotyl explants were placed directly as such (upper part above), very low regeneration was observed on the upper part (Plate.1.e). Callusing was observed in the lower region.

In the case of cotyledonary explants, regeneration started 15 days after inoculation. Sixty per cent regeneration was obtained in the media, MS + BA (5.0 mg $l^{-1}$) + IAA (0.5 mg $l^{-1}$). Callusing was also observed in this medium. The average number of shoot buds per explant was 4.2.

Only the proximal part of the cotyledon showed the regenerative behaviour (Plate.1.b). Adventitious buds were produced on the proximal region of the cotyledonary explants.

Leaf explants inoculated in MS medium containing BA (5.0 mg $l^{-1}$) and IAA (0.5 mg $l^{-1}$) produced 11 per cent regeneration. The average number of shoot buds per explant was 0.3. Callusing was observed in this medium (Plate 1.c).

After 15 days of regeneration, the shoot buds from different explants were cut into 3 to 4 pieces and inoculated in MS medium containing 2 and 3 per cent sucrose. The data is given in Table 4.

Shoot buds from hypocotyl showed 50 per cent elongation in MS medium containing 2 per cent sucrose (without any growth regulators). The average length of shoots was 2.0 cm. Rooting was also observed in this medium. In this media elongation started with the petiolar elongation of one leaf, followed by elongation of one shoot (Plate.2.a). Once a shoot was elongated, it seemed to restrict the subsequent elongation of other shoot buds. By cutting the elongated shoots, further development of other shoot buds from hypocotyl was observed.

Shoot buds from cotyledon explants did not show elongation in this medium. Shoot buds grew into rosettes with well-developed leaves but no elongation was observed (Plate.2.b).

Shoot buds from leaf segments were not elongated in any of the media tested. The shoot buds were remained as such without any change.

After elongation, the shoots formed from hypocotyl explants were cut and given a pulse treatment with IBA (1000 mg $l^{-1}$) and inoculated in MS medium containing 2 and 3 per cent sucrose. The number of days taken for root induction was 10.5 days. The number of roots per shoot is given in Table 5.

Table 3. Response of different explants to *in vitro* regeneration in MS medium

| Explant | Growth regulator BA + IAA ($mg\ l^{-1}$) | Percentage regeneration | No. of shoot buds/explant | Response |
|---|---|---|---|---|
| Hypocotyl | 5.0 + 0.5 | 83.0 | 8.9 | Regeneration + callusing |
| Cotyledon | 5.0 + 0.5 | 60.0 | 4.2 | Regeneration + callusing |
| Leaf segment | 5.0 + 0.5 | 11.0 | 0.1 | Regeneration + callusing |

Average of nine observations

Table 4. Response of different explants to elongation in MS medium

| Explants | Media | | | | | |
|---|---|---|---|---|---|---|
| | MS + 2% sucrose | | | MS + 3% sucrose | | |
| | Shoot elongation (%) | Length of shoots | Response | Shoot elongation (%) | Length of shoots | Response |
| Hypocotyl | 50.0 | 2.0 | Shoot elongation + rooting | 40.0 | 2.3 | Shoot elongation + rooting |
| Cotyledon | nil | 0.0 | Leaf proliferation | nil | 0.0 | Leaf proliferation |
| Leaf segment | nil | 0.0 | No response | nil | 0.0 | No response |

a. Hypocotyl
inverted position

b. Cotyledon

c.Leaf segment

d. Hypocotyl
in horizontal position

e. Hypocotyl

Plate 1. Response of different explants of *Capsicum annuum* L. var. Ujwala to *in vitro* regeneration

Elongation of shoot buds from hypocotyl

Leaf proliferation of shoot buds from cotyledon

Plate 2. Response of regenerated shoot buds in elongation medium

Rooted plantlets were hardened for 15 days in small pots containing sterile sand and then planted in big pots containing potting mixture. The percentage of plants established is given in Table.6. Plants started flowering from 65 days after planting. Complete regeneration of plants from hypocotyl explants is shown in Plate.3.

### 4.1.2 Standardization of basal media

The data on the effect of media on regeneration of hypocotyl explants are represented in Table 7. The MS medium containing 5.0 mg $l^{-1}$ BA + 0.3, 0.5 and 1.0 mg $l^{-1}$ IAA produced 100, 83 and 55.5 per cent regeneration respectively. The SH medium containing same growth regulator combinations produced 44, 22 and 11 per cent regeneration. B5 medium produced 33, 11 and 11 per cent regeneration respectively. The response of hypocotyl explants in MS, SH and B5 media containing BA (5.0 mg $l^{-1}$) and IAA (0.3 mg $l^{-1}$) is shown in Plate 4.

### 4.1.3 Standardization of growth regulators

Hypocotyl explants were cultured in MS medium supplemented with different combinations of BA (1.0, 2.0, 3.0, 5.0, 7.0 mg $l^{-1}$) and IAA (0.2, 0.3, 0.5, 0.7, 1.0 mg $l^{-1}$). The data is given in Table 8.

The media combinations involving 1.0 mg $l^{-1}$ BA produced callusing and rooting of the hypocotyls. No regeneration was observed in these media combinations. When the concentration of BA was 2.0 mg $l^{-1}$, 22 per cent (2.0 mg $l^{-1}$ BA + 0.2 mg $l^{-1}$ IAA), 33 per cent (2.0 mg $l^{-1}$ BA + 0.3 mg $l^{-1}$ IAA), 33 per cent (2.0 mg $l^{-1}$ BA + 0.5 mg $l^{-1}$ IAA), 11 per cent (2.0 mg $l^{-1}$ BA + 0.7 mg $l^{-1}$ IAA) and 11 per cent (2.0 mg $l^{-1}$ BA + 1.0 mg $l^{-1}$ IAA) regeneration was noted. The growth regulator combination involving 3.0 mg $l^{-1}$ resulted 58 per cent (3.0 mg $l^{-1}$ BA + 0.2 mg $l^{-1}$ IAA), 56 per cent (3.0 mg $l^{-1}$ BA + 0.3 mg $l^{-1}$ IAA), 50 per cent (3.0 mg $l^{-1}$ BA + 0.5 mg $l^{-1}$ IAA), 44 per cent (3.0 mg $l^{-1}$ BA + 0.7 mg $l^{-1}$ IAA) and 33 per cent (3.0 mg $l^{-1}$ BA + 1.0 mg $l^{-1}$ IAA) regeneration. The media combinations MS + BA (5.0 mg $l^{-1}$) + IAA (0.2 mg $l^{-1}$) and MS + BA (5.0 mg $l^{-1}$) + IAA (0.3 mg $l^{-1}$) produced 100 per cent regeneration. When the concentration of BA was 7.0 mg $l^{-1}$,

Table 5. Effect of sucrose on rooting of elongated shoots regenerated from hypocotyl

| Sucrose (%) | Number of roots per shoot |
|---|---|
| 2 | 13.5 |
| 3 | 13.25 |

Table.6. Establishment of TC regenerants from hypocotyl

| Sl. no. | Total no. of hardened plants | No. plants survived | Percentage of establishment |
|---|---|---|---|
| 1 | 20 | 18 | 90 |
| 2 | 17 | 16 | 94 |

**Plate 3. *In vitro* regeneration of chilli var. Ujwala from hypocotyl**

a) **Chilli seedling (16-21 days old)**
b) **Hypocotyl in inverted position**
c) **Regeneration from hypocotyl**
d) **Initiation of elongation**
e) **Elongation**
f) **Rooting**
g) **Plants in hardening stage**
h) **Plants at flowering stage**


Plate 3. *In vitro* regeneration of chilli var. Ujwala from hypocotyl

Table 7. Effect of basal media on *in vitro* regeneration of hypocotyl explants

| Basal media | Growth regulator BA+IAA ($mg\ l^{-1}$) | Percentage regeneration | Average no. of shoot buds/explant | Response |
|---|---|---|---|---|
| MS | 5.0 + 0.3 | 100.0 | 11.2 | Regeneration |
| | 5.0 + 0.5 | 83.0 | 8.9 | Regeneration + callusing |
| | 5.0 + 1.0 | 55.5 | 4.9 | Regeneration + callusing |
| SH | 5.0 + 0.3 | 44.0 | 0.5 | Regeneration + callusing |
| | 5.0 + 0.5 | 22.0 | 0.2 | Regeneration + callusing |
| | 5.0 + 1.0 | 11.0 | 0.2 | Regeneration + Callusing |
| B5 | 5.0 + 0.3 | 33.0 | 0.3 | Regeneration + callusing |
| | 5.0 + 0.5 | 11.0 | 0.1 | Regeneration + callusing |
| | 5.0 + 1.0 | 11.0 | 0.1 | Regeneration + callusing |

Average of nine observations

Table 8. Effect of growth regulators on *in vitro* regeneration of hypocotyl

| Growth regulator BA + IAA (mg $l^{-1}$) | Percentage regeneration | No. of shoot buds/explant | Response |
|---|---|---|---|
| 1.0 + 0.2 | Nil | 0.0 | Callusing + rooting |
| 1.0 + 0.3 | Nil | 0.0 | Callusing + rooting |
| 1.0 + 0.5 | Nil | 0.0 | Callusing + rooting |
| 1.0 + 0.7 | Nil | 0.0 | Callusing + rooting |
| 1.0 +1.0 | Nil | 0.0 | Callusing + rooting |
| 2.0 + 0.2 | 22.0 | 0.4 | Regeneration +callusing |
| 2.0 + 0.3 | 33.0 | 0.5 | Regeneration +callusing |
| 2.0 + 0.5 | 33.0 | 0.5 | Regeneration +callusing |
| 2.0 + 0.7 | 11.0 | 0.2 | Regeneration +callusing |
| 2.0 + 1.0 | 11.0 | 0.1 | Regeneration +callusing |
| 3.0 + 0.2 | 58.0 | 3.2 | Regeneration +callusing |
| 3.0 + 0.3 | 56.0 | 5.2 | Regeneration +callusing |
| 3.0 + 0.5 | 50.0 | 5.3 | Regeneration +callusing |
| 3.0 + 0.7 | 44.0 | 3.0 | Regeneration +callusing |
| 3.0 + 1.0 | 33.0 | 2.3 | Regeneration +callusing |
| 5.0 + 0.2 | 100.0 | 11.0 | Regeneration |
| 5.0 + 0.3 | 100.0 | 11.2 | Regeneration |
| 5.0 + 0.5 | 83.0 | 8.9 | Regeneration +callusing |
| 5.0 + 0.7 | 72.0 | 6.2 | Regeneration +callusing |
| 5.0 + 1.0 | 55.5 | 4.9 | Regeneration +callusing |
| 7.0 + 0.2 | 33.3 | 3.8 | Regeneration +callusing |
| 7.0 + 0.3 | Nil | 0.0 | Callusing |
| 7.0 + 0.5 | Nil | 0.0 | Callusing |
| 7.0 + 0.7 | Nil | 0.0 | Callusing |
| 7.0 + 1.0 | Nil | 0.0 | Callusing |

Average of nine observations

only the combination involving 0.2 mg $l^{-1}$ IAA produced 33.3 per cent regeneration (Plate.5). Other combinations showed callusing.

After 15 days of regeneration, the shoot buds were cut into 4 to 5 pieces and inoculated in MS medium containing different growth regulators and without any growth regulators. The data is given in Table 9. MS medium containing 2 per cent sucrose without any growth regulators resulted in 50 per cent elongation. In this media elongation started with the petiolar elongation of one leaf followed by elongation of one shoot.

The media containing 0.025 and 0.05 per cent activated charcoal resulted in 26.6 per cent and 31.3 per cent elongation respectively. In these media, petiolar elongation of the first leaf was not observed. When GA was used for elongation, no change was noted. In the media containing 1.0 mg $l^{-1}$ IAA, 21.75 per cent elongation was observed.

## 4.2 *AGROBACTERIUM* MEDIATED GENETIC TRANSFORMATION

### 4.2.1 Sensitivity of explants to antibiotics

Sensitivity of hypocotyl and cotyledonary explants to four different antibiotics at five different concentrations was tested. A control was also maintained in antibiotic free medium.

#### *4.2.1.1 Sensitivity of explants to Kanamycin*

The response of explants to kanamycin at 50, 100, 200, 300 and 400 mg $l^{-1}$ were tested. The data is given in Table 10.

The buds of explants were successfully induced on kanamycin free regeneration medium (control). Kanamycin at the concentration of 50 mg $l^{-1}$ induced buds in 35 per cent of the hypocotyl explants tested. When the level of kanamycin was 100 mg $l^{-1}$ or higher, buds could not be induced from hypocotyl explants. The explants were bleached (Plate.6). Hence kanamycin 100 mg $l^{-1}$ was considered as minimal lethal.

**Plate 5. Effect of basal media on *in vitro* regeneration of hypocotyl**

a) **B5 + BA 5.0 mg $l^{-1}$ + IAA 0.3 mg $l^{-1}$**

b) **MS+ BA 5.0 mg $l^{-1}$ + IAA 0.3 mg $l^{-1}$**

c) **SH + BA 5.0 mg $l^{-1}$ + IAA 0.3 mg $l^{-1}$**

**Plate 4. Effect of growth regulator on *in vitro* regeneration of hypocotyl**

a) **MS+ BA 1.0 mg $l^{-1}$ + IAA 0.2 mg $l^{-1}$**

b) **MS+ BA 2.0 mg $l^{-1}$ + IAA 0.2 mg $l^{-1}$**

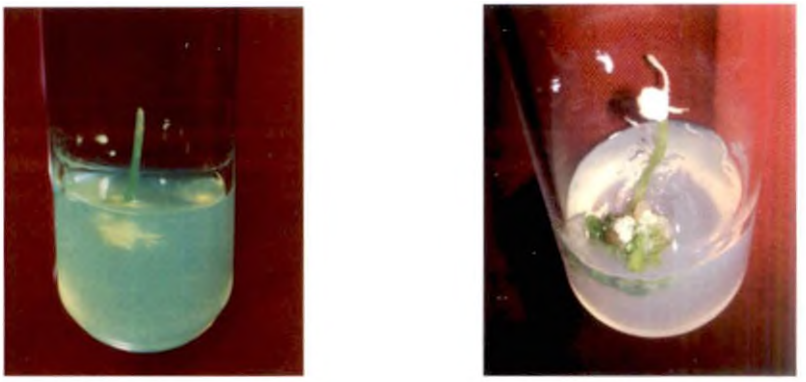
c) **MS+ BA 3.0 mg $l^{-1}$ + IAA 0.2 mg $l^{-1}$**

d) **MS+ BA 5.0 mg $l^{-1}$ + IAA 0.2 mg $l^{-1}$**

e) **MS+ BA 7.0 mg $l^{-1}$ + IAA 0.2 mg $l^{-1}$**

a b c

Plate 4. Effect of basal media on *in vitro* regeneration of hypocotyl



a b

c d e

Plate 5. Effect of growth regulators on *in vitro* regeneration of hypocotyl

Table 9. Effect of growth regulators on elongation of shoot buds from hypocotyl

| Growth regulator | Shoot elongation (%) | Length of shoots (cm) | Response |
|---|---|---|---|
| 2%sucrose | 50.0 | 2.0 | Shoot elongation + rooting |
| 3%sucrose | 40.0 | 2.3 | Shoot elongation+ rooting |
| 1 IAA (mg $l^{-1}$) | 21.75 | 1.8 | Shoot elongation |
| 0.5 IAA (mg $l^{-1}$) | nil | 0.0 | Rooting |
| 0.5 GA (mg $l^{-1}$) | nil | 0.0 | No response |
| 1.0 GA (mg $l^{-1}$) | nil | 0.0 | No response |
| 0.025% AC | 26.6 | 2.1 | Shoot elongation |
| 0.05% AC | 31.3 | 4.25 | Shoot elongation |
| Half MS | nil | 0.0 | No response |

Average of nine observations
MS + 3 % sucrose was used.

For cotyledon, in presence of kanamycin all the explants were bleached and no regeneration was observed.

#### *4.2.1.2 Sensitivity of explants to rifampicin*

Hypocotyl and cotyledonary explants were tested for their sensitivity to various concentrations (50, 100, 200, 300 and 400 mg $l^{-1}$) of rifampicin. The data is given in Table 11.

After first week the cotyledonary leaves were dark at the sides but had light coloured live tissues in the centre. After three weeks the tissues were completely dark and appeared almost dead.

For hypocotyls, complete suppression of regeneration was noticed in all the four different concentrations tested. The explants were brown in colour and looked almost dead.

#### *4.2.1.3 Sensitivity of explants to cefotaxime*

Sensitivity of hypocotyl and cotyledonary explants to varying concentrations of cefotaxime was tested. The data is given in Table 12. Shoot buds were induced from the explants at all concentrations tested (Plate.8). The number of shoot buds per explant was less compared to that of control (without any antibiotics).

#### *4.2.1.4 Sensitivity of explants to carbenicillin*

The effect of various concentrations of carbenicillin on hypocotyl and cotyledonary explants is shown in Table 13. Callusing of the explants was observed in all concentrations of the carbenicillin tested (Plate.7). Shoot buds were induced from the control (without antibiotics) explants.

