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Genetic Transformation of Cauliflower (Brassica oleracea var.botrytis) using Agrobacterium Tumefaciens as a Vector for Improved Stress Resistance

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Genetic transformation of cauliflower (*Brassica oleracea* var.*botrytis)* using *Agrobacterium tumefaciens* as a vector for improved stress resistance.

By

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Genetic transformation of cauliflower *(Brassica oleracea* var*.botrytis)* using *Agrobacterium tumefaciens* as a vector for improved stress resistance.

Fadil Al-Swedi

Cauliflower (*Brassica oleracea* var. *botrytis*) is described as a recalcitrant plant to genetic transformation processes especially *Agrobacterium*-mediated and as an extremely low frequency event then it requires a large amount of explants for this procedure to succeed. This thesis describes the development and refinement of a mass propagation system for cauliflower micropropagation and its use for overcoming recalcitrance to genetic transformation.

Shoot meristematic tissue was taken from the curd of cauliflower and used to establish *in-vitro* cultures in liquid medium. Explants were cultured in a Murashige and Skoog (MS) medium containing various plant growth regulators combinations to induce shoot regeneration and which were optimised to be 2 mg L^{-1} (9.29 µM) kinetin and 1 mg L^{-1}(4.9 µM) IBA. Shoots were cultured for 4–6 weeks to obtain rooted plants, which were then suitable for weaning and subsequently produce fully-developed in-vivo plants in pots in soil with a 95%+ success rate.

A procedure for detection of the presence of insert DNA in recombinant plasmids in individual *Agrobacterium tumefaciens* strains was refined. Cauliflower was transformed using the EHA105 strain of *A. tumefaciens* harboring the binary vector pPRTL2 plasmid carrying the antioxidant gene Ascorbate peroxidase (APX) for increased stress resistance coupled with neomycin phosphotransferase II (nptII) for resistance to kanamycin and β-glucuronidase (GUS) as a marker gene. Selection was carried out in MS medium containing kanamycin (50 mg L^{-1}), and surviving tissues were then tested by histochemical GUS assay.

Agrobacterium-mediated plant genetic transformation requires a two-step process for its success: selection and regeneration of transformed tissues, and the elimination of the transformation vector (*Agrobacterium)*. This study used carbenicillin and cefotaxime in MS media to eliminate *A. tumefaciens*, at selection levels of 25 and 50 mg L^{-1} kanamycin. Kanamycin severely reduced explant growth and regeneration of control cultures at concentrations as low as 10 mg L^{-1} and completely inhibited shoot organogenesis at 50 mg L^{-1}.

The integration of APX gene into putative transformant lines was confirmed using GUS and leaf disc assays. Genomic integration of the gene cassette was optimised using PCR analysis with primers flanking npt II and CaMV promoter regions. The stable integration of the APX gene in the putative transgenic plants was detected using PCR at 478bp. The result confirmed the first report of transformation with APX gene in *Brassica oleracea*. Thus, a protocol for effective *Agrobacterium*-mediated genetic transformation of cauliflower was optimized.

Transformed and control lines were sub-cultured many times on maintenance medium over 2 years without any loss of the transgene and then tested for salt resistance as *in-vitro* and *in-vivo* plants using a leaf disc assay. Control plants had little or no NaCl resistance whilst transformed plants showed varying degrees of resistance. Analysis of APX gene expression under salt treatment showed that putative transgenic cauliflower survived salinity stress compared with control plants. Non-acclimated and acclimated *in-vivo* plants were also assessed for resistance to frost. Both non-acclimated and acclimated APX transformed lines showed improved frost resistance compared to controls. The results clearly confirmed that NaCl and frost resistance were stable traits attributable to improved APX expression.

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List of Abbreviations

- **2, 4-D:** 2, 4-dichlorophenoxyacetic acid
- **AS;** acetosyringone
- **APX;** Ascorbate peroxidase
- **ANOVA:** Analysis of variance
- **BLAST:** Basic Local Alignment Search Tool
- **cDNA:** Complementary deoxyribonucleic acid
- **COSHH**; Control of Substances Hazardous to Health
- **C**; Control
- **Carb**; Carbenicillin
- **Cef**; Cefotaxime
- **ddH2O:** Double-distilled water
- **DNA:** Deoxyribonucleic acid
- **DNTPs:** Deoxynucleoside Triphosphates
- **EC:** Electrical Conductivity
- **EDTA:** Ethylenediaminetetraacetic acid
- **EtBr:** Ethidium Bromide
- **Gent**; Gentamycin
- **GM1:** Genetically modified 1
- **H_2O_2:** Hydrogen peroxide
- **HCl:** Hydrogen chloride
- **H;** Hours
- **IBA:** Indole-3-butyric acid
- **Kan;** Kanamycin
- **K_2HPO_4:** Dipotassium Hydrogen Phosphate

- **KH_2PO_4:** Potassium Dihydrogen Phosphate
- **KPO_4:** Potassium Phosphate
- **LSD:** Least Significant Difference
- **MS:** Murashige and Skoog
- **MSU:** Michigan State University
- **Na_2HPO_4:** Sodium Hydrogen Phosphate
- **NaCl:** Sodium chloride
- **NaH_2PO_4:** Sodium Dihydrogen Phosphate
- **NaOH:** Sodium Hydroxide
- **NCBI**: National Centre for Biotechnology Information
- **OH:** Hydroxyl radicals
- **PBS:** Phosphate Buffer Saline
- **PBST:** Phosphate Buffer Saline Tween
- **PCR:** Polymerase Chain Reaction
- **RNA:** Ribonucleic acid
- **ROS:** Reactive Oxygen Species
- **SOP**; Standard Operation Procedure
- **TA**: Transformant plant with APX
- **Tetr**; Tetracycline

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Finally, my entire deep thanks to Massive God, Who blessed me with the strength, confidence and determination needed for the completion of my research project.

Dedication

I would like to dedicate my thesis to my beloved parents (May Almighty God bless them).

Author's Declaration

This study was financed by Iraq Ministry of Higher Education and Scientific Research (MOHESR) for the award of scholarship that provided the financial support for this research project.

I declare that the work submitted in this thesis is the results of my own investigations except where reference is made to published literature and where assistance is acknowledged.

Word account of main body of thesis: 58,343 words (71.798 including references and appendices).

Signed …… Fadil Al-Swedi …….. ……Date ……

Research publications;

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1. **Al-Swedi F.G**., Al Shamari, M. A., Rihan, H.Z., Lane. S and M.P Fuller. (2012) Micro-propagation and genetic transformation of *Brassica oleracea var. botrytis*. Acta Hort. 961:167-174.

2011

2. **Al-Swedi F.G.,** Lane. S and Fuller M.P. (2011). An improved micro propagation system for successful transformation of cauliflower (*Brassica oleracea var. botrytis*). Acta Hort. (ISHS) 961:167-174.

3. **Al-Swedi F.G**., Lane. S and Fuller M.P. (2011). A micropropagation technique for cauliflower (*Brassica oleracea var. botrytis*) to facilitate agrobacterium transformation. Aspects of Applied Biology 110:105-110.

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- Presentation of MPhil project in symposium (plant physiology) March 9, 2010 in school of Biomedical and Biological Sciences, Plymouth University (Module Bio 5124).
- Presentation in lab based teaching course (Module ENV 5101) November 27, 2009, Plymouth University.
- Presentation in Student Associate Scheme (SAS) teaching training (June 15, 2010) Topic, Plymouth University.
- Presentation in Learning and Teaching for General teaching Associates (GTA course) April 27 2010, Plymouth University.
- Presentation in "Presenting to an audience: part 2" 7th December 2011, Plymouth University.
- Presentation at joint SEB & JEB conference. London (1st Annual symposium Plant Physiology) 20th December 2011.
- Lecture on "Tissue Culture" given to undergraduate students in module BIO 2001. 1st March 2012 Plymouth University.
- Presentation at 12th Congress of the European Society for Agronomy (ESA12), 20-24 August 2012, Helsinki, Finland, pp 252-253.

Presentations (poster)

- Presentation for SEB in London.1st Annual symposium Plant Physiology 20.12.2011.
- 2 Poster presentations for GM; “From basic research to application”, Association for Applied Biology, June 2011, Rothamsted, London, UK.
- Presentation for Annual SEB Symposium, 1st July 2011, Glasgow, UK.
- Presentation for SCI Symposium, 9th July 2011 Reading University, UK. (Prize for best poster competition).
- Presentation for 2nd Symposium on Horticulture in Europe, ISHS, 1st to 5th of July 2012 Anger (France).

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- Postgraduate Society Short Conference 09 March 2010.
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- Postgraduate Society Short Conference 17 March 2011.
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- Principles and Applications in Electron Microscope (Bio5102) Plymouth University 2010-2011.
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- Real-time PCR training course - theory and optimisation 23.11.2011 Dartington Hall conference centre, Totnes.

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- Plant Transforming Technologies II. VIPCA II, Vienna, February 2011.
- The postgraduate society short conference series 2009, 2010, 2011 and 2012, University of Plymouth, UK.
- GM; from basic research to application, Association for Applied Biologists, June 2011, Rothamsted, London, UK
- SEB Annual Meeting, July 2011Glasgow, UK.
- SCI July 2011 Reading, UK. (Prize for poster competition).
- 2nd Symposium on Horticulture in Europe, ISHS, July 2012 Angers (France)
- 12th Congress of the European Society for Agronomy, (ESA12), 20-24 August 2012, Helsinki, Finland.

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- 2011 - to date; Society of Chemical Industry (SCI).
- 2011 - to date; Association of Applied Biologists (AAB).
- 2012- to date; European Society for Agronomy (ESA).
- 2010 -to date - Postgraduate Society at University of Plymouth

1. Chapter one: General introduction

1.1 Plant Genetic Improvement

1.1.1 Plant Breeding

Since the earliest domestication of crop plants nearly 10,000 years ago farmers and growers have improved their crops by selection to create higher yielding, more disease resistant Land Races (Fischer and Edmeades, 2010). During the 20th century the understanding of genetics emerged and plant breeding techniques have been used which systematically recombine the genetic traits within a species to develop specific varieties with even more yield potential and disease resistance (Brown, 2002).

Plant breeding has been a remarkably successful strategy to increase the yield of crops (Stuber et al., 1999). This includes increased resistance to disease, pests and tolerance to stress. It has been estimated that 50% of the yield increase in field crops during the last 50 years is attributable to the improvement of genetic factors and the other 50% has resulted from agronomic practices comprising chemical treatment (pesticides), fertilizers and other husbandry practices (Tanksley and McCouch, 1997).

However, there are many limitations to plant breeding for instance; genetic linkage, complex traits, poly genes and as for time scales, many breeding experiments such as back crossing and pedigree selection need a very long time of several generations or several years to identify useful selections with the potential to become a new varieties (Devi, 2003). Furthermore, many plant breeders are reporting that that annual crop yield gain from plant breeding is slowing down and there is a fear that, for the staple crops, yields are plateauing. This has turned the attention of plant breeders towards new molecular breeding techniques including genetic modification.

Molecular breeding is the term used to represent the breeding methods that are coupled with genetic engineering techniques. These breeding methods have been used to make plant breeding more efficient and targeted in order to meet the food demands of the growing world population and the challenges of the need for improved crop production and yields. The future development in agricultural yields is going to rely heavily on the use of molecular breeding programmes. Linkage analysis which deals with the studies to correlate the link between molecular markers and a desired trait is an important aspect of molecular breeding programmes and molecular breeding involves breeding using molecular markers linked to defined traits. A molecular marker is a DNA sequence in the genome which can be located and identified and is linked (closely situated) to a gene of interest in the breeding programme; therefore molecular markers can be used to identify particular locations in the genome. Gene flow is the transfer of genetic materials between plants and is also known as introgression. One of the main concerns of the use of GM crops is the worry that new genes in GM crops will escape by introgression and affect the natural plant populations in the environment. Research indicates that such gene transfer is affected by many factors such as the stability of genes, hybrid fertility and size of crop and the presence of close relative weed populations in the field (Barton and Dracup, 2000).

Since 1998 agriculture has been exposed in various parts of the world to bitter debates about the cultivation of genetically modified crops. These debates have included discussion of political, scientific, economic, and religious institutions and moreover the debates have been applied to areas such as the research laboratory, legislature and companies. In 1995-96, transgenic potato and cotton plants were used commercially for the first time in the USA. By the year 1998-99, five other major

transgenic crops cotton, maize, canola, soybean, and potato were introduced to the farmers. These varieties accounted for about 75% of the total area planted with crops in the USA. There are still a lot of concerns regarding the harmful environmental and hazardous health effects of transgenic plants and there is continued resistance to their wide-scale introduction in Europe (Andow and Zwahlen, 2006).

1.1.2 Mutations

In the absence of desired agronomic characteristics within a cultivated species, a common and efficient tool to create new genetic variability is mutagenesis. Mutants have been included in germplasm collections or used in breeding programs to improve the yield or eliminate yield reducers. As a complementary approach to breeding activities, this method provides an opportunity to improve a cultivar for a particular trait without disrupting the genotype or breaking desirable linkages between existing genes.

Mutation is a basic source of variation in most breeding material which may occur in a modern cultivar, a landrace, a plant accession, and wild related species or in an unrelated organism. Among the mutants released as cultivars, 12% were resistant to biotic stress (pathogen and parasites) and only 6% were tolerance to abiotic stress (freezing, salinity, drought, alkalinity, high humidity and high soil fertility)(Chen and Murata, 2002).

Mutation can be induced by the use of mutagens which may be either physical or chemical, and both have been used in conventional plant programmes often in conjunction with *in-vitro* selection methods. The majority of chemicals used to induce mutation in plant cell cultures can be placed in two groups, base analogous and

alkylating agents. Alkylating agents include N-nitrose-N-ethyl urea (NEU), N-nitrose-N-methyl urea (NMU), alkyl sulphate and nitrogen mustard. NEU or NMU is a bio-functional agent that requires metabolic activation to a reactive metabolite (Charlotee, 1976). NEU or NMU alkylate nucleophilic organic macromolecules include DNA where they can induce depurination and depyrimination as well as mono-adduct formation. Both NEW or NMU have been shown to induced gene mutation(deletion), transition mutation, unscheduled DNA synthesis (UDS), sister chromatid exchange(SCE) and they can also induced DNA-DNA and DNA protein crosslink(Kamra, 1971, Collin, 1990).

1.1.3 Plant Tissue Culture

Plant tissue culture can be defined as the culture of plant cells, tissue and organs under aseptic condition (Smith, 2012). Tissue culture involves placing decontaminated excised small pieces of plants (explants) into growth promoting media. Explants can be taken from various sites on a plant or pieces of a plant (propagule). Typically explants contain a meristem (apical, lateral) which is able to be broken from dormancy in the tissue culture process. The artificial sterile media used in tissue culture contain minerals, sugar, vitamins, hormones and typically agar(James et al., 1996). Cell and tissue culture techniques offer a number of advantages not available in conventional selection and breeding procedures currently used to enhance tolerance of plants to stress environments and have tremendous potential for crop improvement. The use of tissue culture is now a well-established technique in plant breeding and selection (George et al., 2008). The collection of tissue culture techniques can be directed either towards the production of identical plants (cloning or micropropagation) or to induce variability (soma-clonal variation and mutation induction). Plants can be propagated from numerous explants

including leaf sections, anthers, meristems, or even isolated single cells and protoplasts and whole plants, callus or liquid cell suspension cultures can be established.

Techniques of plant tissue culture are generally useful in crop improvement programs through: 1- propagation *in-vitro*; 2-meristem culture for virus elimination; 3-secondary product synthesis; 4- production of haploid plants from cultured anthers; and 5- development of new varieties via cellular or molecular genetics (Brown and Thorpe, 1995). Tissue culture gives a chance to study various morphological, biochemical and molecular genetic effects on abiotic stress because the plant material can be treated uniformly in a controlled environment with controlled nutrient conditions (Epstein and Rains, 1987).

A key factor in the application of plant tissue culture techniques to plant improvement is the development of efficient protocols for the regeneration of plants from cells, tissue and organs and their subsequent transition to *in-vivo* plants(Debergh, 1990). Plants can be regenerated from plant cells or tissues through organogenesis or embryogenesis depending on the plant developmental state, culture medium and conditions. Organogenesis involves the differentiation of organs, initially as root or shoot primordia.

Several factors have been reported to affect regeneration *in-vitro* such as culture medium, explant source, growth regulators and the use of antibiotics. Each of these factors and their relationship to regeneration will be discussed in detail.

Plant growth regulator is the common term applied to some biochemical products produced in particular tissues or organs of plants and which, despite their small amount, play an important role in plant morphogenesis and development. Auxin,

cytokinins and gibberellins are the three major groups of plant growth regulator and synthetic versions of these are used in different stages of plant tissue culture such as callus induction, differentiation, shoot or root initiation, shoot elongation and plant regeneration.

Growth regulators are used to support a basic level of growth but are equally important to direct the developmental response of the explant or its derived cell lines. Early in tissue culture research it was established that a relative high auxin: cytokinin ratio induced root formation in tobacco callus, while a high cytokinin: auxin ratio favoured shoot production (Skoog and Miller, 1957). The balance between the two sorts of regulator that are required for different purposes can be generally represented as in (Plate1.1). Relative proportions of auxin and cytokinin do not, however, always produce the typical results shown in (Plate1.1) and require empirical optimization for each species and sometimes for each variety within a species. A balance between auxin and cytokinin growth regulators is most often required for the formation of adventitious shoot and root meristems (Berleth and Sachs, 2001, Jordan et al., 2006). The requisite concentrations of each type of regulant differ greatly according to the type of plant being cultured, the culture conditions and the compounds used.

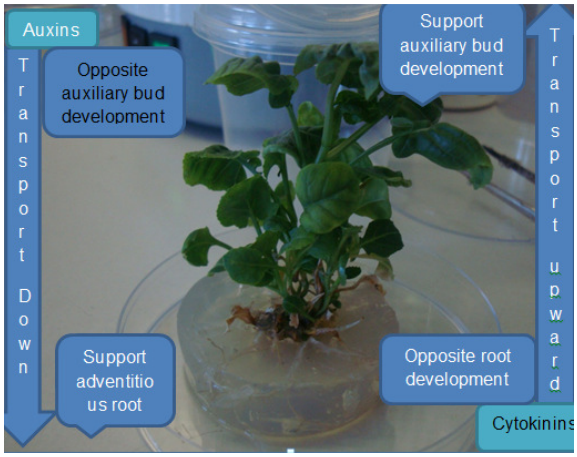


Plate 1.1. Relative amount of auxin and cytokinin that is often required to bring about some kinds of morphogenesis. Source: (Fakhrai 1990).

Many observations of organ formation in cultured tissues support the hypothesis that localized meristematic activity precedes the organized development of shoot and roots (Torrey, 1966, Jiang and Feldman, 2005). In general, buds can be initiated from callus or from cut edges of explants in the presence of high cytokinin: auxin ratio (Helena V., 1976, Banno et al., 2001, Sharma and Anjaiah, 2000, Helenice et al., 2003). In some species, the addition of cytokinins to the medium fails to induce shoots, suggesting that the accumulation of endogenous auxin or other plant growth regulators may result in an inhibitory effect on organogenesis which cannot be reversed by exogenously applied hormones (Khalid M, 2002). In these cases additional hormones may be required or the culture conditions changed, including nutritional and physical factors to unblock the onset of the process. The culture conditions for root production varies considerably; usually a high concentration of auxin is favored to enhance root production, but in some cases exogenous auxin is inhibitory (Thomas and Street, 1970, Hou et al., 2004).

Ethylene is gaseous plant growth regulators that can be produced during plant tissue culture and accumulate in culture vessels and influence growth and development.

Production rates and concentrations may be critical and may have varying effects on different stages of tissue culture (Chi et al., 1994). Work with Brussels sprout (*Brassica oleracea* var*. gemmifera*) suggests that endogenous ethylene may inhibit callus growth and silver nitrate was found to be essential for maintaining callus cultures as well as improving regeneration (Sethi et al., 1990). In the same way, (Kuvshinov et al., 1999) found that high concentrations of silver nitrate led to partial necrosis of turnip rape explants during long culture periods and silver ions have bacterial activity. However, even concentrations as high as 15 mg L^{-1} silver nitrate did not have a negative effect on transformation of *Brassica rapa* ssp*. oleifera*.

Explant tissue is another important factor affecting regeneration. For some species, only certain parts of the plant can respond in culture. In these plants, regions of actively dividing cells seem to respond more readily than others e.g. immature embryos of strawberry have been extremely useful plantlets have been regenerated Wang et al. (Wang et al.). However, in some other plants, e.g. excised embryos, hypocotyls young roots, petioles, peduncles and protoplasts are the most responsive explants (Ammirato, 1986). In many cases, younger tissues have been found to produce more uniform regeneration from differentiated cells (Karp, 1995). A variety of explants including curd, hypocotyls and cotyledons has been tested for their ability to induce shoots of cauliflower. Meristems are the basic unit used in plant micro-propagation; they are most genetically stable part of a plant and consequently the most suitable for production of true-to-type propagules. Micro-propagation using curd meristems has been used for some time to maintain cauliflower parent lines (Crisp and Walkey, 1974), for the production of virus free cauliflower and for the early screening of curd quality (Crips and Gray 1979), for chemical mediated mutagenesis (Fuller et al., 2006a), the development and induction of mutants (Eed, 2001), and

transgenic cauliflower plants (Kieffer, 1996). The technique of curd meristem micro-propagation for cauliflower clonal propagation is now well established but extensive studies have led to the development of a new technique for rapid mass production of cauliflower propagules from fractionated and graded curd (Torres, 1980a, Kieffer et al., 1995b, Kieffer et al., 1995a).