Table 10.Response of explants to different concentrations of kanamycin

| Kanamycin concentration (mg $l^{-1}$) | Explants | | | |
|---|---|---|---|---|
| | Hypocotyl | | Cotyledon | |
| | Response | Remarks | Response | Remarks |
| 50 | Regeneration | Resistant | Bleaching | Sensitive |
| 100 | Bleaching | Sensitive | Bleaching | Sensitive |
| 200 | Bleaching | Sensitive | Bleaching | Sensitive |
| 300 | Bleaching | Sensitive | Bleaching | Sensitive |
| 400 | Bleaching | Sensitive | Bleaching | Sensitive |

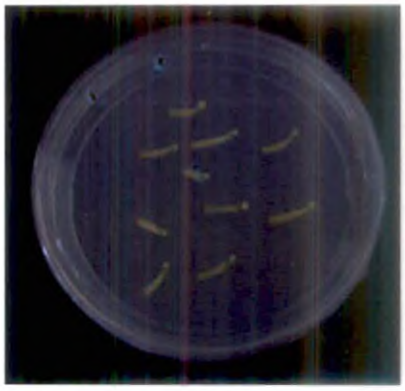
Culture duration 6 weeks: Sub culturing 2 weeks interval

Table 11. Response of explants to different concentrations of rifampicin

| Rifampicin concentration (mg $l^{-1}$) | Explants | | | |
|---|---|---|---|---|
| | Hypocotyl | | Cotyledon | |
| | Response | Remarks | Response | Remarks |
| 50 | Browning | Sensitive | Browning | Sensitive |
| 100 | Browning | Sensitive | Browning | Sensitive |
| 200 | Browning | Sensitive | Browning | Sensitive |
| 300 | Browning | Sensitive | Browning | Sensitive |
| 400 | Browning | Sensitive | Browning | Sensitive |

Culture duration 6 weeks; sub culturing 2 weeks interval

50



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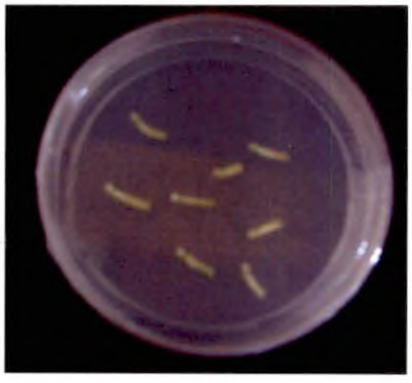
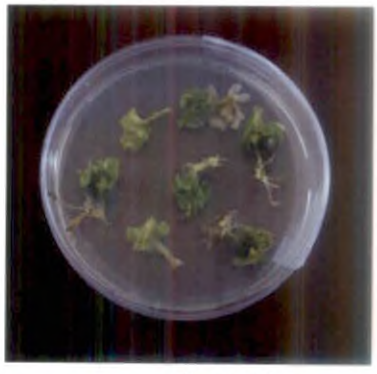


200

300

400



Control

Plate 6. Sensitivity of hypocotyl explants to kanamycin (conc. mg $l^{-1}$)

Table 12. Response of explants to different concentrations of cefotaxime

| Cefotaxime concentration ($mg\ l^{-1}$) | Explants | | | |
|---|---|---|---|---|
| | Hypocotyl | | Cotyledon | |
| | Response | Remarks | Response | Remarks |
| 50 | Regeneration | Resistant | Regeneration | Resistant |
| 100 | Regeneration | Resistant | Regeneration | Resistant |
| 200 | Regeneration | Resistant | Regeneration | Resistant |
| 300 | Regeneration | Resistant | Regeneration | Resistant |
| 400 | Regeneration | Resistant | Regeneration | Resistant |

Culture duration 6 weeks; Sub culturing 2 weeks interval

Table 13. Response of explants to different concentrations of carbenicillin

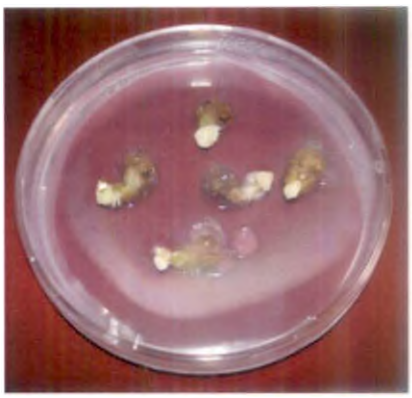
| Carbenicillin concentration ($mg\ l^{-1}$) | Explants | | | |
|---|---|---|---|---|
| | Hypocotyl | | Cotyledon | |
| | Response | Remarks | Response | Remarks |
| 50 | Callusing | Resistant | Callusing | Resistant |
| 100 | Callusing | Resistant | Callusing | Resistant |
| 200 | Callusing | Resistant | Callusing | Resistant |
| 300 | Callusing | Resistant | Callusing | Resistant |
| 400 | Callusing | Resistant | Callusing | Resistant |

Culture duration 6 weeks; Sub culturing 2 weeks interval

50

100

200



300

400

control

Plate 7. Sensitivity of hypocotyl explants to carbenicillin (conc. mg $l^{-1}$)

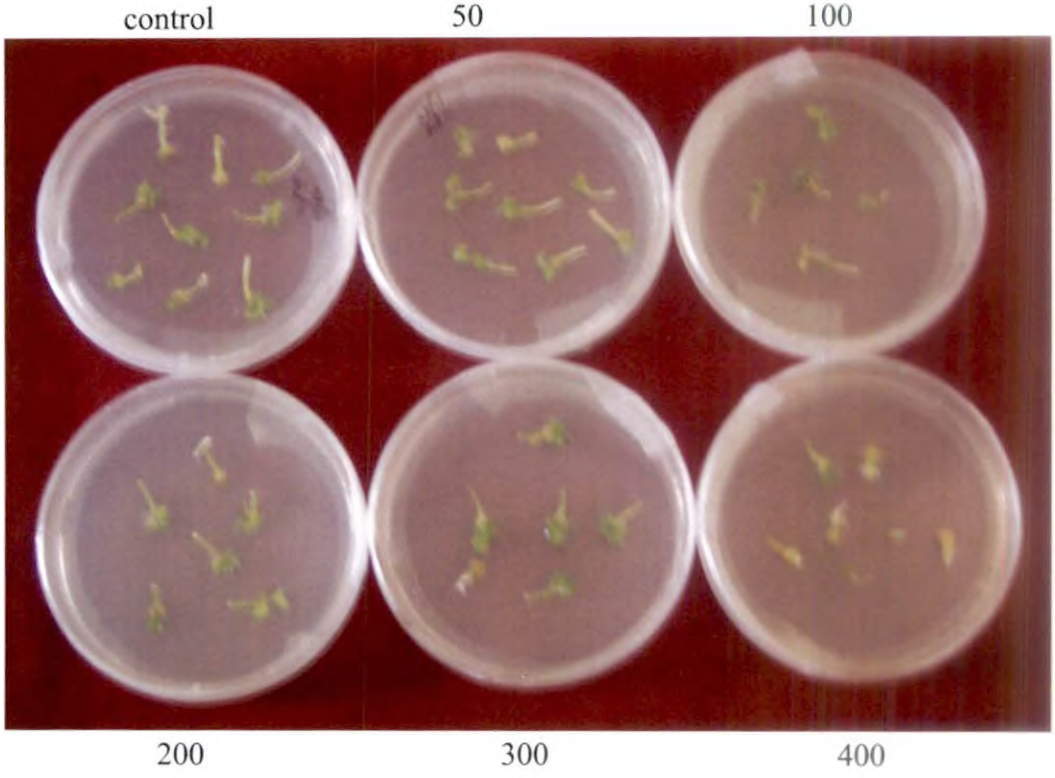



Plate 8. Sensitivity of hypocotyl to cefotaxime (conc. mg $l^{-1}$)

### *4.2.2* Morphological characteristics of *Agrobacterium*

Gram staining of the Agrobacteria was done. It revealed the shape of the bacteria. The results revealed that *Agrobacterium* is gram negative, rod shaped and motile bacteria when viewed under microscope (Plate.10).

### *4.2.3* Cultural characteristics of *Agrobacterium*

The *Agrobacterium* strains EHA 105 and GV 2260 were streaked on Yeast Extract Mannitol (YEM) medium. Bacterial colonies were appeared two days after streaking (Plate.9). Colonies were round with smooth margin, convex, colourless and mucoid in nature.

### 4.2.4 Sensitivity of *Agrobacterium tumefaciens* strains to different antibiotics

Sensitivity of *Agrobacterium* strains EHA 105 and GV 2260 to different antibiotics at varying concentrations is shown in Table 14. The *Agrobacterium* strain EHA 105 was sensitive to cefotaxime and carbenicillin (Plate.13 and 14); and resistant to kanamycin (Plate.12) and rifampicin. The *Agrobacterium* strain GV2260 showed resistance to kanamycin, rifampicin and carbenicillin at all the concentration tested and was sensitive to cefotaxime. Among the two strains, EHA 105 showed good growth in kanamycin and rifampicin containing media.

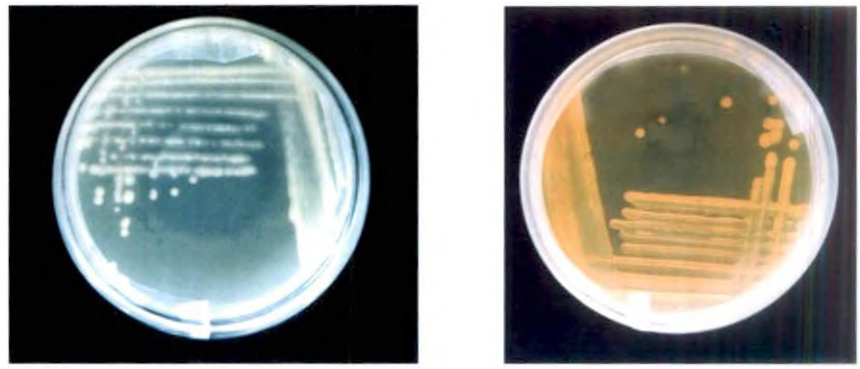
### *4.2.5* Plasmid isolation from *Agrobacterium*

Plasmid DNA was isolated from two *Agrobacterium* strains following the alkali lysis method. Electrophoresis of plasmid DNA of the two strains, EHA 105 and GV 2260, on 0.7 per cent agarose gel showed a single DNA band having a relative molecular weight of approximately 14 kb. The molecular weight of plasmids from EHA 105 was slightly less than that of GV 2260. There was no RNA contamination (Plate.11).

Table 14. Effect of antibiotics on growth of *Agrobacterium tumefaciens* strains

| *Agrobacterium tumefaciens* strains | Response | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Kanamycin (mg $l^{-1}$) | | | | | Rifampicin (mg $l^{-1}$) | | | | | Cefotaxime (mg $l^{-1}$) | | | | | Carbencillin ( mg $l^{-1}$) | | | | |
| | 50 | 100 | 200 | 300 | 400 | 50 | 100 | 200 | 300 | 400 | 50 | 100 | 200 | 300 | 400 | 50 | 100 | 200 | 300 | 400 |
| EHA 105 | + | + | + | + | + | + | + | + | + | + | - | - | - | - | - | - | - | - | - | - |
| GV 2260 | + | + | + | + | + | + | + | + | + | + | - | - | - | - | - | + | + | + | + | + |

- Sensitive
+ Resistant



EHA 105 GV 2260

Plate 9. *Agrobacterium* strains on YEM medium

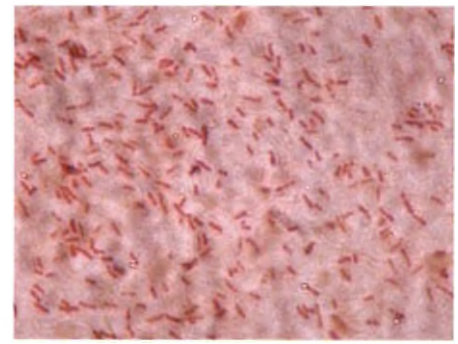
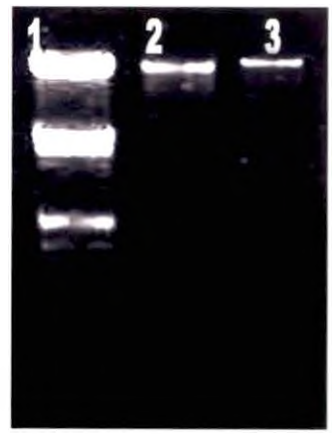


Plate 10. Morphological characters of *Agrobacterium* (strain EHA 105)

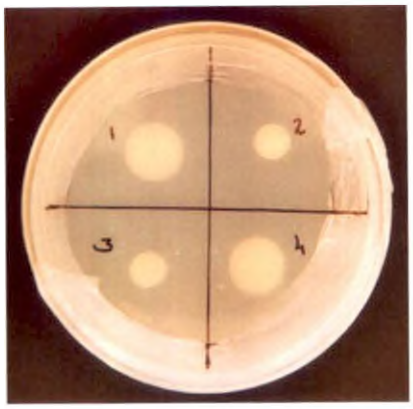



Lane 1-Molecular weight marker (lambda DNA/*Hind*III/*Eco*RI)

Lane 2-p35SGUSINT

Lane 3-pGV2260

Plate 11. Plasmid profile

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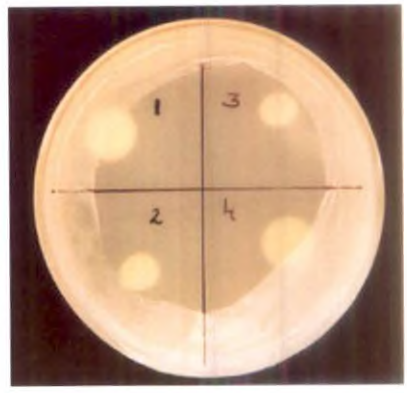


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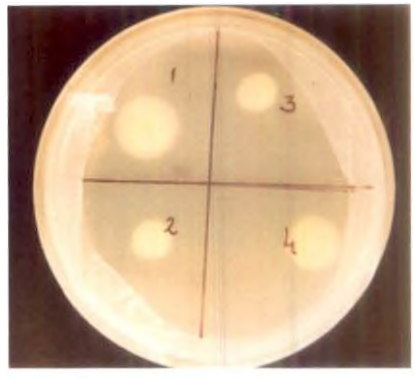


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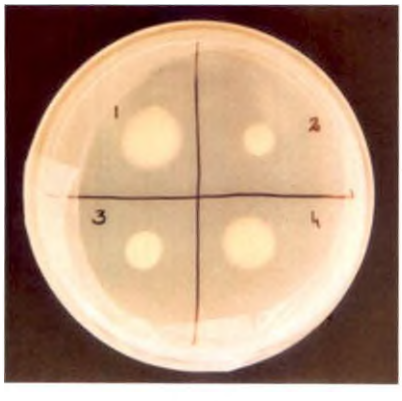


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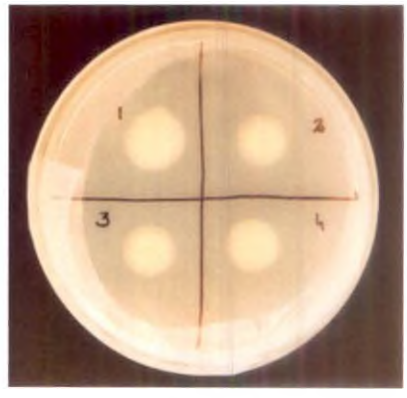
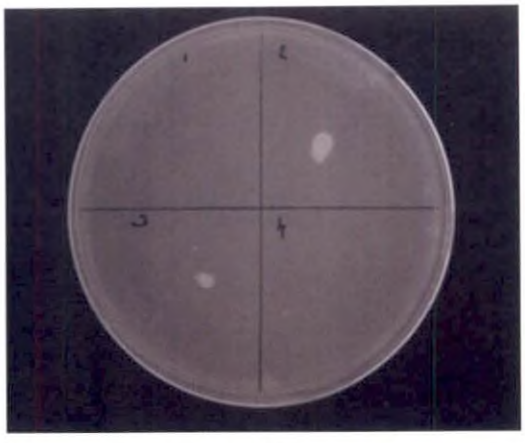



control

1&4-EHA 105, 2&3-GV 2260

Plate 12. Sensitivity of *Agrobacterium* to Kanamycin (conc. mg $l^{-1}$)



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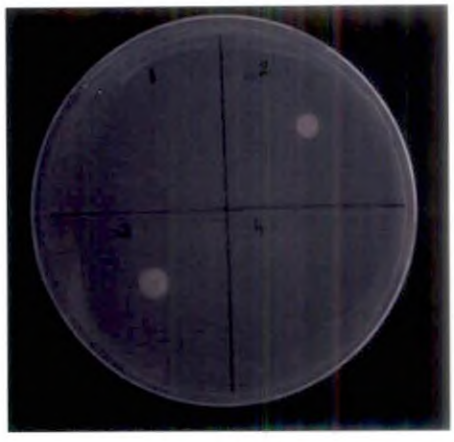