To initiate growth of the explants it is important to provide basic nutrients within the media which usually contains a mixture of salts which provide the essential macro and micro elements as well as a carbon source (sugar). The most widely used of the formulations available is that Murashige and Skoog media (Murashige and Skoog 1962; Jansen et al., 1989). Culture medium effects on regeneration have been demonstrated by numerous studies. MS medium satisfies the nitrogen requirement in many species because of the presence of high concentrations of inorganic nitrate (Reinert. et al., 1967).

One of the biggest challenges to plant tissue culture is the suppression of microbial growth in the nutrient rich medium. Avoiding such contamination requires the use of contaminant-free explants and dissection and culture procedures which are aseptic. Nevertheless, contamination can still occur and antibiotics may be used. The use of antibiotics in culture media has recently become more widespread with the emergence of antibiotic resistance genes as selectable markers in transformation experiments and transformation systems. In addition, co-cultivation of *A. tumefaciens* requires the use of an antibiotic to kill the bacteria at the end of the infection stage of the process. The antibiotics kanamycin, gentamycin and tetracycline have been found to be inhibitory to plant cell or tissue growth at comparatively low concentrations. Fiola et al. (1990) indicated that the addition of 10 mg L^{-1} or higher of kanamycin sulfate to *Rubus* cotyledon regeneration medium drastically reduced the

growth and organogenesis of explants. The use of rifampicin at 50 mg L^{-1} in tissue culture medium effectively controlled bacterial contaminants without affecting the growth of explants culture of *Helianthus tuberosus* (Phillips et al., 1981). Mathias and Mukasa, (1987a) investigated the effect of Cefotaxime on callus initiated from immature embryos of four barley cultivars. Callus growth was up to 45% greater on Cefotaxime than on control medium and the frequency of regenerating calli was increased by up to 80%.

In summary, the ability of explant tissues to regenerate plantlets is primarily related to the growth regulator composition of the culture medium although the other factors which have been discussed in this section should also be considered in the development of protocols for plant regeneration. Further details are overviewed specifically for tissue culture of cauliflower later in this thesis.

1.1.4 Plant Genetic Transformation

Until recently, the only available techniques to genetically improve agricultural crops were sexually hybridization and induction of mutations. In the last 2 decades, dramatic progress has been made in the application of recombinant DNA technology, including direct gene transfer to plants (Puddephat et al., 1999) and (Robledo-Paz et al., 2004). Plant genetic transformation opens new discovery techniques in crop science to regenerate modified plants from a single cell or organ tissue (Abdul et al 2009).

Transformation was first achieved by inoculating plants with a wild-type *Agrobacterium tumefaciens* strain and a disarmed strain consisting of nptII and hpt genes. Cauliflower mosaic virus (CaMV) derived sequences were used to promote the expression of that gene transfer in an attempt to measure the resistance ability to

CaMV infection. Integration of the bacterial T-DNA was detected and whilst CaMV gene transcripts were detected in all plants the amount of RNA transcripts seemed to be very low in most transgenic plants (Ding et al., 1998). Most GM plant research aims for the addition of genes of interest with the expressed aim of heterologous gene expression to improve protection against disease, weeds and pests (Hassanali et al., 2008).

A genetically modified (GM) crop is defined as a new technique of modern biotechnology applied especially in the agricultural sector and involves the introduction of one or more desirable genes added to the target existence plants complement of thousand genes to obtain a new plant with desirable characters. Genetically modified crops provide many probabilities to the future as new knowledge increases and more production of food with high quality and quantity widespread over the whole over the world is demanded (Ken *et al*., 2005).

The science of genetic engineering is described as the characteristic of organisms modified by manipulation of genetic engineering (DNA) and transformation of specific gene(s) to target plants to obtain a new variety (Uzogara 2000).

GM strategies are being developed to overcome problems caused due to biotic stress (viral, bacterial infections, pests and weeds) and abiotic stresses (drought, frost, heat, salt, U.V.). Increased plant tolerance to environmental stresses by increased resistance to abiotic stresses includes stress induced by herbicides, temperature (heat, chilling and freezing), drought, salinity, ozone and intense light. These environmental stresses result in the destruction or deterioration of crop plants which leads to reduced crop productivity. Several strategies have been used and developed to build resistance in plants against these stresses.

Genetic engineering of plants is much easier than that of animals. There are several reasons for this: (1) there is a natural transformation system for plants (the bacterium *Agrobacterium*), (2) plant tissue can dedifferentiate (a transformed piece of the leaf may be regenerated to a whole plant), and (3) plant transformation and regeneration is relatively easy to achieve.

The soil bacterium *Agrobacterium tumefaciens* is closely related to *Agrobacterium rhizogenes,* which induces hairy root disease of dicotyledonous plants. (*A. tumefaciens* (meaning tumour-making) can infect wounded plant tissue, transferring a part of a large plasmid, the T-DNA region of the Ti (tumour-inducing) plasmid, to the plant cell. The T-DNA randomly integrates into the chromosome of the plant. The integrated part of the plasmid contains genes for the synthesis of (1) food for the bacterium (opines), and (2) plant hormones (tumour inducing). Genes from the Ti plasmid that are integrated in the plant chromosome are expressed at high levels in the plant because they have constitutive promoters. The overproduction of the plant hormones leads to continued growth of the transformed cells, causing plant tumours or galls. Rapid, cancerous growth of the transformed plant tissues has obvious advantageous to the bacterium because each tumour cell produces more opines which is a growth substrate for the bacterium.

The Ti plasmid can be genetically modified by deleting the genes involved in the production of opine and the plant hormones ("disarmed"), and inserting a gene that can be used as a selectable marker. Selectable marker genes that are commonly used generally code for proteins involved in the breakdown of plant toxic antibiotics, such as kanamycin or hygromycin and thereby impart antibiotic resistant to a transformed plant. Genes of interest that has been characterised, isolated and

synthesised can theoretically also be inserted into the Ti plasmid in series with the antibiotic resistance genes. In principle, one can thus transform any plant tissue, and select transformed plants carrying the gene of interest by screening for antibiotic resistance. However, unfortunately there are some complications: (1) it has proven difficult to transform some species of plant and these are termed to be recalcitrant to transformation by *Agrobacterium* and monocots (grasses, etc.) are commonly recalcitrant, and (2) regeneration of whole plants from tissue cultures of all species is not always possible.

A number of genetically engineered plant varieties have been developed and common traits that have been introduced by transformation include herbicide resistance, increased virus tolerance, or decreased sensitivity to insect attack (most notably by transformation with the Bt toxin) (Dutton et al., 2002). At the beginning of this area of science, most of such genetically engineered plants were tobacco, petunia, or similar species with a relatively limited agricultural application. However, during the past two decades it has now become possible to transform major staples such as corn and rice and to regenerate them to a fertile plant (Christou, 1995). Increasingly, the transformation procedures used do not depend on *A. tumefaciens*. Instead, DNA can be delivered into the cells by small, µm-sized tungsten or gold particles coated with DNA (Christou et al., 1988). The particles are fired from a device that works similar to a shotgun (Messing et al., 1981). The modernized device uses a sudden change in pressure of Helium gas to propel the particles, but the principle of "shooting" the DNA into the cell remains the same (Sanford, 1990). This DNA-delivery device is nicknamed the "gene gun", and has also been shown to work for DNA delivery into chloroplasts as well ('Neill.O et al., 1993). Over the last several years, use of the "gene gun" has become a very common method to transform

plants, and has been shown to be applicable to virtually all species investigated. For example, transformation of rice by this method is now routine (Christou, 1997). This is a very important development as rice is the most important crop in the world in terms of the number of people critically dependent on it for a major part of their diet (Khush, 2005).

Another method to insert foreign genes into cereals is by electroporation: a pulse of electricity is used to produce self-repairing holes in protoplasts and DNA can penetrate through these holes. However, it is often very difficult to regenerate fertile plants from protoplasts of many species including cereals. Nonetheless, significant advances in overcoming these practical difficulties have been made more recently. Now even transgenic trees have been created (Petri and Burgos, 2005) for example, the gene for a coat protein of the plum pox virus has been introduced into apricot (Câmara Machado et al., 1994).

Essentially all major crop plants can be (and have been or are being) genetically engineered and the procedures are now becoming routine and the frequency of success is very high. Even though genetically engineered crops are more costly than conventionally bred ones, they have been readily accepted by some governments such as the USA, Canada, Brazil and China for more than 10 years and their farmers are provided with the tangible benefits can be demonstrated. Most of the traits marketed in these countries are for herbicide resistance and insect resistance (using Bt). However, it is questionable whether the farmers in poorer countries can come up with the funds to "try out" and use these new crops and legislation restricting their use is common across the world. Another issue in this respect is how genetically engineered crops are perceived by the consumer. Even though in the US there is

little resistance to such crops as long as the products can be shown to be safe and advantageous, in other countries (for example in Europe) genetically modified foods are received poorly by the consumer. It is unlikely that there is a rational sound basis for this rather hostile reaction of the consumer, as most crop plants are the result of human manipulation through centuries of breeding and may have been treated with harmful herbicides and pesticides. Time and education will need to be invested to provide consumers and consumer advocates with a balanced opinion on the acceptability of the origin of their foods. One area of particular concern for some people is the lack of labelling of genetically engineered foods, and legislation may be introduced to address this issue (Klintman, 2002). On the other hand, as so many commodity plants such as soybean and corn are genetically modified and the nature of the genetic modification is not necessarily easy to explain, it may be simpler to label those foods that are guaranteed free of "genetically modified organisms" or their products. Several plant biotechnology companies have increased their efforts to provide information regarding the full, global scope of impacts of plant biotechnology (Brookes and Barfoot, 2007).

The *Agrobacterium* genus includes primarily saprophytic bacterial species that live in the soil micro flora, where they usually occur in the rhizosphere (Escobar and Dandekar, 2003). Four *Agrobacterium* species cause neoplastic diseases on numerous plants: “crown gall” caused by *Agrobacterium tumefaciens* (Plate1.2), “cane gall” caused by *A. rubi,* “hairy root” caused by *Agrobacterium rhizogenes* and lastly, a new species, *A. vitis*, was recently described to cause tumors and necrotic lesions on grape vine and a few other plant species (Gelvin, 2003, Matthysse, 2006). Virulent *Agrobacterium* species infect some hundreds of different plant species. These are commonly woody and herbaceous dicotyledons, but moreover

monocotyledons can be infected (Otten et al., 2008). *A. rhizogenes* has captured some attention and has found its use as a tool for genetic transformation (Nilsson and Olsson, 1997, Taylor, 2006) for the production of secondary metabolites linked to pharmaceutical manufacturing, in the hairy root structures (Guillon et al., 2006, Srivastava and Srivastava, 2007). Amongst the four above-mentioned species, *A. tumefaciens* is by far the greatest essential and most studied. Crown gall was first defined in 1853 as a neoplastic disease affecting some plant species. Ten years later, Smith and Townsend (1907) reported *Bacterium tumefaciens* (now *A. tumefaciens*) as the causal agent of crown gall disease in the perennial species *Bellis perennis*. For a long period, scientists could not understand the mechanisms of crown gall tumor formation. Early theories were finally rejected, and the interest in crown gall generally decreased until the tumorous structures were proposed to form as a result of a "tumor-inducing principle" (TIP). Altering the common host cells to tumor cells happened when TIP was transferred from the *Agrobacterium* to the host cells (Braun, 1947). Later, in the 1970's it was confirmed that TIP in fact is the T-DNA (transferred-DNA) region of the *Agrobacterium* Ti (tumor-inducing) plasmid that is integrated into the host genome in the crown gall tumor cells (Chilton et al., 1977). The discovery that *Agrobacterium* is "transforming" its host in order to create crown gall tumors represented a defining moment in *Agrobacterium* study, and following detections ultimately established this bacteria as a model system for horizontal gene transfer and, most significant, as a device for plant transformation.

Plate 1.2. *A. tumefaciens* is a soil borne pathogen responsible for Crown Gall disease, affecting many higher species of plant ("Courtesy Missouri Botanical Garden"). (Copyright permission granted-copyright clearance centre).

The molecular basis of genetic transformation of plant cells by *Agrobacterium* has now been mostly determined and it is well known that a specific region, namely the T-DNA from the tumor-inducing (Ti) plasmid, is transferred and stably integrated into the plant nuclear genome (Gelvin, 2003).

In order to become appropriate for laboratory purposes, the *Agrobacterium* strains used today required to be engineered from selected wild-type strains. Some of the natural features of the Ti plasmid had to be entirely removed (e.g. genes responsible for tumor development and opine biosynthesis in planta), whereas the characteristics of some transformation machinery components had to be increased and further improved.

The basis for the biotechnological use of *Agrobacterium* in genetic transformation lays in the T-DNA structure and functions. In this way the natural wild type oncogenes and opine synthase genes from the T-DNA can be exchanged by genes of interest (Lacroix et al., 2006, Klee et al., 1987). Consequently, any DNA located between the borders will be transferred to the host cell. Though, because the T-DNA is not able to mediate its own transfer, being only the load vehicle, other bacterial features needed also to be altered. The vir genes, residing on the virulence region of the Ti plasmid, are required for the T-DNA transfer and integration. Altering their

regulation (Ankenbauer, 1991) and copy number (Rogowsky et al., 1987) proved to be suitable for increasing transformation efficiency (Klee et al., 1987). Thus the size of the T-DNA that can be mobilized into plants could be enlarged (Hamilton et al., 1996). While induction and expression level of vir genes could be a limiting step for efficient transformation of some plant species (Klee et al., 1987), new data show that transformation efficiency does not always associate with the vir gene expression, suggesting a more complex correlation (Gelvin, 2003).

The ability of vir genes to act in trans led to the development of binary and super-binary transformation vectors, as a major step toward increasing the range of species that are agreeable to *Agrobacterium*-mediated transformation (Lee et al., 2008). Despite these successes, there are still many economically essential crop species and trees that remain recalcitrant to *Agrobacterium*-mediated transformation. The transformation process is a result of a "cooperative project" between *Agrobacterium* and its host, much effort is now directed toward understanding the host's influence.

Plant transformation becomes more important because of cultivar improvements and a study of genetic features as well as tissue culture improvement. Rao et al., (2009) suggest that plant genetic engineering opens new discovery techniques in crop science to regenerate plants from a single cell or organ tissue. Plant transformation becomes more important because of cultivar improvements and a study of genetic features as well as tissue culture improvement. There are many innovative avenues using transformation of genes to the plants (Plate 1.3) by A) *Agrobacterium tumefaciens*, C) Gene gun as described before. In addition to transformation of genes by chemicals (Plate 1.3B), the uptake and expression of DNA is greatly enhanced by polybrene–spermidine combination treatment and therefore the

recovery of non-chimeric germ line transgenic cotton plants (Wagdy and Sawahel, 2001). To deliver plasmid DNA into cotton suspension culture obtained from cotyledon-induced callus, polybrene and/or spermidine treatments were used. Main transformant cotton plants were regenerated and evaluated by β-glucuronidase assay and DNA hybridization by using transforming plasmid (pBI221.23) contained the selectable *hpt* gene for Hygromycin resistance and the screenable Gus gene. Finally transformation of pollen grains (Plate 1.3D) has shown the great function in agriculture molecular breeding by transformation method via the pollen-tube pathway (Song X, 2007). The pollen-tube pathway (PTP) can be applied to foreign DNA to cut styles shortly after pollination and DNA reaches the ovule by flowing down the pollen-tube. The reported transformation of rice was applied by PTP (Luo and Ray, 1988). Wheat(Mu et al., 1999), soybean (Hu and Wang, 1999).

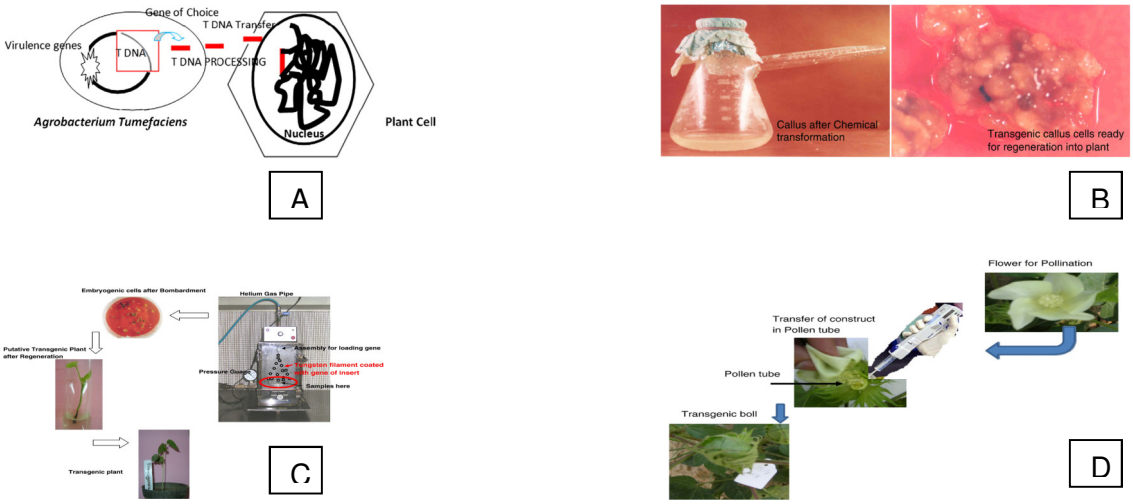


Plate1.3. Schematic overview of gene transformation through a) Agrobacterium, B) Chemical, C) gene gun, D) pollen-tube Cited by (Rao et al., 2009).(copyright permission granted-copyright clearance centre).

An advantage of gene transfer is the potential of adding a single new character to a proven genotype and thus avoiding the slow process of classical breeding.

Furthermore, it allows movement of genes across sexual barriers and from one life form to another i.e., from bacteria or animal to plants.

Although several techniques have been published, the *Agrobacterium*-mediated transformation system has proven to be the most commonly used protocol for the introduction of new genes into dicotyledonous plants (Kuginuki and Tsukazaki, 2001). Using this system, several genes have been successfully introduced and expressed in Brassica plants (Cardoza and Stewart, 2003a, Cao and Earle, 2003a, Cheng et al., 2003a). Genetically engineered cotton plants containing the oxidative stress-related gene for the production of the enzyme ascorbate peroxidase (APX) have already been tested in field trials and dry land agriculture, the altered plants showed increased production.

The ability to manipulate the level of specific enzymes of this pathway using gene transfer technology can be used to improve the stress tolerance of economically important plants.

1.1.5 Reporter genes

All transformation system requires a reporter gene to aid selection of transformation and to confirm the transfer of the T-DNA and a widely used non-selectable reporter marker is the GUS gene. This gene was constructed by attaching the beta-glucuronidase gene to either a 35S CaMV, nopaline synthase or lacz promoter, and to a fragment containing the polyadenlation signal of the nopaline synthase gene of the *A. tumefaciens* Ti plasmid (R A Jefferson et al., 1987, Jefferson, 1987). The GUS gene is one of the most frequently used reporter genes because it is extremely stable and has sensitive assays for the enzyme (Jefferson, 1987). It may be assayed at any physiological pH, with an optimum between 5.2 and 8.0. The GUS gene is

usually used in a gene fusion and this means that GUS coding sequence is under the direction of the controlling sequence of another gene. Usually the GUS gene is under control of the promoter of the 35S small ribosomal subunit of the cauliflower Mosaic virus. The GUS gene has proven to be a useful reporter as its enzyme activity can be easily detected by formation of a blue precipitate in the presence of the substrate X-Gluc (5-bromo-4-chloro-3-indoyl-D glucuronic acid) in histochemical assays, by fluorometry in the presence of 4-methylumbelliferyl glucuronide (MUG), or spectrophotometrically using p-nitrophenylglucuronide as substrate. However, the GUS gene is not ideal for all plants as in some cases the background beta-galactosidase level is high enough to make it difficult to detect chimeric β-galucuronidase by enzymatic methods.

Details about using PCR as a technique for the detection of desired genes in *A. tumefaciens* and evaluate the integration of resistance genes (APX and SOD) in cauliflower by using GUS gene will be discussed later. The unique interaction between the bacterium and its host plant has been well studied at the transcriptome, but not at the metabolic level (Plate 1.4).

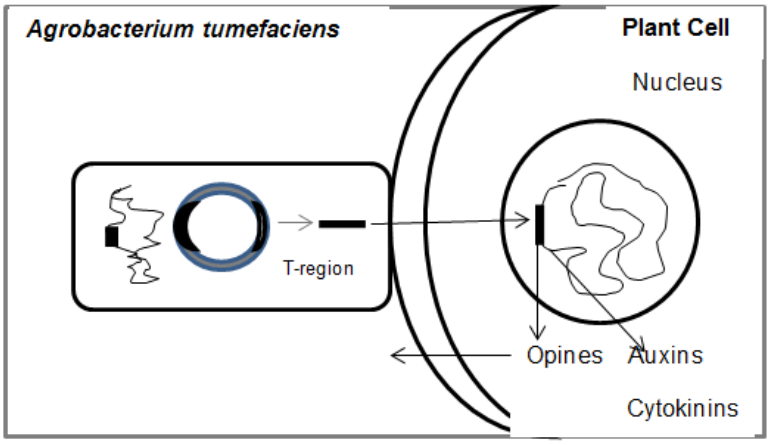


Plate 1.4. *Brassica rapa* to infection disarmed and tumor-inducing strains of *A. tumefaciens* (Simoh et al., 2009).

The presence of phenolic compounds exuded by wounded cells induced transcription of genes in virulence (vir) region of plasmid (which is referred to transfer (T) region). The T-DNA transferred to the plant genome using the mechanisms in fig (1. 4) Ti plasmid contains genes that code production amino acids called opines and plant hormones (auxin and cytokinin). In the same way, *A. rhizogenes* transfers Ri T-DNA include formation of hairy roots in infected sites.