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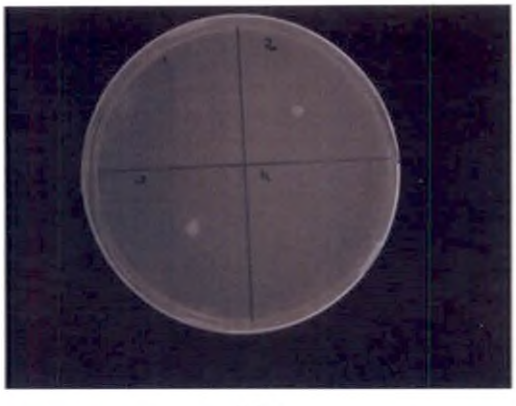

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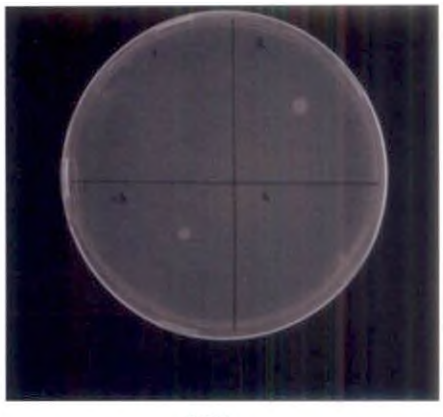

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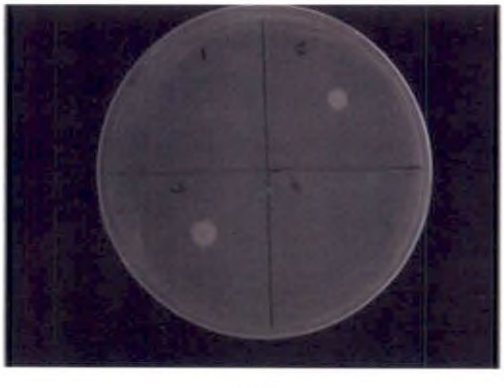

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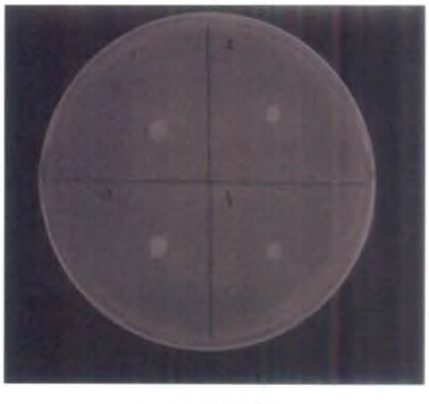
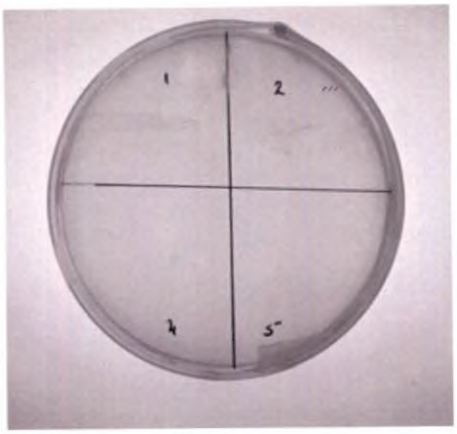


control

1&4-EHA 105, 2&3-GV 2260

Plate 13. Sensitivity of *Agrobacterium* to carbenicillin (conc. mg $l^{-1}$)

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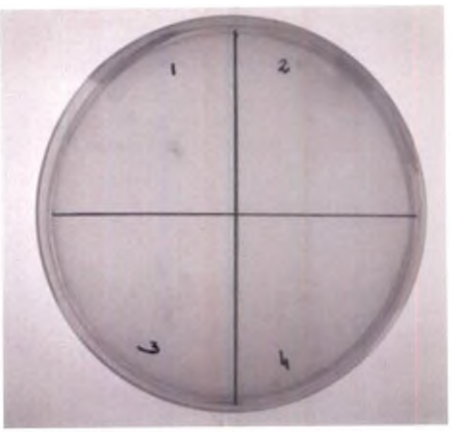


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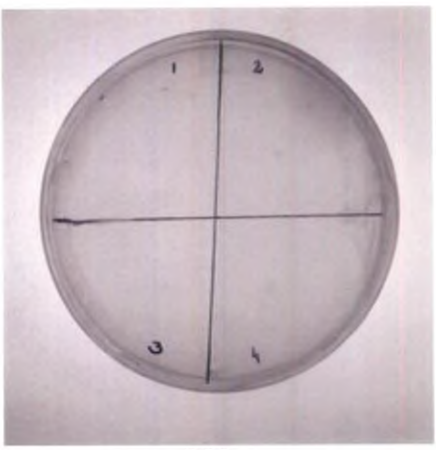


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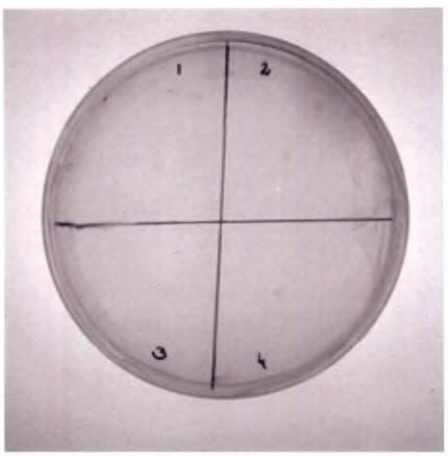


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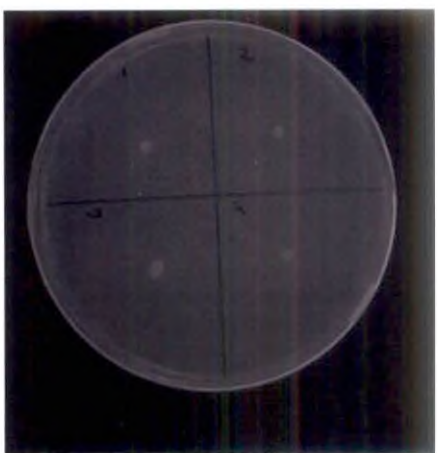



control

1&4-EHA 105, 2&3-GV 2260

Plate 14. Sensitivity of *Agrobacterium* to cefotaxime (conc. mg $l^{-1}$)

### 4.2.6 Standardization of *Agrobacterium* mediated genetic transformation

*Agrobacterium* mediated genetic transformation of *Capsicum annuum* L. var. Ujwala was standardized with EHA 105 having *gus* reporter gene. The coding region of the gus reporter gene contains an intron that prevents translation by *Agrobacterium tumefaciens*. The time of infection, density of bacterial population and co-cultivation period were standardized. Transformants were screened in selection medium containing 100 mg $l^{-1}$ kanamycin and 200 mg $l^{-1}$ cefotaxime. Transformants were subjected to X-gluc assay for confirmation of transformation.

#### *4.2.7.1 Standardization of inoculum density*

Transformation was tried at different inoculum densities (0.1, 0.25, 0.5 and 1.0 $OD_{600}$nm). The hypocotyl explants were used for the experiment. A 2 day period for co-cultivation and 10 minutes infection time were fixed for the study. Control plants were also maintained in both kanamycin containing (negative control) and kanamycin free media (positive control). The results of the experiment are given in Table 15.

The histochemical GUS assay was performed 3 days after co-cultivation. Both control and transformed plants were assayed. The results showed blue coloured areas on the surface of the transformed explants. When these blue coloured areas were examined under microscope, clear blue specks were observed. The blue coloured areas were more when the bacterial density was 1.0 $OD_{600}$nm. The control plants (non transformed) did not show any blue coloured areas (Plate.16). When the explants were subjected to GUS assay after 60 days of co-cultivation, no blue staining was observed.

When the bacterial density was 0.1 $OD_{600}$ nm, 80 per cent of the explants were survived even after 60 days of co-cultivation (Plate.15). When the bacterial density was 1.0 $OD_{600}$ nm, none of the explants were survived after 30 days. Control plants did not show any regeneration in kanamycin containing media

(negative control). Control plants showed 100 per cent regeneration in kanamycin free media (positive control).

#### *4.2.7.2 Standardization of infection time*

Different infection time's viz. 2, 5, 10 and 15 minutes were tried for the transformation study. A 2-day period for co-cultivation and a bacterial density of 0.1 $OD_{600}$ nm were fixed for the study. Control plants were also maintained in both kanamycin containing (negative control) and kanamycin free media (positive control). The data is given in Table 16.

Transient GUS assay was performed 3 days after co-cultivation. Blue coloured areas were present on the surface of transformed explants. These explants when observed under microscope blue specks were seen indicating transformation. The blue coloured areas were more on the transformed explants that were co-cultivated for 5 minutes. No blue staining was detected in control plant tissues. No blue staining was detected when the transformed explants were subjected to GUS assay after 2 months of co-cultivation.

The survival rate of transformed plants after 60 days of co-cultivation was 85 per cent in case of 5 minutes infection time, 70 per cent in 2 minutes, 80 per cent in10 minutes and 50 per cent in 15 minutes infection time tested. The control plants showed 100 per cent regeneration in kanamycin free media (positive control).

#### *4.2.7.3 Standardization of co-cultivation period*

The duration of co-cultivation with *Agrobacterium* is one of the factors affecting transformation efficiency. The effect of co-cultivation duration on chilli (*Capsicum annuum* L. var. Ujwala) was examined using hypocotyl explants. A 5 minutes infection time and a bacterial density of 0.1 $OD_{600}$ nm were fixed. The explants were co-cultivated with *Agrobacterium* for 1, 2, 3 and 4 days. Control plants were also maintained in both kanamycin containing (negative control) and kanamycin free media (positive control). The data is given in Table 17.

Table 15. Effect of inoculum density on survival rate of explants

| Pre-culture (days) | Bacterial density ($OD_{600}$nm) | Infection time (min) | Co-cultivation period (h) | GUS expression | Survival rate (%) |
|---|---|---|---|---|---|
| 2 | 0.1 | 10 | 48 | + | 80.0 |
| 2 | 0.25 | 10 | 48 | + | 70.0 |
| 2 | 0.5 | 10 | 48 | + | 40.0 |
| 2 | 1.0 | 10 | 48 | + | - |
| Positive control | - | - | - | - | 100.0 |

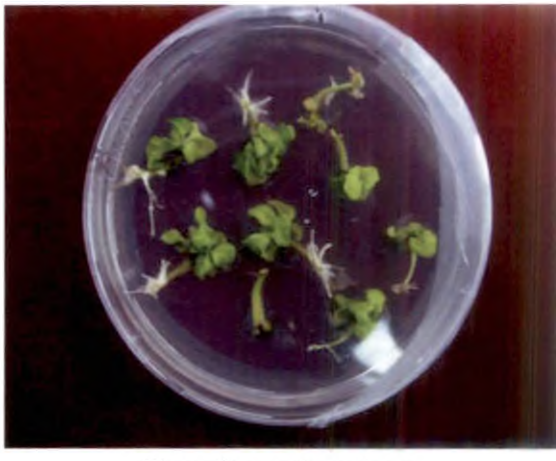
Survival rate after 60 days of co-cultivation

Table 16. Effect of infection time on survival rate of explants

| Pre-culture (days) | Bacterial density ($OD_{600}$nm) | Infection time (min) | Co-cultivation period (h) | GUS expression | Survival rate (%) |
|---|---|---|---|---|---|
| 2 | 0.1 | 2 | 48 | + | 70.0 |
| 2 | 0.1 | 5 | 48 | + | 85.0 |
| 2 | 0.1 | 10 | 48 | + | 80.0 |
| 2 | 0.1 | 15 | 48 | + | 50.0 |
| Positive control | - | - | - | - | 100.0 |

Survival rate after 60 days of co-cultivation

Control



Transformed explants

Plate 15. Transformation of chilli var. Ujwala with EHA 105


T – Transformed hypocotyl
C – Control

Microscopic view

Plate 16. Histochemical GUS assay

Table 17. Effect of co-cultivation period on survival rate of explants

| Pre-culture (days) | Bacterial density ($OD_{600}$nm) | Infection time (min) | Co-cultivation period (h) | GUS expression | Survival r (%) |
|---|---|---|---|---|---|
| 2 | 0.1 | 5 | 24 | + | 55.0 |
| 2 | 0.1 | 5 | 48 | + | 85.0 |
| 2 | 0.1 | 5 | 72 | + | 55.0 |
| 2 | 0.1 | 5 | 96 | + | 40.0 |
| 2 | 0.1 | 5 | - | - | 25.0 |
| Positive control | - | - | - | - | 100.0 |

Survival rate after 60 days of co-cultivation

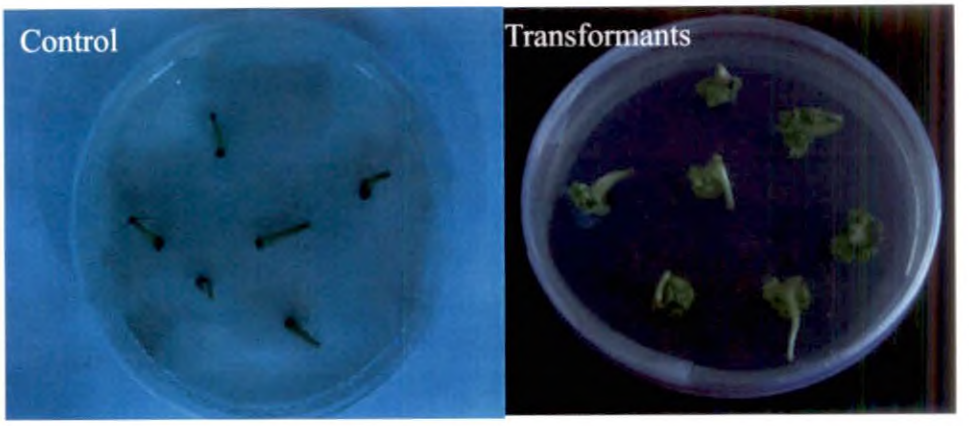
Transient GUS assay was performed 3 days after co-cultivation. Blue coloured areas were present on the surface of explants that were co-cultivated for 1, 2, 3 and 4 days. When these blue coloured areas were examined under microscope, distinct blue specks were observed. Blue coloured areas were not present on the surface of explants that were immediately transferred to the pre selection medium after co-cultivation (without co-cultivation). Control plants also did not show any blue specks. When the explants were subjected to GUS assay after 60 days of co-cultivation, no blue staining was observed.

The survival rate of transformed plants was 85 per cent for 48h co-cultivation period; this was followed by 24 and 72h and then, 96h co-cultivation period. Bacterial overgrowth was seen when there is a prolonged co-cultivation period of more than 2 days. The bacterial overgrowth was removed by culturing the explants in selection medium containing 500 mg $l^{-1}$ cefotaxime.

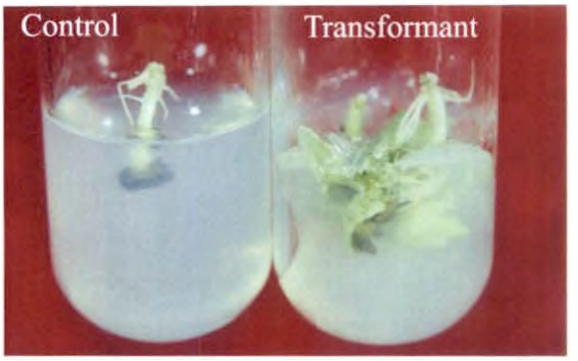
### 4.2.8 Transformation with *Osmotin* gene

The *Agrobacterium* strain GV 2260 carrying the plasmid pGV 2260 was used as the vector system for transformation. The best parameters from the previous experiments were used for the study. Hence, hypocotyl explants, the regeneration media - MS + 5.0 mg $l^{-1}$ BA + 0.3 mg $l^{-1}$ IAA, inoculation time - 5 minutes, bacterial density - 0.1 $OD_{600}$ nm and co-cultivation period - 48 h were used for the transformation study.

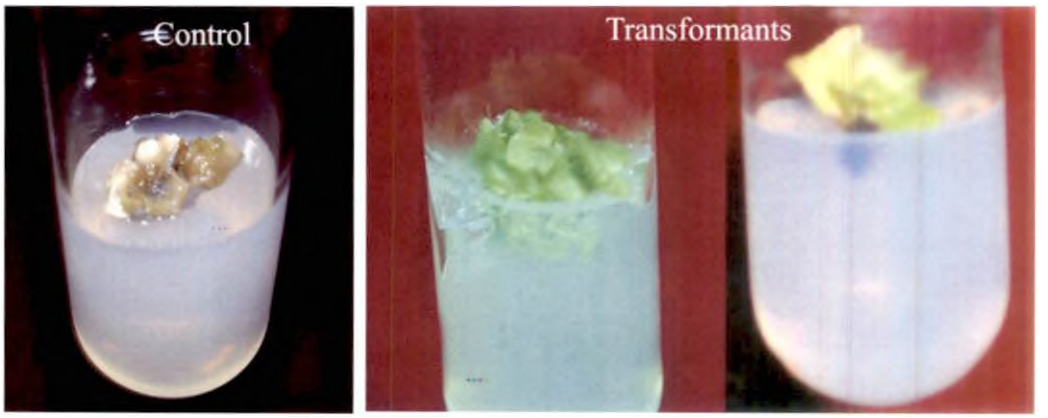
For transformation, 250 hypocotyl explants were co-cultivated with *Agrobacterium* for 48 h. The shoot bud induction was observed after 15 days of infection and regeneration continued on the selection medium. The growth was very less compared to the non co-cultivated explants. The number of shoot buds per explant was also less. The explants with shoot buds were sub cultured in the fresh selection medium after every 15 days. After 45-50 days culture, the transformed shoot buds were transferred to the elongation medium containing 100 mg $l^{-1}$ kanamycin and 200 mg $l^{-1}$ cefotaxime. The transformed explants at different stages of growth along with control are shown in Plate.17. 52 explants with shoot




Initiation of regeneration in selection medium




Transformants in selection medium after third sub culture




Transformants in elongation medium

Plate 17. Transformation of chilli var. Ujwala with *osmotin* gene

buds resistant to kanamycin at 100 mg $l^{-1}$ were transferred to elongation media. But none of the buds were elongated. Prolonged culture of regenerated buds in the selection medium caused vitrification of tissue. The shoot buds were then transferred to elongation medium without any antibiotics. But there was no change.