1.1.6 Antibiotics

Antibiotics have been used to eliminate *Agrobacterium* after co-cultivation, meanwhile antibiotics specifically inhibit prokaryotic cell wall synthesis and kill bacteria (Ogawa and Mii, 2004) and show little harmful effect on eukaryotic plant cells (Pollock et al., 1983). Mathias and Mukasa (1987) investigated the effect of Cefotaxime on callus initiated from immature embryos of four barley cultivars. Callus growth was up to 45% greater on Cefotaxime than on medium and frequency of regenerating calli were increased by up 80%. In addition, co-cultivation of *Agrobacterium tumefaciens* requires the use of an antibiotic to kill the bacteria. Until now, standard antibiotics, e.g., Carbenicillin and Cefotaxime have commonly been used for elimination of *Agrobacterium*. A number of researchers presented other antibiotics, such as amoxicillin coupled with other antibiotics, singly or together with clavulanic acid (Timentin) (Schroeder et al., 1993), Later they selected highly active antibiotics against *A. tumefaciens* strains EHA105 have been selected *in vitro* (Ogawa and Mii, 2004). However, the toxicities of these antibiotics to plant tissues in addition to *A. tumefaciens* have rarely been reported (Okkels and Pedersen, 1988). Furthermore the effects of these antibiotics on *Agrobacterium* used *in planta* as well as their effects on shoot regeneration of cauliflower leaf explants has been examined. As the final goal of the research series, successfully introduced the new antibiotics

into the *Agrobacterium*-mediated transformation of cauliflower. The results suggest that application of new antibiotics for *Agrobacterium* mediated transformation is a simple and efficient strategy to improve and create transformation systems for a wide range of plant species possibly with reduced costs for eliminating the bacterium.

The use of antibiotics in culture media has led to the emergence of antibiotic resistance genes as selectable markers in transformation systems. Kanamycin, gentamycin and tetracycline have been found to be inhibitory to plant cell or tissue growth at relatively low concentrations. Fiola et al., (1990) indicated that the addition of 10 mgL^{-1} or higher kanamycin sulphate to Rubus cotyledon regeneration medium extremely reduced the growth and organogenesis of explants.

The use of rifampicin at 50 mgL^{-1} in tissue culture medium effectively controlled bacterial contaminants without affecting the growth of explants culture of *Helianthus tuberosus* (Jin Kim 1993).

1.1.7 Commercial Crop Biotechnology

In 1994 the Company Calgene produced the first commercial type of genetically modified crop, the FlavrSavr™ tomato. Since then other types of GM crops have appeared usually with the characteristics of herbicide resistance or insect resistance and the production of these genetically modified crops has increased year on year at an annual rate of over 10% per annum (Figure 1.1). 1n 1994 the cultivated area of GM crops was 1.7 million hectares and this increased to 11 million hectares by 1997 and to 44.2 million hectares in 2000 and it exceeded 200 million hectares in 2012. The countries which cultivate genetically modified crops included Argentina, Australia, Bulgaria, Canada, China, France, Germany, Mexico, Romania, Spain, South Africa, Ukraine and the United States of America (James 2011).

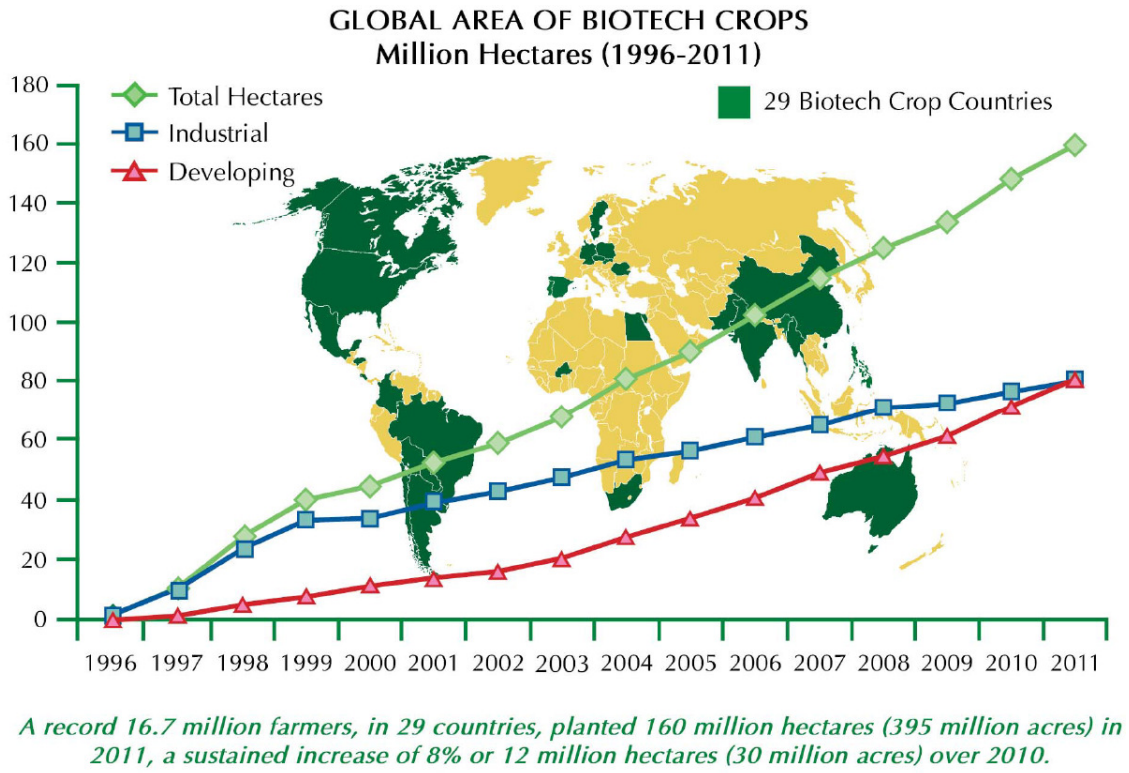


Figure 1-1. Global area of biotech crops cultivation (James 2011) (copyright clearance granted).

The development of crops resistant to biotic and abiotic stress will be critical for food production in developing countries by the year 2025, if not now. Thomson (2003) has a potential development in both transgenic and other technology. In developing countries the discussion about the importance of GM crops that leads to an increase in food production with good quality and lower price. The scarcity of food in developing countries with high prices which affect most people's income which is a real concerned; therefore the application of new crop varieties needs a comfortable biological security legislations for those crops and economical expertise, but the availability of organizations working for the establishment for management, and watching the GM crop applications. In conclusion most GM crops are concerned with herbicides tolerance, the resistance of GM crops to biotic and abiotic stress

conditions. GM crops are reducing the cost of crop production (Hayes, 2005). The techniques applying to different types of plant depending depends on the type of plants if dicotyledonous or monocotyledon (Fromm et al., 1985).

It is predicted that there are many possible advantages of using genetically modified plants in developing countries; firstly higher productivity of plants, secondly reduction of agriculture input costs, thirdly farmer income increases, fourthly improvements in human health and environmental conditions. The first generation group of genetically modified crops improved the ability of these crops to decrease the costs and increase nutritional value. Furthermore there is intense interest in the production of rice rich in iron and vitamin A as well as production of potatoes with high starch content and the production of corn and potatoes with vaccines through oral intake and the production of corn which can grow in very stressful conditions, and the production of safe oils for health from soybeans and canola.

The risk from the production of genetically modified crops occurred by an application of any type of new technology. There are many expected risks for instants; the risk produce from entrance of material causes the allergic and decrease the nutritional value of foods as well as possibility of transfer the genes from GM crops in cultivated area to the wild crops. In addition to the possibility of increase the resistance of insects and pest to the pesticides and insecticides that produced from GM crops. The poisoning probably extended to unexpected living organisms therefore the importance of establishment or produce legislations and regulator rules which in this case prevent or reduce. The responsibility of scientists who discovered this technology also the workers on this field who responsible from health security, community and environmental protection. There are many risks produced not from this technology exactly but the gap extended to developing new technology

comfortable with the needed of poor people and make them using easy of this technology (i.e. the gap between developed and developing countries).

What are the advantages of GM foods?

The world population has now reached 6 billion people and is expected to double in the next 50 years. GM foods promise to meet this need in a number of ways:

- The production of high-quality food must increase with reduced inputs because of the increasing in people living on this world (Tester and Langridge, 2010).
- Poor people depend on rice as the main staple diet in many third world countries but often suffer from malnutrition (Khush et al., 2012). However, rice does not contain adequate amounts of all necessary nutrients to prevent malnutrition. Genetically engineered rice can contain additional vitamins, minerals and nutrient deficiencies could be improved. The common problem in the third world countries is blindness due to vitamin A deficiency. "Golden" rice containing an unusually high content of beta-carotene (vitamin A) (Tang et al., 2012) is an example of how GM crops can be used against malnutrition. Since this rice was funded by the Rockefeller Foundation, a non-profit organization, the Institute offers the golden rice seed free to any third world country that requests it.
- Pharmaceuticals Medicines and vaccines frequently are costly to produce and sometimes in third world countries require special storage conditions. Edible vaccines developed by researchers are working in tomatoes and potatoes (Daniell et al., 2001). These vaccines will be much easier to store, ship, and manage than traditional injectable vaccines.

- Herbicide tolerance for some crops, physical means such as tilling it is not cost-effective to remove weeds, thus farmers will regularly spray large quantities of different herbicides to destroy weeds, a time consuming - and expensive process. Genetically-engineered crop plants resistant to herbicide could help prevent environmental damage by reducing the amount of herbicides needed as in soybeans (Preston, 2002) .
- Pest resistance; insect pests can cause staggering crop losses, leading to financial loss for farmers and starvation in developing countries. Annually, many tons of chemical pesticides are used by farmers. Consumers do not wish to eat food that has been treated with pesticides because of potential health hazards, and run-off of agricultural wastes from extreme use of pesticides and fertilizers can be toxic to the water supply and cause harm to the environment. Growing GM foods such as B.t. corn can help eliminate the application of chemical pesticides and reduce the cost of bringing a crop to market (Barton et al., 1987, Daniell et al., 2001).
- Disease resistance; Plant biologists are working to create plants with genetically-engineered resistance to many diseases (viruses, fungi and bacteria) that cause plant diseases. (Dahleen et al., 2001, Scorza et al., 2001). Caiy (2012) described one of the most serious diseases in cotton Verticillium dahliae (a soil-borne pathogen) which causes *Verticillium* wilt, deleteriously influencing crop's quality and production.
- Cold tolerance Sensitive seedlings can be destroyed by unexpected frost. An antifreeze gene from cold water fish has been introduced into plants such as tobacco and potato (Kenward et al., 1999).

- ❖ Drought tolerance/salinity tolerance; as the world population grows and more land is used for housing instead of food production; farmers will need to grow crops in locations previously unsuited for plant cultivation. Creating plants that can withstand long periods of drought or high salt content in soil and groundwater will help people to grow crops in formerly inhospitable places (HX Zhang, 2001).

How does genetic engineering compare to traditional breeding?

Although the goal of both genetic engineering and traditional plant breeding is to improve an organism's traits, there are some key differences between them.

I. While genetic engineering manually moves genes from one organism to another, traditional breeding moves genes through crossing to obtaining offspring with the desired combination of traits.

II. Using traditional breeding. The product is half from each original. Therefore, half of the genes in the offspring of a cross come from each parent.

III. Breeding together with the generation of transgenic plants, has led to the production of commonly used transgenic cultivars in several main cash crops, such as cotton, maize, soybean, and canola. The opportunity to generate unique genetic variation in Genetic engineering that is either has very low heritability or absent in the sexually compatible gene pool as described by (Wang and Brummer, 2012).

IV. Traditional breeding is effective in improving traits; however, when compared with genetic engineering, it does have disadvantages. Since breeding relies on the ability to mate two organisms to move genes, trait improvement is basically limited to those traits that already exist within that species.

I. Genetic engineering, on the other hand, physically removes the genes from one organism and places them into the other. This eliminates the need for mating and allows the movement of genes between organisms of any species. They have a high potential to pass their genes to adjacent plants compared with inbreeding (Wang and Brummer, 2012). Genetic engineering, though, permits for the movement of a single, or a few genes.

II. Breeding is also less precise than genetic engineering. In breeding, half of the genes from each parent are passed on to the offspring. This may include many undesirable genes for traits that are not wanted in the new organism.

1.1.8 What are the limitations?

Plant transformation is at a threshold. Over 3000 field trials of transformed plants are in progress or completed in at least 30 countries. (Birch, 1997a) involving over 120 diverse plant species modified for various economic traits.

- In tissue culture systems for plant transformation, what is most important is a large number of regenerable cells that are accessible to the gene transfer treatment, and that will retain the capacity for regeneration for the duration of the essential target preparation, cell proliferation, and selection treatments (Birch, 1997a). A high multiplication ratio from a micropropagation system does not necessarily indicate a large number of regenerable cells accessible to gene transfer (Livingstone and Birch, 1995).
- Gene transfer into potentially regenerable cells may not allow recovery of transgenic plants if the capacity for efficient regeneration is short-lived (Grando et al., 2002).

- Some tissue culture systems based on features affecting suitability or efficiency, comprising ready availability of explants, and minimal time in tissue culture (Birch, 1997a).
- Limiting factors remain the ability to mechanically prepare the explants, transfer genes into regenerable cells, and select or screen for transformants at an efficiency sufficient for practical use in cultivar improvement (Birch, 1997b).
- For *Agrobacterium*, it is considered more efficient to first establish the conditions for gene transfer and then work on conditions for regeneration of transformed cells (Hood et al., 1993). This contrast may be biologically well-founded, because of the more complexity and lesser understanding of the biological communication preceding the gene transfer event from *Agrobacterium.*
- Unfortunately, there is no guarantee that a transformable plant cell type will prove regenerable of the most successful tissue culturist (Potrykus, 1991).
- If initial transformation experiments using techniques successful in similar plant systems are not successful, the best advice is to establish by histological studies the precise cellular origins and timing of events leading to plant regeneration within the explants to be used as targets for gene transfer (Potrykus, 1991). This is likely to avoid much wasted time and prevention from optimizing gene transfer and regeneration within the same region of the explant, but potentially in different cells, so that transformed plants are unlikely to result. Work in sunflower is a fine example of the value of this relatively simple check to explain and potentially overcome difficulty in transformation of a recalcitrant species (Laparra et al., 1995, Birch, 1997b).

- Assays for transient expression of introduced reporter genes in plant cells can provide unequivocal evidence of gene transfer. A great deal of time can be wasted unless this analysis is focused on regenerable cells (Laparra et al., 1995, Birch, 1997b).
- There is little information on forms of DNA or sequences that may increase the frequency of stable transformation (Benediktsson et al., 1995, Buising and Benbow, 1994, Staskawicz BJ, 1995).
- There is considerable batch-to-batch variation in the frequency of transient expression events following cocultivation with *Agrobacterium* (Janssen and Gardner, 1990). The correlation between transient expression and stable transformation has not been thoroughly tested.
- *Agrobacterium* employs a highly evolved and still incompletely understood gene transfer and integration system that appears optimized for efficient nuclear targeting and integration of a protein-complexed single stranded DNA introduced as a small number of copies per cell (Zupan and Zambryski, 1995).
- It may be essential to introduce a considerable number of genes into plants for some purposes. The limits of available gene transfer techniques have not yet been defined.
- Other key variables in *Agrobacterium*-mediated gene transfer include *Agrobacterium* and plant genotype, treatment with *vir* gene inducers such as acetosyringone, wounded cell extracts, feeder cells or sugars; pH, temperature, cell concentration, light conditions, and duration of cocultivation; explant type, quality, preculture (van et al., 1992), hormone treatment (Gafni et al., 1995), wounding, or infiltration (Bidney et al., 1992); and use of appropriate antibacterial agents (Lin et al., 1995), antioxidants(Peri et al.,

1996), ethylene antagonists(De Block et al., 1989), and/or methylation inhibitors (Palmgren et al., 1993) to reduce damage and/or gene silencing in treated plant cells.

1.2 Environmental stresses

Environmental stress effects on plant growth and development determine the yield and reproductive success. Microorganisms must be able to identify environmental change and respond with metabolic modifications through appropriate changes in protein activity and gene expression (Hecker et al., 1996, Sonenshein, 2000). Bacterial cells respond to physical and chemical challenges that include shifts in temperature, salinity, oxygen concentrations and nutrient deficiency (Colwell, 2000, Oliver, 2005). Biotic and abiotic stresses are considered as limiting factor for growth and productivity (Boyer, 1982, Rathinasabapathi, 2000, Gill et al., 2003, Seki et al., 2003). Selye, (1936) developed the frost concept of stress and (Levitt, 1980) defined eight stresses: chilling, freezing, heat, salt, drought, flooding UV and X-rays (Figure 1.2).

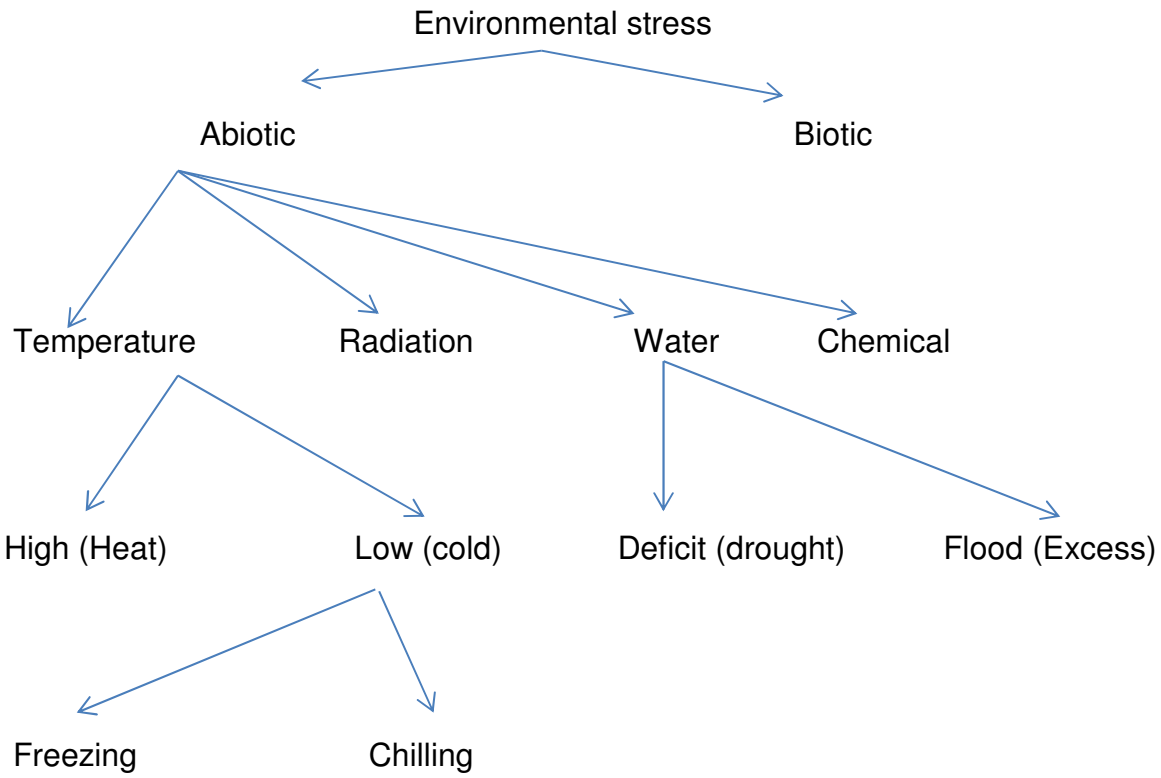


Figure 1-2. The classification of the kinds of environmental stresses to which organism may be subjected.

Abiotic environmental stresses are common threats and present major limitations to plant growth and crop production system all over the world. Paramount among these stresses is excessive salt, freezing and drought. Various scientists define stress differently according to their mode of studies. According to (Lichtenthaler, 1998), any condition or substance that blocks a plant's metabolism, growth, or development is regarded as stress. Farhatullah; et al (2002) defined stress as any factor that decreases plant growth and reproduction below the genotype's potential. Adjustments made by plants such as changes in physiology, biochemistry and metabolite build up caused by stress can be regarded as the stress responses. Plant stress responses can be divided into two principal components, adaptation and acclimation.

Adaptation is typically a response to long-term environmental changes and alteration in the genome are stable and retained in the population over generations by natural (or non-directed selection). In contrast, acclimation is normally a temporary response induced by an environmental change that causes phenotypic alterations without changes in the genetic complement. Acclimation is the term used to describe the transition from a less hardy status to a more hardy status. Acclimation is usually initiated in response to a mild stress response mechanism that is characterized by transient, physiological, biochemical and molecular perturbation (Levitt, 1980, Sakai, 1987, Kerepesi et al., 2004).

Based on the original concept of stress proposed by (Selye, 1936) and extended by ideas given by (Lichtenthaler, 1998), responses to stress can be differentiated into four phases.

1. Response phase: beginning of stress
   - deviation from functional average
   - decline of vitality
   - catabolic process exceeds anabolism
2. Restitution phase :continuing stress(acclimation)
3. End phase (long term stress)
   - stress intensity too high
   - overcharge of the adaptation capacity
   - chronic disease or death
4. Regeneration phase; partial or full regeneration of physiological function, when the stressor is removed and the damage is not severing.

At the beginning of stress, plants respond with a decline in physiological function such as the performance of photosynthesis. Acute damage and senescence will occur rapidly in those plants that possess only low or no stress tolerance mechanisms and thus have a low resistance. At this alarm phase most plants will, however, activate their stress coping mechanism such as acclamatory metabolic and morphological adjustments. This general alarm syndrome will cause a hardening of the plants by establishing a new physiological standard until an optimum stage of physiology under impact of the stressor has been achieved and corresponds to the plants maximum resistance.