4.2.8.1 ***Confirmation of transformation***

The transgenic nature of the explants was confirmed by the PCR analyses of the *npt*II gene. PCR was used to demonstrate the presence of T-DNA in the transgenic plants.

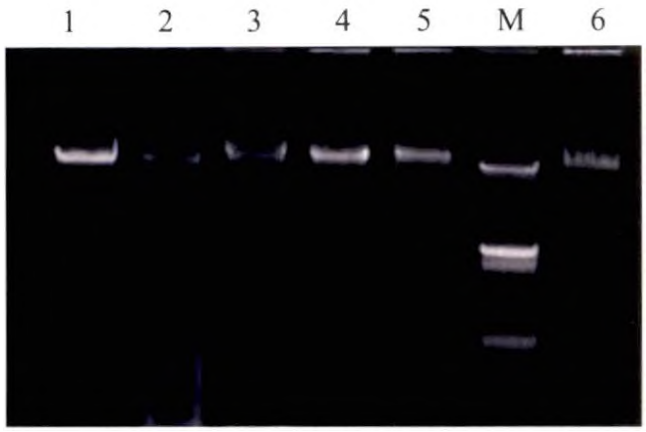
***4.2.8.1.1 Isolation of genomic DNA***

Genomic DNA from independently obtained transgenic plants and non-transformed plant was isolated using Doyle and Doyle method. Upon electrophoresis on 1.0 per cent agarose gel, intact DNA was observed in all the wells (Plate.18).

***4.2.8.1.2 PCR analysis of nptII gene***

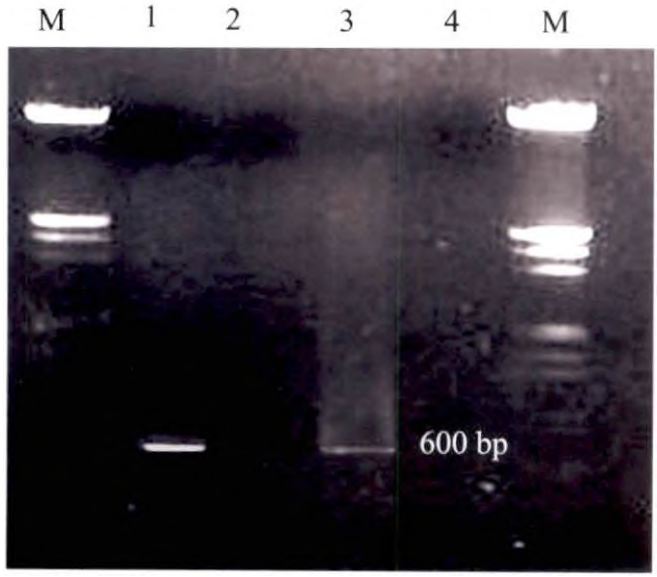
The kanamycin resistant transgenic shoots were screened by PCR. PCR was conducted with genomic DNA from transformed and from non-transformed plants as negative control. The plasmid DNA pGV2260 was used as positive control. Two specific primers derived from *npt*II gene sequences were used to detect a 600 bp fragment. The amplified DNA samples were electrophoresed on 0.7 per cent agarose gel (Plate.19). The results showed that only DNA from the transformed plants and the positive control showed amplification of the 600bp fragment. The control plants did not amplify a 600 bp fragment corresponding to *npt*II gene. Thus, PCR analysis of genomic DNA confirmed the introduction of *npt*II gene in the genome of transgenic chilli plants.

The assay revealed that out of fifty two putative transgenic plants only three showed amplification of the *npt*II gene co-migrating with amplified product from plasmid. Thus the transformation frequency assayed by PCR analysis of *npt*II gene was 5.7 per cent.




Lane 1, 2, 3, 4, 5, 6- Genomic DNA
Lane M- Molecular weight marker
(lambda DNA/*Hind*III/*Eco*RI)

Plate 18. Gel electrophoresis of genomic DNA of plants




Lane M- molecular weight marker
(lambda DNA/*Hind*III/*Eco*RI)
Lane 1- Positive control (plasmid)
Lane 2- Control (without DNA)
Lane 3- Transformed plant
Lane 4- Negative control (nontransform

Palte 19. PCR analysis of *npt* II gene

# *Discussion*

# 5. DISCUSSION

Chilli is highly valued throughout the world both as a vegetable and spice crop for its intrinsic qualities like pungency, flavour, appealing colour and nutrient content. The crop faces several biotic and abiotic stresses apart from the considerable loss due to post harvest damages. In Kerala chilli is mainly cultivated during the summer months of the year. So, it is highly prone to water stress. Besides this, the crop is susceptible to pathogens like *Ralstonia solanacearum*, *Collectotrichum capsici* and viruses. Genetic improvement by conventional breeding in this crop is not as rapid as envisaged to meet the demands of increasing population. There is an urgent need to improve several commercially grown varieties in India and elsewhere. With the rapid development of genetic engineering techniques based on the knowledge of gene structure and function, plant breeding has been dramatically broadened. Tools of genetic engineering can be exploited as an additional method for the introduction of agronomically useful traits into established cultivars. *In vitro* gene transfer is the laboratory technique of transferring refined desirable genes across taxonomic boundaries into plants and animals from other plants, animals and microbes, or even to introduce artificial, synthetic or chimeric genes into plants. To increase the tolerance to these biotic and abiotic stresses its high time that defense genes are cloned and transformed into chilli. *Agrobacterium tumefaciens* mediated transformation is an effective and widely used approach to introduce desirable genes into plants. Osmotin is a pathogenesis related protein produced by plants in response to several biotic and abiotic stresses. The *osmotin* gene is known to impart tolerance to salinity, drought and fungal attack. Hence, transformation of crops with this gene is expected to impart tolerance to biotic and abiotic stresses. In the present study an attempt was made to transform chilli (*Capsicum annuum* L.) var. Ujwala with *osmotin* gene. The results obtained in the study are discussed in this chapter based on the earlier reports and possible interpretations.

## 5.1 *IN VITRO* REGENERATION

An essential step towards development of transgenic plants through *Agrobacterium* mediated transformation is the development of an efficient and reproducible regeneration protocol. Many reports on regeneration of plants *in vitro* from various explnts of chilli via organogenesis are available (Philips and Hustenburger, 1985; Agrawal *et al.*, 1989). In chilli, the regeneration is highly genotype specific. In some cases this may vary from laboratory to laboratory (Shivegowda *et al.*, 2002). The intervarietal differences in regeneration from various explants and species are highly pronounced (Christopher and Rajam, 1996). Therefore, cultivars, species and tissue specific media have been devised to optimize regeneration for various species and cultivars.

In view of these facts, the standardization of *in vitro* regeneration technique in *Capsicum annuum* L. var. Ujwala was attempted.

### 5.1.1 Standardization of explant

The best explant for direct regeneration in *Capsicum annuum* L. var. Ujwala was found to be hypocotyls. This is in confirmation with the studies of Arroyo and Revilla (1991) in *Capsicum annuum* L. cvs. Pico and Piquillo and Cui *et al.*(2000) in snapdragon. The percentage regeneration and the average number of shoot buds per explant was higher when hypocotyl explants were used. Only the upper part of the hypocotyls showed the ability to differentiate shoot buds, whereas as roots were produced from the lower and middle regions. Similar differential morphogenic responses were observed by Fari and Czako (1981) in the capsicum cultivar "T. Hatvani". They related this phenomenon to a hypothetical gradient of endogenous growth substances along the axis between the cotyledonary node and root neck. The growth substances administered exogenously through the culture medium complemented the variable endogenous levels in step-wise shifts. Under the conditions of the present experiment, the most favourable ratio for shoot initiation occurred on the upper sections of the hypocotyl. In this respect it is better to concentrate on the gradient of natural IAA, as its relative importance in the organization of chilli has already been established (Gunay and Rao, 1978).

According to their observations, concentrations of endogenous IAA diminish from the top towards base of the hypocotyl. Concentrations of IAA were insufficient for shoot organization in the middle and base, but adequate for rooting and callus formation.

Cotyledonary explants also showed direct regeneration. Only the proximal part of the cotyledon showed the regenerative behaviour. The increased gradient of growth substances from the base of the hypocotyl could be the reason for this behaviour. In case of leaf explants, direct regeneration was very low.

It is thus quite evident that different explants vary in their regenerative behaviour. The explant cells differ in their DNA synthesis and cell division ability due to the difference in physiological maturity of the cells. The seedling hypocotyls and cotyledons are juvenile in nature. These cells are competent for regeneration and have the highest rate of cell division and DNA synthesis, as a consequence of which they show higher regeneration ability. The leaf explants showed lower regeneration ability. This may be due to the difference in physiological maturity of the tissues.

Although regeneration was obtained from the hypocotyl and cotyledonary explants, only buds that originated from hypocotyls were elongated. 50 per cent shoot elongation was obtained in MS medium containing 2 per cent sucrose (without any growth regulators). The same media for shoot elongation has been reported in bell pepper (Girija *et al.*, 2004). The buds regenerated from the cotyledon and leaf explants did not elongate in any of the media tested. Similar observations were earlier reported by Arroyo and Revilla (1991). In their study also, the shoot buds grew into rosettes with numerous well-developed leaves but did not elongate. Several attempts to elongate the rosettes, such as culture in hormone free medium, reduction of cytokinin concentration, addition of gibberellic acid or dark and cold treatment were unsuccessful. The major obstacle to chilli regeneration is the elongation of newly formed buds. This has been reported by several workers (Manoharan *et al.*, 1998; Shivegowda *et al.*, 2002; Jayapadma *et*

*al.*, 2005). Defects in shoot meristem differentiation or primordial organization is the reason for this behaviour. This justifies the observation made by Steintz *et al.* (1999) that the genus *Capsicum* is highly recalcitrant for regeneration especially at the shoot elongation stage.

Elongated shoots were easily rooted by placing them in MS medium containing 2 per cent sucrose after pulse treatment with IBA at 1000 mg $l^{-1}$. This was earlier reported by Girija *et al.* (2004) in bell pepper. Root formation is an energy demanding process and thus exogenous supply of carbohydrate is required. However, this being the last stage of *in vitro* culture it is important to transform the plant from heterotrophic to autotrophic mode of nutrition. Thus the supply of exogenous sugars should be reduced at this time. In most species, reducing sugar from 3 per cent to 2 per cent level does not make any difference (Anilkumar and Nair, 2004). Hence in the present investigation hormone free MS medium containing 2 per cent sucrose was used for rooting.

### .5.1.2 Standardization of basal medium

Different plant tissue culture media like MS medium, SH medium and B5 were used for the *in vitro* regeneration study and MS medium was found to be the best. In SH and B5 medium the percentage regeneration and the number of shoot buds per explant was less.

Generally MS media is used for the *in vitro* studies in most of the solanaceous crops. In the present study, the percentage regeneration and the number of shoot buds per explant was higher in Murashige and Skoog (MS) medium compared to SH and B5. The superiority of MS medium over other media was earlier reported in *Holostemma* (John, 1996), Gurmar (Gholba, 2000) and *Tinospora* (Kalimuthu, 2002).

### 5.1.3 Standardization of growth regulators

Growth regulator concentration in the culture is critical for morphogenesis. The ratio of auxins to cytokinins influences the balance between root and shoot

organogenesis from cultured tissues. A high cytokinin/auxin ratio results in the formation of shoots and a low cytokinin/auxin, results in roots.

MS medium supplemented with the auxin, IAA and the cytokinin, BA was used for obtaining regeneration from hypocotyl explants. Enhanced regeneration of *Capsicum annuum* L. on media containing BA and IAA was previously reported by many workers (Arroyo and Revilla, 1991; Valera-Montero and Ochoa-Alejo, 1992; Christopher and Rajam, 1996).

Benzyl adenine (BA) is an important cytokinin, widely used for inducing multiple shoot formation in many species. The inclusion of auxin in the medium was found to be beneficial for shoot production in some cases (Rajmohan, 1985). The proper balance between the auxin and cytokinin is believed to be the key factor for various patterns of *in vitro* regeneration. In the present investigation, the percentage of regeneration was higher in the medium containing BA 5.0 mg $l^{-1}$ and IAA 0.3 mg $l^{-1}$. Average number of shoot buds was also higher in this medium. The same growth regulator combination for shoot bud induction from the hypocotyl explants was earlier reported in different cultivars of Capsicum by Valera-Montero and Ochoa-Alejo (1992) and Girija *et al.* (2004). The combination with the lowest level of BA (1.0 mg $l^{-1}$) resulted in callus formation in all the cultures tested. With the increasing concentration of BA (1.0 to 5.0 mg $l^{-1}$) shoot buds were formed directly from the hypocotyl segments. When the concentration of BA (7.0 mg $l^{-1}$) was further increased there was a reduction in the percentage of regeneration. Similar effect of BA at higher concentration has been reported in *Solanum nigrum* (Jabeen *et al.*, 2005).

## 5.2 *AGROBACTERIUM* MEDIATED GENETIC TRANSFORMATION

### 5.2.1 Sensitivity of different explants to antibiotics

Selection of the transformed cells is a key factor in developing successful methods for genetic transformation, otherwise the untransformed cells would overgrow leading to the loss of transformed cells. This is done by the use of certain selectable marker genes that are present in the vector along with gene of interest.

Genes conferring resistance to antibiotics are generally used as selection markers. Once the tissue is transformed with the antibiotic resistance genes, it is grown in the medium containing antibiotic resistance genes. The cells which are transformed with the antibiotic resistance gene would multiply and grow normally, whereas, the untransformed cells are killed.

The usefulness of a particular antibiotic depends on several factors. The best selection agents are those that either inhibit growth or slowly kill the non transformed cells so that the dying cells do not overwhelm the transformed ones. Optimal selection pressure will use the lowest level of antibiotic needed to kill untransformed tissues. This necessitates the evaluation of sensitivity of plant tissue to antibiotics and the minimum concentrations of antibiotics required to inhibit growth (Cheriyan, 2000).

In the present study, sensitivity of hypocotyls and cotyledon explants of *Capsicum annuum* L. var. Ujwala to different antibiotics at different doses were evaluated.

Kanamycin is the most commonly used selecton agent for plant transformation (Owens, 1981). Sensitivity of the explants to kanamycin at five different concentrations was tested. The application of kanamycin to the regeneration medium above 50 mg $l^{-1}$ concentration strongly inhibited the development of shoot buds. Hence, kanamycin 100 mg $l^{-1}$ was selected as cut off level for the selection of transformants. In previous investigations on *Capsicum* transformation also, kanamycin resistance was used as the selection marker but kanamycin levels were different. Li *et al.* (2003) used 50 mg $l^{-1}$ kanamycin as the selection level. Manoharan *et al.* (1998) found that kanamycin at 50 mg $l^{-1}$ in the shoot induction medium and 25 mg $l^{-1}$ in the shoot elongation and rooting medium was optimum for transformation. Shiwegowda *et al.* (2002) used 100 mg $l^{-1}$, 50 mg $l^{-1}$ kanamycin in the shoot induction and rooting media respectively. The differences in selective kanamycin levels are due to the different genotypes tested. In contrast, Mihalika *et al.* (2000) reported that kanamycin resistance is not an

optimal selection marker in producing transgenic capsicum plants, and in their experiments nontransformed cotyledons could tolerate kanamycin concentration as high as 150 mg $l^{-1}$.

Rifampicin at all concentrations completely inhibited the regeneration. This indicates that the explants are sensitive to rifampicin.

Complete elimination of the bacteria from the explant after co-cultivation is very essential, otherwise it will interfere with the growth and organogenesis of the explant. Overgrowth of the bacteria causes death of the explant and disrupts the experiment. Elimination of the bacteria from the explant is done by the use of antibiotics. The antibiotic chosen should be such that it efficiently kills the bacteria, at the same time it does not affect the growth and morphogenesis of the explants. The most commonly used antibiotics for this purpose are carbenicillin and cefotaxime. However, its effect on the explants has to be studied before choosing any one of them as they are also reported to have detrimental effect on some species (Cheriyan, 2000).

The antibiotics cefotaxime and carbenicillin were not toxic to explant tissues of chilli within the range of concentrations tested. Carbenicillin induced callusing in all the explants at all the concentrations tested. Similarly callusing of the tissue induced by carbenicillin was reported in *Antirrhinum majus*, *Nicotiana* (Holford and Newbury, 1992; Pullock *et al.*, 1983). Holford and Newbury (1992) concluded that the breakdown product of carbenicillin with auxin activity, phenylactic acid, was responsible for stimulatory effects in *Antirrhinum majus* callus growth. Such a mechanism could partly explain the results observed with carbenicillin in the present study. The antibiotic cefotaxime induced shoot buds from all the explants at all concentrations tested even though the number of shoot buds per explant was less. This is in contrast to the reports in tea. In tea the cefotaxime was found toxic to the somatic embryos. The cefotaxime containing media gradually turned yellow in colour and subsequent bleaching of the tissue was noticed. However the increased organogenetic effect in tea by cefotaxime was

also reported (Tosa *et al.*, 1996). The reason for this difference may be the difference in genotype, species, explant and the source of the chemicals used for the study.

### 5.2.2 Sensitivity of *Agrobacterium* strains to antibiotics

The prerequisite steps in *Agrobacterium* mediated transformation are an efficient system to produce transformed tissue and subsequent killing of *Agrobacterium* effectively. Kanamycin is the most commonly used antibiotic for the transformation experiments (Owens, 1981). On the contrary, cefotaxime and carbenicllin have been most widely used for killing *Agrobacterium* because of their broad-spectrum bactericidal activity. The elimination of *Agrobacterium* is important because the continued presence of *Agrobacterium* can present a problem for identifying transformants or interferes with the growth and development of the transformed plant cells or cause the death of the cultures (Tang *et al.*, 2000).

Strain EHA 105 harbouring the construct p35SGUSINT was found resistant to kanamycin and rifampicin and sensitive to cefotaxime and carbenicillin at all the concentrations tested. This was expected because the construct has genes for kanamycin and rifampicin resistance.

The *Agrobacterium* strain GV 2260 contains the antibiotic resistance genes for kanamycin, rifampicin and carbenicillin and therefore, is resistant to these antibiotics even at a high concentration of 400 mg $l^{-1}$. However, it was sensitive to cefotaxime indicating that this antibiotic could be used for killing the bacteria after co-cultivation.

### 5.2.3 Standardization of *Agrobacterium* mediated transformation

*Agrobacterium* mediated genetic transformation of *Capsicum annuum* L. var. Ujwala was standardized with the *Agrobacterium* strain EHA 105 having *gus* reporter gene. Humara *et al.* (1999) and Fatima *et al.* (2005) also used the same strain for standardization of transformation experiments in *Pinus pinea* and *Capsicum* respectively. The selectable marker gene present in the T-DNA was *npt*

*II* (neomycin phosphotransferase II), which confers resistance to kanamycin. The T-DNA also harbours genes conferring resistance to rifampicin and this acts as bacteria selection agent to prevent contamination of bacterial strain. Once the T-DNA is transferred to the plant genome, the transformed plant cells are expected to develop resistance to kanamycin and will be capable of normal growth in the medium containing kanamycin, in contrast, the non-transformed cells will be susceptible to kanamycin. The transformed cells thus developed will also show GUS activity, which can be detected by histochemical GUS asay.

The bacterial gene *gusA* encoding β-gucuronidase (GUS) is the most frequently used reporter gene for the analysis of plant gene expression. There is little or no detectable endogenous β glucuronidase activity in most of the higher plants allowing the easy detection of chimaeric gene expression. Glucuronidase activity can be detected easily and sensitively. Histochemical localization is possible in cells and tissues due to the blue colour developed with the substrate X-gluc (5-bromo-4-chloro-3-indolyl glucuronide). The substrate is cleaved by the enzyme within the plant cells (transformed/transgenic), and the product is converted into an insoluble blue precipitate at the site of enzyme activity. The colour so developed is visible to the naked eye and even under microscope in sectioned tissues.

*Agrobacterium* strain EHA 105 contains the plasmid p35SGUSINT. The *gus*A gene present in the plasmid has the PIV2 intron of the gene ST-L1 from potato within its coding sequence, preventing its expression in *Agrobacterium*. Early detection of transformants is invariably based on the histochemical analysis of expression of the gus as a reporter gene. A major limitation to this approach is the expression of this reporter gene in *Agrobacterium* despite the use of plant promoters. The problem was overcome by inserting introns, which are processed in plants but not in *Agrobacterium*. Vancanneyt *et al.* (1990) placed the intron ST-LS1 gene in the coding sequence of gus gene. In another study, Ohta *et al.* (1990) placed the castor bean catalase gene intron within the N-terminal part of the coding

sequence of gus. Thus the GUS activity was limited to transformed tissues and not detected in *Agrobacterium*.

### *5.2.3.1 Standardization of inoculum density*

Concentration of bacterial cells in the inoculation medium is an important factor to be considered for efficient transformation. Among the different inoculum densities tried, $OD_{600}$ nm = 0.1 was found optimum(Fig. 3). Transient GUS expression after 3 days of co-cultivation showed more blue areas when bacterial density was 1.0 ($OD_{600}$ nm). But none of the transformed explants were survived after 60 days. This is in confirmity with the observations of Humara *et al.* (1999). Very high bacterial density leads to necrosis and death of the explant. This is due to the hypersensitive response of the tissue as a part of the plant defense against pathogen.

### *5.2.3.2 Standardization of infection time*

The time of infection of hypocotyl explants of chilli var. Ujwala was standardized with *gus* construct. The infection times viz, 2, 5, 10 and 15 minutes were tried and 5 minutes was found optimum (Fig. 4). Sarmento *et al.* (1992) also reported that an infection time of 5 minutes was optimum for transformation of pickling cucumber. With the prolonged exposures, explants became necrotic and died or were colonized by *Agrobacterium* even in the presence of 500 mg $l^{-1}$ carbenicillin. In the present study, there was a positive GUS expression in all the infection times tested. The low survival rate in an infection time of 2 minutes is due to the low rate of transformation. Low survival rate of the tissues in 10 and 15 minutes infection time is due to the hypersensitive response of the tissue.

### *5.2.3.3 Standardization of co-cultivation period*

Co-cultivation period plays an important role in the success of the transformation. It is during this period that the *vir* genes are activated and the T-DNA transferred into the plant cell. However, increasing the co-cultivation period might lead to necrosis and death of the explant due to the hypersensitive response of the tissue. Hence the length of co-cultivation period should always be the

shortest interval necessary to obtain the maximum frequency of transformation in terms of the number of transgenic plants recovered following co-cultivation.

In the present study, a co-cultivation period of 48 h was the best for transformation when compared to 0, 24, 72 and 96h (Fig. 5). These differences could be explained by the fact that the concentration of *Agrobacterium* was optimum during 48 h co-cultivation than after 24 h and that could considerably increase the probability of gene transfer. Similar results were also reported by Pawlicki *et al.* (1992) in carrot.

Bacterial cells multiplied in the co-culture medium and after 48 h, the optimum quantity of bacteria were available for transformation and hence a higher survival rate in the selection medium was achieved. After 48 h, the level of bacterial cells reached supra optimum level and competitive inhibition of competitive bacterial cells resulted in inhibition of transformation. This accounts for the low survival rate of explants after 48 h of co-cultivation (Hari, 2001).

#### *5.2.3.4 Histochemical GUS assay*

β-glucuronidase enzyme activity was used to monitor the transformation. Blue staining was evident when the explants were examined 3 days after co-cultivation with EHA 105. Microscopic examination showed that indigo dye precipitates were localized within cells. No blue sectors were present when the explants were examined 2 months after co-cultivation. This may be due to several factors including transient GUS expression, lack of division of transformed cells or sensitivity to kanamycin under these conditions.

### 5.2.4 Transformation with *osmotin* gene

The *Agrobacterium* strain GV2260 carrying the plasmid pGV2260 was used as the vector system for transformation. The plasmid contains osmotin gene tagged with 35S *CaMV* promoter. The selectable marker gene present in the T-DNA is *npt II* gene which confers resistance to kanamycin. Besides, these genes mentioned above, the T-DNA also harbours genes, which confers resistance to

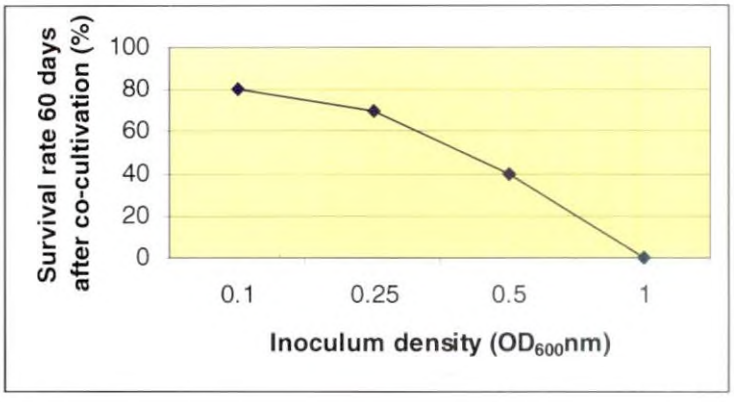



Fig. 2. Effect of inoculum density on survival rate of transformed explants

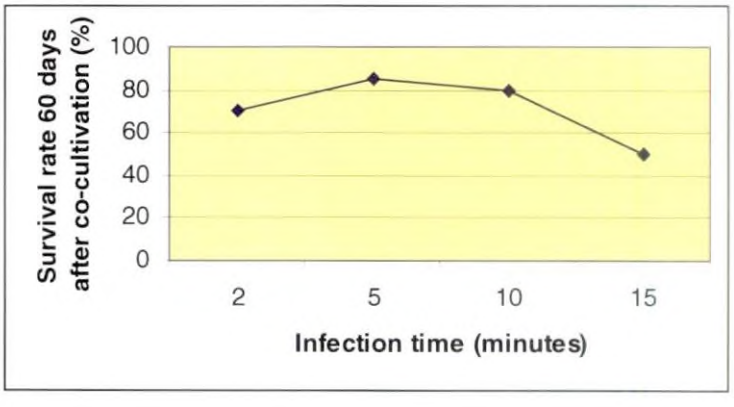



Fig.3. Effect of infection time on survival rate of transformed explants

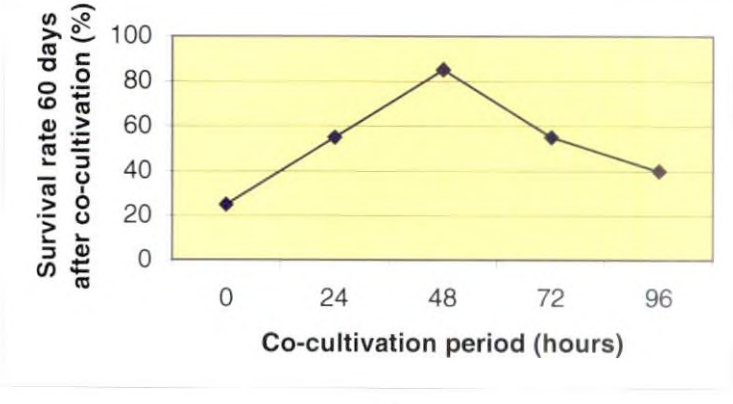



Fig. 4. Effect of co-cultivation period on survival rate of transformed explants

carbenicillin and rifampicin and acts as a bacterial selection gene to prevent contamination of the bacterial strain. Once the T- DNA is transferred to the plant genome the plant cells are expected to develop resistance to kanamycin and will be capable of normal growth in the medium containing kanamycin, in contrast, the non-transformed cells will be susceptible to kanamycin.

The explants after co-cultivation was transferred to preselection and then to selection medium.The transformed shoot buds were maintained in continuous antibiotic medium. The growth was very slow, compared to the non co-cultivated cells. Similar observations were made by Belarmino and Mii (2000) and Swarnapiriya and Rajmohan (2005) in orchids. The transformed shoot buds did not show any elongation in the elongation medium containing selection agent. Hence, the shoot buds were then transferred to the elongation medium without any selection agent. Even in this medium, no elongation was observed even after 60 days. Prolonged culture of transformed shoot buds in selection medium lead to vitrification of shoot buds. The major obstacle to chilli pepper plant transformation and regeneration has been the lack of elongation of newly formed buds. The frequency of bud elongation reported in literature was generally too low to permit production of respectable amount of transformants (Liu *et al.*, 1990; Jayapadma *et al.*, 2005). The failure of elongation of the transformed buds may be due to inhibitory effect of kanamycin on plants.

Jun *et al.* (1995) during their transformation work in Chinese cabbage had to transfer the explants soon after induction of shoots from screening media to rooting medium without kanamycin, as kanamycin inhibited root formation.

During transformation of passion fruit, Manders *et al.* (1994) found that using 86μM of kanamycin in screening media allowed good percentage of untransformed shoots and hence had to raise the concentration to 172 μM. However, prolonged culture of regenerated shoots in a medium with 172 μM

kanamycin caused vitrification of shoots. Hence after initial screening the regenerated shoot were grown at 86 μM of kanamycin.

### *5.2.4.1 Confirmation of transformation*

To assess whether these shoot buds were transformed or not, PCR analysis of the *npt II* gene was done. Polymerase Chain Reaction (PCR) is a powerful technique for confirming DNA insertion in transgenic plants. Primers can be designed which simultaneously amplify specific regions on the T-DNA that are expected to be integrated into the genome of plants. Advantages include the rapid manner in which large collections of transgenic plants can be analysed and the very small amount of plant tissues required (Lassner *et al.*, 1989). The forward and reverse primers of *nptII* gene were designed in such a manner that PCR amplification using these primers will amplify a 600bp fragment corresponding to *nptII* gene. The presence of a 600bp fragment confirms the transgenic nature. This indicated that the plasmid gene was successfully integrated into the genome of the chilli plant. The plasmid DNA was used as the positive control as it contained the construct having *nptII* gene.

The assay revealed that out of the fifty two plants only three showed the amplification of *nptII* gene. This indicates that even after growing the infected explant in selection media containing suitable selectable markers, some untransformed cells may also show growth (escapes or false positives). This could be because of the cross protection provided by the transformed cells. Hence all tissues showing growth in the selective medium cannot be considered as transgenic and further confirmatory tests are to be done to confirm the transfer and stable integration of the foreign genes.

The presence of a selectable marker allows selection of transgenic cells but does not guarantee 100 co-transmission and/or expression of the other genes on the same section of T-DNA. Independently selected transgenic plants often show varying degrees of gene expression. Several factors other than the absolute effects

of the regulatory sequences can influence the magnitude of gene expression. These include copy number, position effects resulting from the site of insertion and methylation of the transferred genes. Hence molecular analysis of transformed plants using Southern hybridization, RT-PCR analysis and Western blotting are to be done to know the stable integration and expression of transgenes.

# *Summary*

# 6. SUMMARY

The study entitled 'genetic transformation of chilli (*Capsicum annuum* L.) with osmotin gene' was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara. The variety Ujwala was used for the study. The salient features of the study are summarized below:

1. Among the different explants tried for *in vitro* regeneration, the hypocotyl explants showed highest percentage of regeneration and the average number of shoot-buds per explant. Only the shoot buds originated from hypocotyl explants showed elongation. Shoot buds originated from the cotyledon and leaf explants remained in rosette form and did not elongate.

2. MS basal medium was found to be the best for *in vitro* regeneration in chilli, followed by SH and B5.

3. The growth regulator combination, BA (5.0 mg $l^{-1}$) and IAA (0.3 mg $l^{-1}$) produced a higher percentage of regeneration and maximum number of shoot-buds.

4. Kanamycin at 100 mg $l^{-1}$ completely inhibited the regeneration of explants. Hence it is selected for screening.

5. The antibiotic rifampicin completely inhibited the regeneration of explants at all the concentrations tested.

6. Carbenicillin induced callusing at all concentration tested.

7. The antibiotic cefotaxime induced shoot-buds at all concentrations tested and a concentration of 200 mg $l^{-1}$ was selected for transformation experiments.

8. Two *Agrobacterium* strains EHA 105 and GV 2260 were used for the transformation experiments. Strain EHA 105 was found sensitive to cefotaxime and carbenicillin and resistant to kanamycin and rifampicin. Strain GV 2260 was sensitive to cefotaxime and resistant to kanamycin, rifampicin and carbenicillin. Hence, cefotaxime was used for the elimination of bacteria from the transformed explants.

9. Morphological and cultural caharacterestics of the *Agrobacterium* was studied on YEM medium. Bacterial colonies were round with smooth margin, convex, colourless and mucoid in nature. *Agrobacterium* is gram negative and rod shaped when viewed under microscope.

10. Binary vector constructs were isolated from the *Agrobacterium* strains to confirm its presence. A single plasmid DNA of molecular weight approximately 14 kb was obtained from both strains.

11. The genetic transformation was standardized with the *Agrobacterium* strain EHA 105. Among the different infection times tested, 5 minutes was found optimum. The survival rate of explants 60 days after infection was higher when 5 minutes infection time was tested.