Under long term stress or with a stress dose which overloads the plants stress coping mechanisms the stage of exhaustion becomes apparent in which physiology

and vitality are progressively lost, causing severe damage and cell death, which, when the stressors are removed progresses rapidly to senescence. If the intensity and duration of stress are not too high and long, the plants will orient themselves within the range set by the resistance minimum and maximum and damage symptoms might not be detectable but there will always be some metabolic cost (Lichtenthaler, 1998).

Abiotic stresses such as salinity, drought, freezing, oxidative stresses and chemical treatments are serious threats affected on agriculture production and the environment. Molecular mechanisms control the acclimation response typically through a transcription factor and the subsequent upregulation of an abiotic stress regulon involving hundreds or even thousands of genes and gene products. Many researchers have sought to identify key genes in these pathways and put these genes by genetic modification into susceptible crops to control abiotic stress that to be relevant in agriculture (Zhang et al., 2000). Achievement of genetic engineering approaches to improving plant abiotic stress resistance salinity, drought, and extreme temperature have been recorded in transgenic plants that expressed in genes regulating antioxidants, specific protein, transcription factors and membrane composition.

The dramatic increase in world population with increasing the deterioration of arable land, water scarcity and increasing environmental stress create serious threats to universal agricultural production and food scarcity. Even though focused effort to improve the major food products for abiotic stress resistance has been a goal for many years traditional breeding success has been very limited. Recently, it has been shown that it is possible to improve abiotic stress tolerance in agricultural crops with a few target traits through transgenic approaches (Bhatnagar-Mathur et al., 2008).

1.2.1 Salt stress

Water quantity and quality are major problems for agricultural production. Salinity is principally regarded as an inherited problem of irrigated culture, ultimately restricting crop yield and sustained production in sizeable parts of the world. Soil salinity existed long before humans and agriculture but the problem has been aggravated by irrigation. Salinity affects 7% of the world's land area, which amounts to 930 million ha (Vengosh, 2003). Munns and Rawson,(1999) indicated that 5 % (77million ha) of the 1.5 billion ha of cultivated land is affected by salts. Wang et al., (2001) predicted that increasing salinization of arable land will have devastating effects resulting in a further 3% land loss within the next 25 years and up 50% by the year 2050. Increased salt tolerance in crops is widely recognized as an effective way to overcome the limitation of production in saline area (Epstein, 1983, Flowers, 2004).

The problem of excessive salt accumulation in top soils is enhanced by poor agricultural practices, which include most especially irrigation water which in most cases contains low levels of salt which accumulate with successive irrigation. Most irrigated land, which produces about one third of the world's food, is at risk of the saline problem (Munns et al., 2000). Other factors which contribute to excessive salt accumulation in soils are natural processes and poor drainage conditions. In the semi-arid regions there is frequently insufficient rain to leach away soluble salts. Evaporation from the soil surface and transpiration from crops removes water but leaves the salts in the soil. The soil salinity problem is increasing both because of inadequate irrigation and drainage practices and because the water used irrigation, whether from a river or from wells, is never pure but always contain dissolved salt. All soil contains salt but there are differentially taken by plants and levels of sodium

can accumulate disproportionally to others and it is high levels of sodium that tends to define the salinity problem.

1.2.2 Mechanism of salt resistance

Salt resistance means the ability of plants to grow satisfactory in saline soil (Levitt, 1980), however the term of ' salt resistance' is used in a broad sense, which includes: salt tolerance, when plants respond to salinity stress either by accumulating salt generally in their cells or in specific cells such as in salt glands and salt avoidance, when plants avoid salt stress by maintaining their cell salt concentration unchanged either by water absorption or salt exclusion. Nonetheless, the term ' salt resistance' remains to describe the capacity of plants to grow on salty soils whether they accumulate ions in their cells or exclude the salts. Tolerance seems to be the more evolutionary primitive adoption and avoidance more advanced, and this is reasonable, since tolerance involves an equilibrium state, and avoidance requires development by the plant of a mechanism to avoid equilibrium and replace it by the steady state. Avoidance is also more efficient adaptation; by avoiding the stress, the plant avoids both elastic (reversible) and plastic (non-reversible) strains. Plants are therefore by this mechanism, able not only to survive when exposed to the stress, but also metabolize, develop, and complete their life cycle. Tolerance merely permits the plant to survive until such time that the stress is removed and the plant can recommence its normal metabolism, growth, and development.

Hernández et al.,(2000) defined the determination of salt stress tolerance by the presence of effector molecules (metabolites, proteins or other components of biochemical pathways) that control the amount and timing of resistance. Stress adaptation efforts are categorized as those that mediate ion homeostasis, osmolyte

biosynthesis, toxic radical scavenging (including ROS) and water transport. Osmoregulation is a common response to salinity stress, which allows maintenance of turgor and thus avoidance of cell desiccation and the associated repercussions of turgor loss. Plants usually accumulate specific types of inorganic and organic molecules in response to salt stress. They serve the primary function of maintaining the cytoplasmic osmotic balance and can accumulate to high concentrations without impairment of normal physiological function and are known as compatible solutes. On the other hand, Ashraf et al.,(2001) demonstrated that salt tolerance is very complex in most plant species because of the numerous mechanisms at cellular, tissue, organ or whole plant levels.

Tester,(2003) showed that mechanisms of tolerance show large taxonomic variation. These mechanisms occur in all cells within the plant, or can occur in specific cell types, reflecting adaptations at two major levels of organization: those that contribute to tolerance of cells and those at the whole plant level. High salts disrupt homeostasis in water potential and ion distribution. The disruption of homeostasis occurs both at cellular and the whole–plant levels and drastic changes in ion water homeostasis lead to molecular adjustment growth arrest and even death. The integrity of cellular membranes and their activity, nutrient acquisition and the function of the photosynthetic apparatus are all known to be prone the toxic effect of high salt stress. An important cause of damage is thought to be reactive oxygen species (ROS) generated in response to salt stress (Zhu et al., 1997, Hernández et al., 2000) Plants salt tolerance depends primarily on characteristics that can be broadly grouped in three categories: 1- physical uptake or exclusion of salt followed by transport and compartmentation of salt; 2- structural features and biomass distribution to shoots and roots, which include rates of transpiration and stomata

closure; 3- physiological and metabolic events that counteract the diverse effects of salt at the cellular level. These characteristics could be the primary target for manipulation in molecular engineering of salt stress tolerance. Multicellular adaptation to an increase in the ionic environment implies integrated changes in regulation of gene expression for groups of functionally related genes (Winicov, 1998, Huazhong Shia, 2002).

It is recognized that salt affects virtually all aspect of a plant's physiology from ion transport, selectivity, excretion, nutrition and compartmentation together with growth, water use and water use efficiency (Harkamal Walia and Steve I. Wanamaker, 2005). Several studies have indicated that plants under saline conditions establish physiological mechanisms of salt tolerance such as osmotic adjustment against tissue water loss, ion uptake and transport control against ion toxicity (Kumar et al., 2009, Ashraf, 2004). The synthesis of compatible organic solutes or accumulation of inorganic solute achieves osmotic adjustment in the cells, which prevent internal water loss resulting in the maintenance of water relations.

Garcia et al.,(1997b) demonstrated that physiological characteristics contributing to the resistance of salinity include: 1-reduced salt transport to the shoot which may be a consequence of low transpiration-bypass flow or of high water-use efficiency, 2-plant vigour which acts to dilute, through growth, the salt within the tissue,3-compartmentation of salt away from young expanding or photosynthesizing leaves, 4- tolerance of salt within the tissue which reflect differences in the distribution of salt between apoplast and protoplast or cytoplasm and vacuole.

Since salt is injurious to plants if absorbed in high concentration, ion exclusion at the root would be an effective mechanism for avoiding injury. Schachtman and Munns,

(1992) proposed two mechanisms of salt tolerance, one is lower tare of Na+ accumulation that is independent of the growth of individual leaves and therefore probably regulated by some root process. The second is ion compartmentation within leaves that enhances the ability to tolerate high concentrations of Na+. Yeo and Flowers, (1986) on the other hand, found that old leaves accumulate much higher salt levels than young leaves. A major characteristic of solute transport of plants in saline conditions is the degree of selectivity between Na and K (Greenway and Munns, 1980). Basically, plant membrane channels cannot distinguish between Na and K and an influx of Na limits the uptake of the essential element K leading to reduced metabolic function.

Mahmood,(1991) suggested that for survival in high salt levels a plant has to overcome two main problems A- the solute potential of saline water is very low and to take in water from saline soil a resistant plant must achieve an even lower intracellular water potential. B- The plant should be able to overcome specific ion effects, since high concentrations of ions, particularly Na and Cl, are toxic and after a certain level can be lethal. Glenn,(1987) considered that internal cells and tissues of plants are in general exposed to much lower NaCl level than occurs in the external solution and nutritional and osmotic effects are more common than direct salt damage for a plant growing within its salt tolerance range.

1.2.3 Effect of salinity on plant growth

The most common adverse effects of salinity on plants include reduction in height and size, and the deterioration of growth, yield and physiological function and the reduction in the quality of the harvested product. The first and most common symptom of salt injury is a reduced rate of plant growth (Araus et al., 2002, Tilahun et al., 2003). Plants growing in saline soil are usually smaller than normal, may have darker leaves than normal and will wilt from drought sooner than in a non-saline soil. As salinity increases, plant growth will eventually cease and leaf burn, commencing at the tip will occur. Ultimately there will be plant death in highly saline soil (Chauhan and Prathapasenan, 2000). Plant growth under saline conditions requires additional expenditure of metabolic energy, usually expended for the synthesis of compatible organic solutes and the increase of enzyme activity.

Greenway and Munns,(1980) suggest that deleterious effects of salinity on plant growth were attributed to specific ion toxicity and nutrient ion deficiency. Carcia et al.,1997 indicated that NaCl reduced the growth of plants and caused chlorophyll loss in leaves of rice due to disrupted chloroplast integrity. (Zeng and Shannon, 2000) reported that the reduction in seedling survival rates and growth are major causes of crop loss in salt-affected rice field. Heuer and Nadler,(1995) showed that plant height, leaf area and fresh weight accumulation were significantly affected by salinity whereas stem number, leaf elongation and the content of dry-matter in leaves, stems and tubers were hardly affected.

1.2.4 Frost stress

For 64% of the earth's land mass the mean minimum temperature over the whole year is below 0˚C and for 48% it is below -10˚C (Deane, 1994). Freezing limits the

geographical distribution of plants (Becwar et al., 1981, Steponkus, 1984, Li et al., 2004). In the case of economically important plants, frost can cause significant losses to crop production for example in Cornwall in SW England, severe winter weather years have caused large economic losses (Tapsell et al., 1990). Frost stress resistance is intimately linked with drought and salt stress and many genes that are regulated by frost are also responsive to drought and salt stress (Zhu et al., 1997). These three major abiotic stress factors strongly limit plant productively by reduced the growth of plants caused by chlorophyll loss in leaves due to disrupted chloroplast integrity (Carcia et al., 1997); decrease the content of dry matter (Heuer and Nadler, 1995); dehydration and disruption of the cell (Thomashow, 1999a) membrane damage (Tao et al., 1998) protein denaturation (Guy et al., 1998) and growth arrest and even plant death (Tester, 2003).