12. The inoculum density $OD_{600}$nm 0.1 was found optimum for transformation.

13. A bacterial co-culture period of 48h was optimum for transformation studies in chilli var. Ujwala.

14. Transient GUS assay revealed blue coloured specks on the transformed plants when viewed under microscope. Control plants did not develop blue colour.

15. The transformation of chilli with *osmotin* gene was done using the *Agrobacterium* strain GV 2260. The transformation was confirmed by PCR analyses of *nptII* gene. The results showed that only DNA from the

transformed plants and the positive control showed amplification of the 600bp fragment. The control plants did not amplify a 600 bp fragment corresponding to *npt*II gene. The transformation frequency assayed by PCR analysis of *npt*II gene was 5.7 per cent.

16. The transformed shoot buds did not elongate in the selection medium due to the inhibitory effect of kanamycin. Prolonged culture of shoot-buds in selection medium lead to vitrification of the tissue.

# References

# REFERENCES


Abdin, M.Z. 2005. Development of transgenic chicory (*Chicorium intybus* L.) through *Agrobacterium* mediated genetic transformation with osmotin gene. *In vitro Application in Crop Improvement* (ed. Mujib, M.). CSIRO Publishing, Collingwood, Australia, pp. 134-135

Agrawal, S., Chandra, N. and Kothari, S.L. 1989. Plant regeneration in tissue cultures of pepper (*Capsicum annuum* L. cv. Mathania). *Pl. Cell Tiss. Org. Cult.* 16: 47-55

An, G., Waston, B.D. and Chang, C.C. 1986. Transformation of tobacco, tomato, potato and *Arabidopsis thaliana* using vector system. *Pl. Physiol.* 81: 301-305

Anilkumar, M. and Nair, A.S. 2004. Multiple shoot induction in *Capsicum annuum* L.cv.Early California Wonder. *Pl. Cell Biotech. mol. Biol.* 5: 95-100

Archiletti, T., Lauri, P. and Damiano, C. 1995. *Agrobacterium* mediated transformation of almond leaf pieces. *Pl. Cell Rep.* 14: 267-272

Arroyo, R. and Revilla, M.A. 1991. *In vitro* plant regeneration from cotyledon and hypocotyl segments in two bell pepper cultivars. *Pl. Cell Rep.* 10: 414-416

Barthakur, S., Babu, V. and Bansal, K.C. 2001. Over-expression of osmotin induces proline accumulation and confers tolerance to osmotic stress in transgenic tobacco. *J. Pl. Biochem. Biotech.* 10: 31-37

Battraw, M.J. and Hall, T.C. 1990. Histochemical analysis of *CaMV 35S* promoter-β glucuronidase gene expression in transgenic rice plants. *Pl. mol. Biol.* 15: 527-538

Belarmino, M.M. and Mii, M. 2000. *Agrobacterium* mediated genetic transformation of a Phalenopsis orchid. *Pl. Cell Rep.* 19: 435-442

Bevan, M.W. 1984. Binary *Agrobacterium* vectors for plant transformation. *Nucleic Acid Res.* 12: 8711-8721

Bevan, M. W., Barnes, W. and Chilton, M.D. 1983a. Structure and transcription of the nopaline synthase gene region of T-DNA. *Nucleic Acid Res.* 11: 369-385

Bevan, M.W., Flavell, R.B. and Chilton, M.D. 1983b. A chimaeric antibiotic resistance gene as a selectable marker for plant cell transformation. *Nature* 304: 184-187

Binzel, M.L., Sankhla, N., Joshi, S. and Sankhla, D. 1996a. Induction of direct somatic embryogenesis and plant regeneration in pepper (*Capsicum annuum* L.). *Pl. Cell Rep.* 15: 536-540

Binzel, M.L., Sankhla, N., Joshi, S. and Sankhla, D. 1996b. *In vitro* regeneration in chilli pepper (*Capsicum annuum* L.) from 'half seed explants'. *Pl. Growth Regulation* 20: 287-293

Birnhoim, H.C. and Doly, J. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acid Res.* 7: 1513-1523

Bol, J.F., Linthorst, H.J.M. and Cornellssen, B.J.C. 1990. Plant Pathogenesis related proteins induced by virus infection. *A. Rev. Phytopathol.* 28: 113-138

Brederobe, F.T., Linthorst, H.J.M. and Bol, J.F. 1991. Differential induction of acquired resistance and PR gene expression in tobacco by virus infection, ethephone treatment, UV light and wounding. *Pl. mol. Biol.* 17: 1117-1125

Broeck, V.G., Timke, M.P., Kansch, A.P., Cashmore, A.R. and Herrera-Estrella, L. 1985. Targeting of a foreign protein to chloroplasts by a fusion to transit peptide of ribulose-1,5 biphosphate carboxylae. *Nature* 313: 358-363

Buyukalaca, S. and Mavituana, F. 1996. Somatic embryogenesis and plant regeneration of pepper in liquid media. *Pl. Cell Tiss. Org. Cult.* 46: 227-235

Buyukalaca, S., Mavituana, F. and Gomez-Guillamdu, M.L. 1995. Artificial seeds of pepper somatic embryos. *Acta Hort.* 412: 106-110

Bytebier, B., Deboech, F., De Geeve, H., Van Montagu, M. and Hernalsteens, J.P. 1987. T-DNA organization in tumour cultures and transgenic plants of the monocotyledon *Asparagus officinals. Proc. nat. Acad. Sci. USA* 84: 5345-5349

Callis, J., Fremm, M. and Walbot, V. 1987. Introns increase gene expression in cultured maize cells. *Genes Dev.* 1: 1183-1200

Cervera, M., Pina, J.A., Juarez, J., Navarro, L. and Pina, L. 1998. *Agrobacterium* mediated transformation of citrange: factors affecting transformation and regeneration. *Pl. Cell Rep.* 18: 271-278

Chawla, H.S. 2002. *Introduction to Plant Biotechnology.* Second edition. Oxford and IBH Publishing Co. Pvt. Ltd., New Delhi, 538p.

Cheriyan, H. 2000. *Agrobacterium* mediated genetic transformation in black pepper (*Piper nigrum* L.). M Sc. (Ag.) thesis, Kerala Agricultural University, Thrissur, 125p.

Christopher, T. and Rajam, M.V. 1994. *In vitro* clonal propagation of *Capsicum* spp. *Pl. Cell Tiss. Org. Cult.* 38: 25-29

Christopher, T. and Rajam, M.V. 1995. *Agrobacterium* mediated genetic transformation of red pepper. *In vitro Pl.* 31: 222

Christopher, T. and Rajam, M.V. 1996. Effect of genotype, explant and medium on *in vitro* regeneration of red pepper. *Pl. Cell Tiss. Org. Cult.* 46: 245-250

Clercq, J.D., Zambre, M., Van-Montagu, M., Dillen, W. and Angenon, G. 2002. An optimized *Agrobacterium*-mediated transformation procedure for *Phaseolus acutifolius* H. *Pl. Cell Rep.* 21: 333-340

Coates, D., Talierco, E.W. and Gelvin, S.B. 1987. Chromatin structure of integrated T-DNA in crown gall tumors. *Pl. mol. Biol.* 8: 159-168

Comai, L., Faciolotti, D., Heatt, W.R., Thompson, G., Rose, R.E. and Stalker, D.M. 1985. Expression in plants of a mutant *aro A* gene from *Salmonella typhimurium* conifers tolerance to glyphosate. *Nature* 317: 741-744

Cui, M. T., Takayanagi, K. and Handr, T. 2004. High frequency of shoot regeneration from hypocotyls and stem segments of *Antirrhinum majus* (snapdragon). *Pl. Cell Tiss. Org. Cult.* 78: 51-53

De-Block, M., De-Breuwer, D. and Tenning, P. 1989. Transformation of *Brassica napus* and *Brassica oleracea* using *Agrobacterium tumefaciens* and the expression of the bar and neo-genes in the transgenic plants. *Pl. Physiol.* 91: 694-701

Decleene, M. and De Leney, J. 1976. The host range of crown gall. *Bot. Rev.* 42: 389-466

De-Framond, A.J., Back, E.W., Chitton, W.S., Kayes, L. and Chitton, M.D. 1986. Two unlinked T-DNAs can transform the same tobacco plant cell and segregate in the F1 generation. *Mol. Gen. Genet.* 202: 125-131

De-Greve, H., Dhaese, P., Sevrinck, J., Lemmers, M., Van Montagu, M. and Schell, J. 1982. Nucleotide sequence and transcript map of the

*Agrobacterium tumefaciens* Ti plasmid encoded octopine synthase gene. *J. mol. appl. Genet.* 1: 499-511

Dekeyser, R., Claes, B., Marchal, M., Van Montagu, M. and Caplan, A. 1989. Evaluation of selectable markers for rice transformation. *Pl. Physiol.* 90: 217-223

*Dong, C.Z., Jiang, C.X., Feung, L.X. and Guo, J.Z. 1992. Transgenic pepper plants (*Capsicum annuum* L.) containing CMV sat RNA cDNA. *Acta Hort. Sinica* 19: 184

Doyle, J.J. and Doyle, J.L. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem. Bull.* 19:11-15

Eady, C.C., Wolf, R.J. and Lister, C.E. 2000. *Agrobacterium tumefaciens* mediated transformation and transgenic plant regeneration of onion (*Allium cepa* L.). *Pl. Cell Rep.* 8: 16-20

Eapen, S. and George, L. 1994. *Agrobacterium tumefaciens* mediated gene transfer in pea nut (*Arachis hypogaea* L.). *Pl. Cell Rep.* 13: 582-586

Ediba, A.I.A. and Hu, C.Y. 1993. *In vitro* morphogenetic responses and plant regeneration from pepper (*Capsicum annuum* L. cv. Early California Wonder) seedling explants. *Pl. Cell Rep.* 13: 107-110

Ellis, D., Roberts, D., Sutton, B., Lazaroof, W., Webb, D. and Flinn, B. 1989. Transformation of white spruce and other conifer species by *Agrobacterium tumefaciens. Pl. Cell Rep.* 8: 16-20

Engstrom, P., Zambryski, P., Van Montagu and Stachel, S. 1987. Characterization of *Agrobacterium tumefaciens* virulence proteins induced by the plant factor acetosyringone. *J. mol. Biol.* 197: 635-645

Ezura, H., Nishimiya, S. and Kasumi, M. 1993. Efficient regeneration of plants independent of exogenous growth regulator in bell pepper (*Capsicum annuum* L.). *Pl. Cell Rep.* 12: 676-680

Fari, M. and Czako, M. 1981. Relationship between position and morphogenetic response of pepper hypocotyl explants cultured *in vitro*. *Scient. Hort.* 15: 207-213

Fari, M. and Tury, Z. 1990. Comparative studies on *in vitro* regeneration of seedling explants in chilli pepper (*Capsicum annuum* L.). *Acta Hort.* 280: 131-132

Fari, M., Nagy, I., Csanyi, M., Mytiko, J. and Andrafalvy, A. 1995. *Agrobacterium* mediated genetic transformation and plant regeneration via organogenesis and somatic embryogenesis from cotyledon leaves in egg plant (*Solanum melongena* L. cv. Kecskemet; lila). *Pl. Cell Rep.* 15: 82-86

Fatima, A.G., Girija, D., Meera, C.S., Nazeem, P.A. and Joseph, L. 2005. *Agrobacterium* mediated genetic transformation in bell pepper (*Capsicum annuum* L.). *Proceedings of the ICAR National Symposium on Biotechnological Interventions for improvement of Horticultural Crops: Issues and Strategies, January 10-12, 2005* (eds. Rao, G.S.L.H.V.P., Nazeem, P.A., Girija, D., Keshavachandran, R., Joseph, L. and John, P.S.). Kerala Agricultural University, Thrissur, pp.348-349

Fillatti, J.A., Kiser, J., Rose, R. and Comai, L. 1987. Efficient transfer of glyphosate tolerance gene into tomato using a binary *Agrobacterium tumefaciens* vector. *Biotechnology* 5: 726-730

Fischhoff, D.A., Bowdish, K.S., Perlak, F.J., Marrone, P.G., Mc Cornick, S., Niedermeyer, J.G., Dean, D.A., Kusanokretzmer, K., Mayer, E.J., Rochester, D.E., Roger, S.G. and Fraley, R.T. 1987. Insect tolerant transgenic tomato plants. *Biotechnology* 5: 807-813

Fraley, R.T., Rogers, S.G., Harsch, R.B., Sanders, P.R., Flick, J.S., Adams, S., Bittner, M., Brand, L., Fink, C.L., Fey, J., Gallupi, G., Goldberg, S., Hoffman, N.L. and Woo, S. 1983. Expression of bacterial gene in plant cells. *Proc. nat. Acad. Sci. USA* 80: 4803-4807

Franck-Duchenne, M., Wang, Y., Tahar, S.F. and Brachy, R.N. 1998. *In vitro* stem elongation of sweet pepper in media containing 2,4-epibrassinolide. *Pl. Cell Tiss. Org. Cult.* 53: 79-84

Gamborg, O.L. and Shyluk, J.P. 1981. Nutrition media and characterestics of plant cell and tissue culture. *Plant Tissue Culture: Methods and Application in Agriculture* (ed. Thorpe, T.A.). Academic Press, New York, pp.21-44

Gamborg, O.L., Miller, R. A. and Ojima, K. 1968. Nutrient requirements of suspension cultures of soybean root cells. *Exp. Cell Res.* 50: 148-151

Gholba, D. 2000. *In vitro* callus induction in Gumar (Gymnema sylvestre R. Br.) for secondary metabolite synthesis. MSc. (Ag.) thesis, Kerala Agricultural University, Thrissur, 151p.

Girija, D., Fatima, A.G., Kuriakose, L.S., Nazeem, P.A., Joseph, P.L., Indira, P., Beena, P.S. and Shaju, K.V. 2004. A viable protocol for direct regeneration of bell pepper (*Capsicum annuum* L.) cv. California wonder. *Capsicum Eggplant Newsl.* 23: 97-100

Gunay, A.L. and Rao, P.S. 1978. *In vitro* plant regeneration from hypocotyl and cotyledon explants of red pepper (*Capsicum*). *Pl. Sci. Lett.* 11: 365-372

Gupta, C.G., Lakshmi, N. and Srivalli, T. 1998. Micropropagation studies on male sterile line of *Capsicum annuum* L. at Naganna University. *Capsicum Eggplant Newsl.* 17: 42-45

Hari, K.S. 2001. Genetic transformation and hairy root culture in ada-kodien (*Holostemma adakodien* K.Suchum). MSc. (Ag.) thesis, Kerala Agricultural University, Thrissur, 137p.

Harini, I. and Sita, G.L. 1993. Direct somatic embryogenesis and plant regeneration from immature embryos of chilli (*Capsicum annuum* L.). *Pl. Sci.* 89: 107-112

Helmer, G., Casadaban, M., Bevan, M., Kayes, L. and Chilton, M.D. 1984. A new chimeric gene as a marker for plant transformation. The expression of *Escherichia coli* β galactosidase in sunflower and tobacco cells. *Biotechnology* 2: 520-527

Herrera-Estrella, L., Depicker, A., Van-Montagu, M. and Schell, J. 1983. Expression of chimeric genes transferred into plant cells using a Ti plasmid-derived vector. *Nature* 303-209-213

Hinchee, M.A.W., Connor-Ward, D.V., Nowell, C.A., Mc Donnel, R.E., Sato, S.J., Gasser, C.S., Fischheff, D.H., Re, D.B., Fraley, R.T. and Harsch, R.B. 1988. Production of transgenic soybean plants using *Agrobacterium* mediated DNA transfer. *Biotechnology* 6: 915-922

Holford, P. and Newbury, H. J. 1992. The effect of antibiotics and their breakdown products on the *in vitro* growth of *Antirrhinum majus*. *Pl. Cell Rep.* 11: 93-96

Horsch, R.B., Fry, J.E., Hoffmann, N.L., Eichholtz, D., Rogers, S.G. and Fralley, R.T. 1985. Transferring genes into plants. *Science* 227: 1229-1231

Humara, J.M., Lopez, M. and Ordas, R.J. 1999. *Agrobacterium tumefaciens* mediated transformation of *Pinus pinea* L. cotyledons: an assessment of

factors influencing the efficiency of *Uid* A gene transfer. *Pl. Cell Rep.* 19: 51-58

Iannamico, V., Perrone, D., Alessandro, A.D. and Rotino, G.L. 1993. Preliminary results on regeneration and genetic transformation of *Solanum sodomeum*. *Capsicum Eggplant Newsl.* 12: 90-93

Jabeen, F.T.Z., Venugopal, R.B., Kiran, G., Kaviraj, C.P. and Rao, S. 2005. Plant regneration and *in vitro* flowering from leaf and nodal explants of *Solanum nigrum* L.- an important medicinal plant. *Pl. Cell Biotech. mol. Biol.* 6: 17-22

Jayapadma, P.N., Bhat, S. and Kuruvinashetti, M.S. 2005. Transformation studies in chilli (*Capsicum annuum*). *Proceedings of the ICAR National Symposium on Biotechnological Interventions for improvement of Horticultural Crops: Issues and Strategies, January 10-12, 2005* (eds. Rao, G.S.L.H.V.P., Nazeem, P.A., Girija, D., Keshavachandran, R., Joseph, L. and John, P.S.). Kerala Agricultural University, Thrissur, pp. 346-347

Jayashree, R., Rekha, K., Venkatachalam, P., Uratsu, S.L., Dandekar, A.M., Jayasree, P.K., Kala, R.G., Priya, P., Kumari, S.S., Sobha, S., Ashokan, M.P., Sethuraj, M.R. and Thulaseedharan, A. 2003. Genetic transformation and regeneration of rubber tree (*Hevea brasiliensis* Muell. Arg) transgenic plants with a constitutive version of an antioxidative stress superoxide dismutase gene. *Plant Cell Rep.* 22: 201-209

Jefferson, R.A., Kavanagh, T.A. and Bevan, M.W. 1987. GUS Fusion: ß-glucuronidase on a sensitive and versatile gene fusion marker in higher plants. *EMBO J.* 6: 3901-3907

John, S.M. 1996. Standardisation of *in vitro* techniques for rapid multiplication of *Holostemma annulare* K. Schum. MSc. (Ag.) thesis, Kerala Agricultural University, Thrissur, 234p.

Jones, J.D.G., Dunsmwi, P. and Bedbrook, J. 1985. High level expression of introduced genes in regenerated transformed plants. *EMBO J.* 4: 2411-2418

Jones, J.D.G., Svab, Z., Harper, E.C., Hurwitz, C.D. and Maliga, P. 1987. A dominant nuclear streptomycin resistance marker for plant cell transformation. *Mol. Gen. Genet.* 210: 86-91

Jun, S.I., Kuwon, S.Y., Park, K.Y. and Park, K.H. 1995. *Agrobacterium* mediated genetic transformation and regeneration of fertile plants of Chinese cabbage (*Brassica campestris* spp. Pekinensis cv.Spring flavour). *Pl. Cell Rep.* 14: 620-625

*Kahl, G. 1982. Molecular biology of wound healing the conditioning phenomenon. *Molecular Biology of Plant Tumours* (eds. Kahl, G. and Schell, J.). Academic Press, New York, pp. 211-267

Kalimuthu, M. 2002. Utilization of *in vitro* cultures of *Tinospora cordifolia* Miers. (chittamruthu) for beriberin. MSc. (Ag.) thesis, Kerala Agricultural University, Thrissur, 111p.

Kim, K., Lee, Y., Kim, D., Park, Y., Lee, J., Hwang, Y. and Kim, Y. 2004. *Agrobacterium* mediated genetic transformation in *Perilla frutescens*. *Pl. Cell Rep.* 23: 386-390

Kim, S., Kim, S.Y., An, C.S., Hony, Y.N. and Lee, K.W. 2001. Constitutive expression of Rice MADS box gene using seed explants in hot pepper (*Capsicum annuum* L.). *Molecules Cells* 12: 221-226

Kim, S.J., Lee, S.J., Kim, B.D. and Paek, K.H. 1997. Satellite RNA mediated resistance to cucumber mosaic virus in transgenic plants of hot pepper (*Capsicum annuum* cv. Golden Tower). *Pl. Cell Rep.* 16: 825-830

Kononowicz, A.K., Nelson, D.E., Singh, N.K., Hasegawa, P.M. and Bressan, R.A. 1992. Regulation of the osmotin promoter. *Pl. Cell* 4: 513-524

Koukolikova-Nicola, Z., Shillito, R.D., Hohn, B., Wang, K., Van-Montagu, M. and Zambryski. 1985. Involvement of circular intermediates in the transfer of T-DNA from *Agrobacterium tumefaciens* to plant cells. *Nature* 313: 191-196

Kumar, A., Miller, M., Whitty, P., Lyon, J. and Davie, P. 1995. *Agrobacterium* mediated transformation of five wild *Solanum* species using *in vitro* microtubers. *Pl. Cell Rep.* 14: 324-328

La-Rosa, P.C., Chen, L., Nelson, D.E., Singh, N.K., Hasegawa, P.M. and Bressan, R.A. 1992. Osmotin gene expression is post transcriptionally regulated. *Pl. Physiol.* 100: 409-415

La-Rosa, P.C., Handa, A.K., Hasegawa, P.M. and Bressan, R.A. 1985. Absicic acid accelerated adaptation of cultured tobacco cells to salt. *Pl. Physiol.* 79: 138-142

La-Rosa, P.C., Hasegawa, P.M., Rhodes, D., Clithern, J.M., Watad, A.E.A. and Bressan, R.A. 1987. Absicic acid stimulated osmotic adjustment and its involvement in adaptation of tobacco cells to NaCl. *Pl. Physiol.* 85: 174-185

Lassner, M.W., Petersen, P. and Yoder, J.I. 1989. Simultaneous application of multiple DNA fragments by polymerase chain reaction in the analysis of transgenic plants and their progeny. *Pl. mol. Biol. Rep.* 7: 116-128

Le, Q.V., Bogusz, D., Gherbi, H., Lappartient, A., Duhoux, E. and Franche, C. 1996. *Agrobacterium tumefaciens* gene transfer to *Casuarina glauca*, a tropical nitrogen fixing tree. *Pl. Sci.* 118: 57-69

Lemmers, M., De Beuckeleer, M., Holsters, M., Zambryski, P. and Depicker, A. 1980. Internal organisation, boundaries and integration of Ti plasmid DNA in nopaline crown gall tumours. *J. mol. Biol.* 144: 353-376

Li, D., Zhao, K., Xie, B., Zhang, B. and Luo, K. 2003. Establishment of a highly efficient transformation system for pepper (*Capsicum annuum* L.). *Pl. Cell Rep.* 21: 785-788

Li, R., Wu, N., Fan, Y. and Song, B. 1999. Transgenic potato plants expressing osmotin gene inhibits fungal devlopment in inoculated leaves. *Chin. J. Biotech.* 15: 71-75

Lichtenstein, C. 1987. Bacteria conjugate with plants. *Nature* 328: 108-109

Lin, W., Parrott, W.A., Hilderbrand, D.F., Collins, G.B. and Williams, E.G. 1990. *Agrobacterium* induced gall formation in bell pepper (*Capsicum annuum* L.) and formation of shoot like structures expressing introduced genes. *Pl. Cell Rep.* 9: 360-364

Lindsey, K. 1992. Genetic manipulation of crop plants. *J. Biotech.* 26: 1-28

Linthorst, H.J.M. 1991. Pathogenesis related proteins of plants. *Crit. Rev. Pl. Sci.* 10: 123-150

Liu, W., Parrot, W.A., Hilderbrand, D.F., Collins, G.B. and Williams, E.G. 1990. Agrobacterium induced gall formation in bell pepper (*Capsicum annuum* L.) and formation of shoot like structures expressing introduced genes. *Pl. Cell Rep.* 9: 360-364

Manders, G., Otoni, W.C., Vaz, F.B., Blackhell, N.W., Power, J.B. and Davey, M.R. 1994. Transformation of passion fruit (*Passiflora edulis* fv. Flavcarps Degener) using *Agrobacterium tumefaciens*. *Pl. Cell Rep.* 13: 697-702

Manoharan, M., Vidya, C.S.S. and Sita, G.L. 1998. *Agrobacterium* mediated genetic transformation in hot chilli (*Capsicum annuum* L. var. Pusa Jwala). *Pl. Sci.* 131: 77-83

Mathew, D. 2002. *In vitro* shoot and root morphogenesis from cotyledon and hypocotyl explants of hot pepper cultivars Byadagi Dabbi and Arka lohit. *Capsicum Eggplant Newsl.* 21: 69-72

Matzke, A.J.M. and Chilton, M.D. 1981. Site-specific insertion of genes into T-DNA of the *Agrobacterium* tumor inducing plasmid: an approach to genetic engineering of higher plant cells. *J. mol. appl. Genet.* 1: 39-49

Mavitiuana, F. and Buyukalaca, S. 1996. Somatic embryogenesis of pepper in bioreactors: a study of bioreactor type and oxygen uptake rates. *Appl. Microbiol. Biotech.* 46: 327-333

Mc-Cormick, S., Niedermeyer, J., Fry, J., Branson, A., Horsch, R. and Fralley, R. 1986. Leaf transformation of cultivated tomato (*Lycopersicon esculentum*) using *Agrobacterium tumefaciens. Pl. Cell Rep.* 5: 81-84

Metz, T.D., Dixit, R. and Earle, E.D. 1995. *Agrobacterium tumefaciens* - mediated transformation of broccoli (*Brassica oleracea* var. italica) and cabbage (*B. oleracea* var. capitata). *Pl. Cell Rep.* 15: 287-292

Mihalika, V., Fari, M., Szasz, A., Balazs, E. and Nagy, I. 2000. Optimzed protocols for efficient plant regeneration and gene transfer in pepper (*Capsicum annuum* L.). *J. Pl. Biotech.* 2: 143-149

Mourgues, F., Chevrean, E., Lambert, C. and Bondt, D. A. 1996. Efficient *Agrobacterium* mediated transformation and recovery of transgenic plants from pear (*Pyrus communis* L.). *Pl. Cell Rep.* 16: 245-249

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol. Pl.* 15: 473-497

Nagaraju, V., Srinivas, G.S.L. and Sita, G.L. 1998. *Agrobacterium* mediated genetic transformation in Gerbera hybrids. *Curr. Sci.* 74: 630-634

Nehra, N.S., Chibbar, R.N., Kartha, K.K., Datla, R.S.S., Crosby, W.L. and Stunhnoff, C. 1990. Genetic transformation of strawberry by *Agrobacterium tumefaciens* using a leaf disc regeneration system. *Pl. Cell Rep.* 9: 293-298

Nianiou, I., Karavangeli, M., Zambounis, A. and Tsaftaris, A. 2002. Development of pepper transgenic plants vis *Agrobacterium* mediated and biolistic transformation. *Acta Hort.* 579: 83-87

Novel, G. and Novel, M. 1973. Mutants of *E. coli* K12 unable to grow on methyl-ß-D-glucuronide: Map location of *Uid A* locus of the structural gene of ß-D-glucuronidase. *Mol. Gen. Genet.* 120: 319-335

Ohta, S., Mita, S., Hattori, T. and Nakamura, K. 1990. Construction and expression in tobacco of a β-glucuronidase (GUS) reporter gene containing an intron within the coding sequence. *Pl. Cell Physiol.* 31: 805-814

Ottaviani, M.P., Smita, T. and Cate, H.C. 1993. Differential methylation and expression of the ß-glucuronidase and neomycin phospho transferase genes in transgenic potato cv Bintje. *Pl. Sci.* 88: 73-81

*Otten, L.A. and Schilperoort, R.A. 1978. A rapid microsale method for the detection of lycopine and nopaline dehydrogenase activities. *Biochem. Biophys. Acta* 528: 497-500

Ow, D.W., Wood, K.V., Delnca, M., De-Wet, J.R., Helinski, D.R. and Howell, S.H. 1986. Transient and stable impression of the firstly luciferase gene in plant cell and transgenic plants. *Science* 234: 856-859

Owens, L.D. 1981. Characterization of kanamycin resistant cell lines of *Nicotiana tabaccum. Pl. Physiol.* 67: 1166-1168

Pawlicki, N., Sangwan, R. S. and Norreel, B. S. S. 1992. Factors influencing the *Agrobacterium* mediated transformation of carrot *(Daucus carota L.). Pl. Cell Rep.* 31: 129-139

Philips, G.C. and Hustenburger, J.F. 1985. Organogenesis in pepper tissue cultures. *Pl. Cell Tissue Organ Cult.* 4: 261-269

Potrykus, I., Paszkowski, J., Saul, M.W., Petruska, J. and shilito, R.D. 1985. Molecular and general genetics of a hybrid foreign gene introduced into tobacco by direct gene transfer. *Mol. Gen. Genet.* 199: 169-177

Prakash, A.H., Rao, K.S. and Kumar, M.V. 1997. Plant regeneration from protoplasts of *Capsicum annuum* L. cv. California Wonder. *J. Biosci.* 22: 339-344

Pullock, K., Barfield, D. G. and Shields, R. 1983. The toxicity of antibiotics to plant cell cultures. *Pl. Cell Rep.* 2: 36-39

Puonti-Kaerlas, J., Stabel, P. and Erikson, T. 1989. Transformation of pea (*Pisum sativum* L.) by *Agrobacterium tumefaciens. Pl. Cell Rep.* 8: 321-324

Raghothama, K.H., Lin, D., Nelson, D.E., Hasegawa, P.M. and Bressan, R.A. 1993. Analysis of an osmotically regulated pathogenesis - related osmotin gene promoter. *Pl. mol. Biol.* 23: 1117-1128

Rajmohan, K. 1985. Standardization of tissue/meristem culture technique in important horticultural crops. Ph.D. thesis, Kerala Agricultural University, Thrissur, 242p.

Ramage, C.M. and Leung, D.W.M. 1996. Influence of BA and sucrose on the competence and determination of pepper (*Capsicum annuum* L. var. Sweet Banana) hypocotyl cultures during shoot formation. *Pl. Cell Rep.* 15: 974-979

Ramirez-Malagon, R. and Ochoa-Alejo, N. 1996. An improved and reliable chilli pepper (*Capsicum annuum* L.) plant regeneration method. *Pl. Cell Rep.* 16: 226-231

Riker, A.J. 1930. Studies on infectious hairy root of nursery apple trees. *J. agric. Res.* 41: 507-540

Sambrook, J., Fritisch, E.F. and Maniatis, T. 1989. *Molecular cloning: A Laboratory Manual.* Second edition. Coldspring Harbor Laboratory Press, New York, USA, 1332p.

Sarad, N., Rathore, M., Singh, N.K. and Kumar, N. 2004. Genetically engineered tomatoes: New vista for sustainable agriculture in high altitude regions. *Proceedings for the fourth international crop science congress, September 26-30, 2004* (eds. Fischer, T. and Turner, N.). The Regional Institute Ltd, Gosford, Australia, pp. 118-121

Sarmento, G. G., Alpert, K., Tang, F. A. and Punja, Z. K. 1992. Factors influencing *Agrobacterium tumefaciens* mediated transformation and expression of kanamycin resistance in pickling cucumber. *Pl. Cell Rep.* 31: 185-193

Schenk, R.V. and Hilderbrandt, A. C. 1972. Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. *Can. J. Bot.* 50: 199-204

Scott, R.J., Draper, J., Jefferson, R., Dury, G. and Jacob, L. 1988. Analysis of gene organization and expression in plants. *Plant Genetic Transformation and*

*Gene Expression: A Laboratory Manual* (eds. Draper, J., Scott, R., Amitage, R. and Walden, R.). Blackwell Scientific Publishers, Oxford, UK. pp. 267-337

Shen, H.C., Wang, Z.Y., Jiang, J.Z., Shen, H.L., Wang, Z.Y., Jiang, J.Z. and Meng, L.Y. 1994. *In vitro* plant regeneration and variation of pepper. *Advances in Horticulture: 9. Plantation and Spice Crops, Part 1* (eds. Chadha, K.L. and Rethinam, P.). Malhotra Publishing House, New Delhi, pp. 546-562

Shivegowda, S.T., Mythili, J.B., Anand, L., Saiprasad, G.V.S., Gowda, R. and Gowda, T.K.S. 2002. *In vitro* regeneration and transformation in chilli pepper (*Capsicum annuum* L.). *J. hort. Sci. Biotech.* 77: 629-634

Singh, N.K., Bracker, C.E., Hasegawa, P.M., Handa, A., Bucker, S., Hermodson. M.A., Pfankoch, E., Regneir, F.E. and Bressan, R.A. 1987. Characterization of osmotin. *Pl. Physiol.* 85: 529-536

Singh, N.K., Handa, A.K., Hasegawa, P.M. and Bressan, R.A. 1985. Proteins associated with adaptation of cultured tobacco cells to NaCl. *Pl. Physiol.* 79: 126-137

Siregar, E.B.M. and Sudarsono. 1997. Shoot regeneration from hypocotyl segments of hot pepper mediated by non-disarmed isolates of *Agrobacterium. Capsicum Eggplant Newsl.* 16: 102-105

Smith, E.F. and Townsend, C.O. 1907. A plant tumour of bacterial origin. *Science* 25: 671-673

Spices Board. 2005. April 4, Chilli export touch all-time high. [On-line]. *The Hindu Business Line.* Available: http://www.thehindubusinessline.com [10 July 2005].

Stachel, S.E. and Nester, E.W. 1986. The genetic and transcriptional organization of the vir region of the A6 Ti plasmid of *Agrobacterium tumefaciens*. *EMBO J.* 7: 1445-1454

Steinitz, B., Wolf, D., Matzevitch-Josef, T. and Zelcer, A. 1999. Regeneration *in vitro* and genetic transformation of pepper (*Capsicum spp.*). The current state of the art. *Capsicum Eggplant Newsl.* 15: 9-15

Striekema, W.J., Heidekamp, F., Lanwerse, J.D., Verhoeven, H.A. and Dijkhuis, P. 1988. Introduction of foreign genes into potato cultivars Bintje and Desires using an *Agrobacterium tumefaciens* binary vector. *Pl. Cell Rep.* 7: 47-50

Swarnapiria, R. and Rajmohan, K. 2005. *Agrobacterium* mediated transformation in Dendrobium. *Proceedings of the National Symposium on Biotechnological Interventions for improvement of Horticultural Crops: Issues and Strategies, January 10-12, 2005* (eds. Rao, G.S.L.H.V.P., Nazeem, P.A., Girija, D., Keshavachandran, R., Joseph, L. and John, P.S.). Kerala Agricultural University, Thrissur, pp. 334-336

Szasz, A., Mityko, J., Andrafalvy, A. and Fari, M. 1997. Methodological and genetic aspects of *in vitro* plant regeneration and genetic transformation of the recalcitrant pepper (*Capsicum annuum* L.). *Acta Hort.* 447: 365-366

Szasz, A., Nervo, G. and Fari, M. 1995. Screening for *in vitro* shoot forming capacity of seedling explants in bell pepper (*Capsicum annuum* L.) genotypes and efficient plant regeneration using thidiazuron. *Pl. Cell Rep.* 14: 666-669

Tae, L., Yeun, L., Sook, Y., Jun, P., Nam, S., Chun, Y., Hwa, C., Lim, H.T., Lee, G.Y., You, Y.S., Park, E.J., Song, Y.N. and Yang, D.C. 1999. Regeneration and genetic transformation of hot pepper plants. *Acta Hort.* 483: 387-396

Taj, G., Kumar, A., Bansal, K.C. and Garg, G.K. 2004. Introgression of osmotin gene for creation of resistance against *Alternaria* blight pertubaration of cell cycle machinery. *Indian J. Bact.* 3: 291-298

Tang, H., Ren, Z. and Krezal, G. 2000. An evaluation of antibiotics for the elimination of *Agrobacterium tumefaciens* from walnut somatic embryos and for effects on the proliferation of somatic embryos and regeneration of transgenic plants. *Pl. Cell Rep.* 19: 881-887

Thomashow, M.F., Karlinsey, J.E., Marks, J.R. and Hurlbert, R.E. 1987. Identification of a new virulence locus in *Agrobacterium tumefaciens* that affects polysaccharide composition and plant cell attachment. *J. Bact.* 169: 3209-3216

Torres, A.C., Cautliffe, D.J., Laughner, B., Birniek, M., Nagata, R., Ashraf, M. and Ferl, R.J. 1993. Stable transformation of lettuce cultivar South Bay from cotyledon explants. *Pl. Cell Tiss. Org. Cult.* 34: 279-285

Tosa, A., Pandolfi, R. and Vasconi, S. 1996. Organogenesis in *Camellia x williamsii* : cytokinin requirement and susceptibility to antibiotics. *Pl. Cell Rep.* 15: 541-544

Valera-Montero, L.L. and Ochoa-Alejo, N. 1992. A novel approach for chili pepper (*Capsicum annuum* L.) plant regeneration: shoot induction in rooted hypocotyls. *Pl. Sci.* 84: 215-219

Vancanneyt, G., Schmidt, R., Sanchez, O.C.L., Willmitzer, L. and Rosa, M. 1990. Construction of an intron-containing marker gene: splicing of the intron in transgenic plants and its use in monitoring early events in *Agrobacterium* mediated transformation. *Mol. Gen. Genet.* 220: 245-250

Van-Loon, L.C. and Van Kammen, A. 1970. Polyacrylamide disc electrophoresis of the soluble leaf proteins from *Nicotiana tabaccum* var. Samsun and

Samsun NN, changes in protein constitution after infection with tobacco mosaic virus. *Virology* 40: 199-211

Vasil, V., Clancy, M., Ferl, R.J., Vasil, I.K. and Hannal, L.C. 1989. Increased gene expression by the first intron of maize shrunken locus in grass species. *Pl. Physiol.* 91: 1575-1579

Veluthambi, K., Jayaswal, R.K. and Gelvin, S.B. 1987. The virulence genes A, G and D mediate the double stranded border cleavage of the T-DNA from *Agrobacterium tumefaciens* Ti plasmid. *Proc. nat. Acad. Sci. USA* 34: 1881-1885

Venkataiah, P., Christopher, T. and Subhash, K. 2001. Plant regeneration and *Agrobacterium* mediated genetic transformation in four capsicum species. *Capsicum Eggplant Newsl.* 20: 68-71

Venketachalam, P., Geetha, N., Khandalwal, A., Shaila, M.S. and Sita, G.L. 2000. *Agrobacterium* mediated genetic transformation and regeneration of transgenic plants from cotyledon explants of groundnut (*Arachis hypogaea* L.) via somatic embryogenesis. *Curr. Sci.* 78: 1130-1136

Wang, K., Harrera-Estrella, L., Van-Montagu, M. and Zambryski, P. 1984. Right 25 bp terminus sequence of the nopaline T-DNA is essential for and determines direction of DNA transfer from *Agrobacterium* vir gene expression. *Science* 235: 587-591

*Wang, Y.W., Yang, M.Z., Pan, N.S. and Chen, Z.L. 1991. Plant regeneration and transformation of sweet pepper (*Capsicum frutescens*). *Acta Botanica Sinica.* 33: 780-786

Warren, G. 1991. The regeneration of plants from cultured cells and tissues. *Plant Cell and Tissue Culture* (Ed. Stafford, A. and Warren, G.). Redwood Press Limited, Witshire, U.K. pp. 82-100

Weising, K., Schell, J. and Kahl, G. 1988. Foreign genes in plants: transfer, structure, expression and applications. *A. Rev. Genet.* 22: 421-477

White, D.W.R. and Greenwood, D. 1987. Transformation of the forage legumes *Trifolium repens* L. using binary *Agrobacterium* vectors. *Pl. mol. Biol.* 8: 461-469

Wolf, D., Matzevitch, T., Shiffriss, C., Steinitz, B. and Zelcer, A. 1998. Towards the establishment of a reliable transformation protocol for pepper (*Capsicum annuum*). *Proceedings of the IX International Congress on Plant Tissue and Cell culture, June 14-19, 1998* (ed. Ziv, M.). The International Association for plant tissue culture, Israel, pp.187-188

Woloshuk, C.P., Meulenhoff, J.S., Sela-Buurlaye, M., Elzen, V.P.J.M. and Cornelissen, B.J.C. 1991. Pathogen-induced proteins with inhibitory activity toward *Phytophthora infestans*. *Pl. Cell* 3: 619-628

Wu, H., Sparkes, C., Amoah, B. and Jones, H.D. 2003. Factors affecting successful *Agrobacterium* mediated genetic transformation of wheat. *Pl. Cell Rep.* 21: 659-668

Yanofsky, M.F., Porter, S.G., Youg, C., Albright, L.M., Garden, M.P. and Nester, E.W. 1986. The vir D operon of *Agrobacterium tumefaciens* encodes a site specific endonuclease. *Cell* 47: 471-477

Zambryski, P., Holsters, M., Kruger, K., Depicker, A., Schell, J., Van Montagu, M. and Goodman, H. 1980. Tumour DNA structure in plant cells transformed by *Agrobacterium tumefaciens*. *Science* 209: 1385-1391

Zambryski, P., Joos, H., Genetello, C., Leemans, J., Van Montagu, M. and Schell, J. 1983. Ti plasmid vector for the introduction of DNA into plant cells without alteration of their normal regeneration capacity. *EMBO J.* 2: 2143-2150

Zhu, Y.X., Ouyang, W.J., Zhang, Y.F. and Chen, Z.L. 1996. Transgenic sweet pepper plants from *Agrobacterium* mediated transformation. *Pl. Cell Rep.* 16: 71-75

* Originals not seen

# *Appendices*

# Appendix I

**1. Composition of different tissue culture media**

| Chemical | MS (mg $l^{-1}$) | SH (mg $l^{-1}$) | B5 (mg $l^{-1}$) |
|---|---|---|---|
| Inorganic constituents | | | |
| $(NH_4)\ NO_3$ | 1650 | - | - |
| $KNO_3$ | 1900 | 2500 | 2500 |
| $KH_2PO_4$ | 170 | - | - |
| $MgSO_4.7H_2O$ | 370 | 400 | 250 |
| $(NH)_2\ SO_4$ | - | - | 134 |
| $NaH_2PO_4$ | - | - | 150 |
| $(NH_4)\ H_2PO_4$ | - | 300 | - |
| $CaCl_2.2H_2O$ | 440 | 200 | 150 |
| $Na_2EDTA.2H_2O$ | 37.3 | 15 | - |
| $FeSO_4.7H_2O$ | 27.8 | 20 | - |
| EDTA Na ferric salt | - | - | 43 |
| $MnSO_4.4H_2O$ | 22.3 | - | - |
| $MnSO_4.H_2O$ | - | 10 | 10 |
| $ZnSO_4.7H_2O$ | 8.6 | 1.0 | 2.0 |
| $H_3BO_3$ | 6.2 | 5.0 | 3.0 |
| KI | 0.83 | 1.0 | 0.75 |
| $Na_2MoO_4.2H_2O$ | 0.25 | 0.1 | 0.25 |
| $CuSO_4.5H_2O$ | 0.025 | 0.2 | 0.025 |
| $CoCl_2.6H_2O$ | 0.025 | 0.1 | 0.025 |

| Organic constituents | | | |
|---|---|---|---|
| Glycine | 2.0 | - | - |
| Nicotinic acid | 0.5 | 5.0 | 1.0 |
| Pyridoxine HCl | 0.5 | 0.5 | 1.0 |
| Thiamine HCl | 0.1 | 5.0 | 1.0 |
| Sucrose | 30000 | 30000 | 30000 |
| Myoinositol | 100 | 1000 | 100 |
| pH | 5.8 | 5.8 | 5.8 |

Half MS represents 50 percent concentration of inorganic constituents.

**2. Chemical composition of Yeast Extract Mannitol (YEM) medium used for culturing *Agrobacterium tumefaciens***

| Chemical | $g\ l^{-1}$ |
|---|---|
| $K_2HPO_4$ | 0.5 |
| $MgSO_4.7H_2O$ | 0.2 |
| NaCl | 0.1 |
| Mannitol | 10.0 |
| Yeast Extract | 1.0 |
| Agar | 20.0 |
| pH | 7.0 |

# Appendix II

## 1. Reagents for plasmid DNA isolation

A. Solution I

| | | |
|---|---|---|
| Glucose | : | 50 mM |
| EDTA | : | 10 mM |
| Tris HCl | : | 50 mM pH 8.0 |
| Lysozyme | : | 2 mg $ml^{-1}$ |
| RNase | : | 0.2 mg $ml^{-1}$ |

B. Solution II

| | | |
|---|---|---|
| NaOH | : | 0.2 N |
| SDS | : | 1% |

This solution was prepared fresh, immediately before use.

C. Solution III

| | | |
|---|---|---|
| $CH_3COOK$ | : | 4 M pH 6.0 |

D. Solution IV

| | | |
|---|---|---|
| Na acetate | : | 0.1 M |
| Tris HCl | : | 50 mM pH 8.0 |

E. Solution V

| | | |
|---|---|---|
| $CH_3COONH_4$ | : | 7.5 M |

F. TE Buffer

| | | |
|---|---|---|
| Tris HCl | : | 10 mM pH 7.6 |
| EDTA | : | 1 mM |

**2. Reagents for plant DNA isolation**

A. Extraction buffer (4x)

| | | |
|---|---|---|
| Sorbitol | : | 2.5 g |
| Tris-HCl | : | 4.8 g |
| EDTA 0.25M | : | 0.74 g |
| Distilled water to | : | 100 ml |

The chemicals were dissolved in 60 ml sterile distilled water. The pH was adjusted to 7.5 and final volume was made up to 100 ml with distilled water and then autoclaved.

B. Lysis buffer

| | |
|---|---|
| 1M Tris-HCl (pH 8.0) | : 20 ml |
| 0.25M EDTA | : 20ml |
| CTAB | : 2 g |
| NaCl 5 M | : 40 ml |
| Distilled water to | : 100 ml |

Cetyl Trimethyl Ammonium Bromide (CTAB) was dissolved in 20 ml sterile distilled water. To this solution the required volumes of other stock solutions were added.

1M Tris-HCl (pH-8.0)

Tris-HCl 15.76g was dissolved in 60 ml sterile distilled water. The pH was adjusted to 8.0 and final volume was made up to 100 ml with distilled water and then autoclaved.

0.25 M EDTA

Ethylene Diamine Tetra Acetic acid (EDTA) 9.305 g was dissolved in 100 ml sterile distilled water and autoclaved.

5 M NaCl

Sodium chloride 29.22 g was dissolved in 100 ml sterile distilled water and autoclaved.

C. Sarcosine (5%)

| | | |
|---|---|---|
| Sarcosine | : | 5 g |
| Distilled water to | : | 100 ml |

Sarcosine 5 g was dissolved in 100 ml sterile distilled water and autoclaved.

D. TE buffer

(Tris HCl -10.0 mM; EDTA -1.0 mM)

| | | |
|---|---|---|
| Tris-HCl 1.0 M (pH 8.0) | : | 1.0 ml |
| EDTA 0.25 M (pH 8.0) | : | 0.4 ml |
| Distilled water | : | 98.6 ml |

Autoclaved and stored at room temperature.

E. Chlroform: isoamyl alcohol mixture

| | | |
|---|---|---|
| Chloroform | : | 24 ml |
| Isoamyl alcohol | : | 1 ml |

The mixture was stored in refrigerator before use.

F. Ice-cold Isopropanol

Isopropanol stored in refrigerator was used as such.

G. Ethanol 70 per cent.

To 70 parts of absolute ethanol, 30 parts of double distilled water was added.

# Appendix III

## 1. Reagents for GUS assay

| | |
|---|---|
| X-gluc | : 10 mg |
| Dimethyl sulfoxide (DMSO) | : 100 µl |
| 1M sodium phosphate pH 7 | : 1 ml |
| Triton | : 1 ml |
| Sterile Distilled water | : 18 ml |

Ten mg X-Gluc was dissolved in 100 µl dimethyl sulfoxide (DMSO). Sodium phosphate and triton were added and finally the volume made upto 20 ml with sterile distilled water. Aliquots of 1 ml were taken in storage vial, wrapped with aluminium foil and stored at -20°C.

# GENETIC TRANSFORMATION OF CHILLI (*Capsicum annuum* L.) WITH *OSMOTIN* GENE

By

**RESMY HENRY T.**

## ABSTRACT OF THE THESIS

*submitted in partial fulfilment of the requirement for the degree of*

## Master of Science in Agriculture

**(PLANT BIOTECHNOLOGY)**

*Faculty of Agriculture*

*Kerala Agricultural University, Thrissur*

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**2005**

# ABSTRACT

The study entitled 'genetic transformation of chilli (*Capsicum annuum* L.) with *osmotin* gene' was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara, from December 2003 to September 2005. The study was undertaken to standardize *in vitro* regeneration and *Agrobacterium* mediated transformation of chilli with *osmotin* gene.

Different explants, media and hormonal combinations (auxin and cytokinin) were tried in order to standardize *in vitro* regeneration in chilli var.Ujwala. The best explant for *in vitro* regeneration was hypocotyl. Only the buds originated from hypocotyl explants showed elongation. Shoot buds formed from cotyledon and leaf segments did not elongate in any of the media tested. Based on the percentage regeneration in cultures and no. of shoot-buds per explant, MS medium containing the hormonal combination; BA (5.0 mg $l^{-1}$) + IAA (0.3 mg $l^{-1}$) was found optimum. The regenerated plantlets were transferred to pots for acclimatization so that they can sustain and survive in the natural conditions. The hardened plantlets were planted out.

*Agrobacterium* mediated transformation protocol was optimized considering all the factors for successful transformation. Optimum inhibitory concentration of selectable marker (Kanamycin: 100mg $l^{-1}$) was established. The antibiotic cefotaxime (200 mg $l^{-1}$) was selected for killing the bacteria. *Agrobacterium* strain EHA 105 harbouring the *gus* reporter gene was used for the standardization of transformation. Hypocotyl explants of chilli were co-cultivated with *Agrobacterium* strain (EHA 105). The inoculum density-0.1 $OD_{600}$ nm, infection time-5minutes and co-cultivation period-2 days were found optimum based on GUS assay and survival rate 60 days after co-cultivation.

The hypocotyl explants of chilli were co-cultivated with *Agrobacterium* strain (GV2260) harbouring *osmotin* gene in the plasmid pGV2260 tagged with

35S CaMV promoter. The transformed explants were regenerated on the selection medium optimized for regeneration of chilli. However, none of the transformed buds were elongated in the elongation medium containing selection agent. Hence the transformed plants were transferred to elongation medium containing no antibiotics. Even in this medium, no elongation of shoots was observed even after 60 days. So, further refinement of transformation protocol using an optimal selectable marker is needed for the production of transgenic chilli. After selection of the transformants, the putative transgenics were characterized employing molecular biology techniques viz. PCR-utilizing the gene specific primers of *npt*II. The presence of transgene was confirmed in the transformed plants.