1.2.5 Freezing injury

The primary problem that plants face when exposed to freezing temperatures is ice formation. Ice crystals can cause severe injuries to living cells that can eventually lead to the death of the plant.

The freezing process in plant tissue is affected by the following factors; a)the water saturated gaseous environment surrounding cells; b) the relatively small amount of osmotically available extracellular water (apoplastic 5 -10%) relative to the amount available inside living cells (symplastic 90 - 95%); c) the lower solute concentration of the apoplastic solution compared to the cell; d) the greater freezing point depression of cellular water because of its higher solute concentration; e) a functionally intact cell membrane which is an effective barrier to the propagation of ice crystals; f) liquid water which can freely move across the plasma membrane in

either direction; g) the effectiveness of the cell membrane (which acts as a barrier to ice which may vary with cold acclimation or temperature); h) the presence of heterogeneous nucleators inside the cell which may be minimised, excluded or masked; and i) a portion of the extracellular volume as free air space normally saturated with water vapour. The other major factor in the freezing of plant cells is the rate of cooling. Ice formation may occur and intracellular freezing is suspected to occur when cooling rates are rapid or after significant super cooling has occurred by delayed ice nucleation. Super cooling is where the water is cooled to a temperature below its melting point without ice formation, and the super cooling point is the lowest subfreezing temperature attained before ice formation (Levitt, 1980). In contrast, extracellular freezing occurs in the apoplastic spaces. Ice will spread from the initial nucleated point through the extracellular space in the apoplastic water and as long as the plasma membrane is intact and cooling rate is slow (1 - 2˚C hr^{-1}) ice will remain confined to the extracellular region and will gradually draw water from the cell (ice has a lower water potential than liquid water) until equilibrium of water potential is achieved relative to the temperature and the solute concentration of the cell (Wisniewski and Fuller, 1999).

(Thomashow, 1999a) explained that when the temperature drops below 0˚C, ice formation is generally initiated in the intracellular space due, in part, to the extracellular fluid having a higher freezing point (lower solute concentration) than the intracellular fluid. Because the chemical potential of ice is less than that of liquid water at a given temperature, the formation of extracellular ice results in a drop in water potential outside the cell, leading to a flow of water (as vapour) from the cell to the ice effecting a dehydration of the cell. Steponkus et al., (1993) indicated that membrane systems of the cell are the primary site of freezing injury in plants and

established that freeze-induced membrane damage results from the severe dehydration associated with freezing.

Ice also forms in xylem vessels and then spreads to other parts of the plant through the vessels (Sakai, 1987). Uprooted barely organs under laboratory conditions were reported to freeze in the order of nucleated leaf, roots, older leaves, younger leaves and secondary tillers, respectively (Pearce and Fuller, 2001).

Multiple forms of membrane damage can occur as a consequence of freeze-induced cellular dehydration including expression induced lysis, lamellar-to-hexagonal-phase transition, and fracture jump lesions (Steponkus et al., 1993). Freeze-induced production of reactive oxygen species also contributes to membrane damage. Also, protein denaturation in cells at low temperature, often brought about by the severe dehydration caused by freezing, can potentially result in cellular damage and death (Guy et al., 1998) .

Olien and Smith,(1977) believed that freezing causes extracellular ice adhesion to cell walls and membranes which then causes cell disruption. Bartels and Nelson,(1994) and Zhu et al.,(1997) indicated that the increased osmotic stress due to cellular dehydration by ice formation is the main cause of damage. Ball et al.,(2004) confirmed that freezing in unacclimated tissues caused irreversible tissue death, but acclimated leaves showed reversible symptoms during freezing, including massive extracellular ice formation in specific expansion zones within the mid vein.

1.2.6 Freezing acclimation and tolerance

Freezing acclimation is the positive effect of exposure to mild stress of the plant on subsequent resistance to freezing. During exposure of plant to low but non-freezing temperatures, many changes in physiological and biochemical parameters have been observed such as; modification of carbohydrate concentrations (especially sucrose, increasing cellular osmotic concentration), proteins developing more resistant low temperature forms, increased proline concentration (compatible solute), altered lipid composition of the cell membrane (Bartels and Nelson, 1994), reduced cell size, increased flexibility of cell walls and changed permeability of membranes. Blum,(1988) defined freezing resistance in terms of the internal interaction among chemical and physiological parameters during a freeze-thaw cycle of cells and tissues and believed that the increased osmolality of the cellular solute is an effective mechanism for avoiding intercellular ice formation and reduces cellular dehydration. The resistance of higher plants to freezing has two main components, tolerance and avoidance.

The ability of plants to survive freezing is dependent on many factors including; likelihood of ice nucleation, site of ice nucleation, rate of ice growth, minimum temperature of exposure and duration of exposure to freezing treatments and rate of cooling during crystallization. Levitt (1980) decided that the ability to survive freezing is dictated by the genetic makeup of the plant, stage of development and environmental factors.

Thomashow, (1999a) demonstrated that cold acclimation is characterized by the expression of certain cold-induced genes that function to protect membranes against freeze induced in injury and (Farhatullah; et al., 2002, Guy, 1990)suggested that

acclimation induced cold tolerance is a quantitative character controlled by a number of additive genes.

1.2.7 Molecular responses to acclimation

Many changes in mRNA levels have been observed during preconditioning of plants to water deficit inducing abiotic stresses (salt, cold and drought). Many genes with various functions have now been described (Thomashow, 1999a, Shinozaki et al., 2000, Zhu, 2002) . Shinozaki et al., (2003) revealed that many genes are induced by abiotic stress to function in the stress response mechanisms and modify plant tolerance. The products of these genes are involved not only in the protection of cells against stresses, but also in the further regulation of gene expression and signal transduction pathway in the abiotic stress response.

1.2.8 Reactive Oxygen Stress

ROS are reactive molecules that contain the oxygen atom. They are very small molecules that include oxygen ions and peroxides and can be either inorganic or organic (Plate 1.5). They are highly reactive due to the existence of unpaired valence shell electrons. ROS form as a natural byproduct of the normal metabolism of oxygen and have significant roles in cell signaling. However, during times of environmental stress ROS levels can increase dramatically, which can result in significant damage to cell structures. This cumulates into a situation known as oxidative stress. ROS are also generated by exogenous sources such as ionizing radiation.

Several stresses such as high temperature, high light intensity and osmotic stress lead to overproduction of ROS (e.g. peroxide, superoxide), causing extensive cellular damage and inhibition of photosynthesis. This phenomenon is called oxidative stress

and is known as one of the major consequences of environmental stresses (Sunkar et al., 2003). Plants have evolved systems to combat oxidative stress. Different enzymes aid in reducing the active oxygen species in order to protect the plant cell from damage. To minimize the concentration of ½ O_2 and H_2O_2 with enzymes overproduced or upregulated include superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR) and glutathione-synthesizing enzymes. Several enzymes can efficiently detoxify reactive oxygen, however, during prolonged stress conditions, such detoxification systems get saturated and can damage occur (Van Breusegem et al., 1999). A major hydrogen peroxide-detoxifying system in both plant chloroplasts and cytosol is called the ascorbate-glutathione cycle, in which APX is the key enzyme.(Hernández et al., 2000) report NaCl-induced enhanced mRNA expression and activity of Mn-SOD, APX, GR and mono-dehydro-ascorbate reductase (MDHAR) in tolerant pea cv. Granda, whilst in the salinity-sensitive cv. Chillis, no significant changes in activity and mRNA levels of the above enzymes were observed suggesting considerable genetic availability which may be amenable to genetic selection or manipulation through biotechnology.

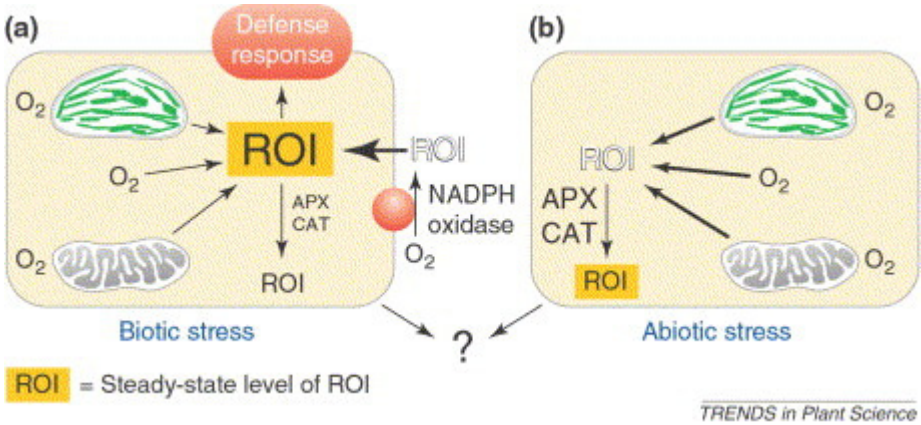


Plate1.5. Differences in the steady- state levels of reactive oxygen intermediates (ROI) during biotic stress and abiotic stress. Source (Mitter, 2002).

Sato et al.,(2001) reported that, when rice seedling were kept at 42℃ for 24 h before being exposed to 5℃ for 7 days, chilling injury did not occur - i.e. chilling resistance was enhanced in parallel with the period of heat-treatment (heat stress). The level of APX activity was found to be higher in the heat treated rice (*Oryza sativa L.*) seedlings whilst there was no significant difference in SOD activity between heated and unheated seedlings. Allen, (1995) showed that the physiological and genetic evidence clearly indicates that the ROI scavenging systems of plants are an important component of the stress productive mechanism.

APX exists as isoenzymes and plays an important role in the metabolism of H_2O_2 in higher plants. APX is also found in eukaryotic algae. The characterization of APX and the sequence analysis of their clones have led to a number of investigations that have yielded interesting and novel information on this enzyme. Interestingly, APX isoenzymes of chloroplasts in higher plants are encoded by only one gene, and their mRNA are generated by alternative splicing of the gene's two 3'-terminal exons. Manipulation of the expression of the enzymes involved in the ROI-scavenging systems by gene-transfer technology has provided a powerful tool for increasing the present understanding of the potential of the defense network against oxidative damage caused by environmental stresses. Transgenic plants expressing *E. coli* catalase to chloroplast with increased tolerance to oxidative stress indicate that ROI, such as scavenging enzymes, especially chloroplasts, APX isoenzymes are sensitive under oxidative stress conditions. It is clear that a high level of endogenous ascorbate is essential effectively to maintain the antioxidant system that protects plants from oxidative damage due to biotic and a biotic stresses (Shigeoka et al., 2002, Gupta et al., 1993).

1.2.9 Damaging effects

Cells are normally able to protect themselves against ROS damage through the use of enzymes such as superoxide dismutase, catalases lactoperxidases, glutathione peroxides and peroxiredoxins. Small molecule antioxidants such as ascorbic acid (vitamin C), tocopherol (vitamin E), uric acid, and glutathione also play important roles as cellular antioxidants. Likewise, polyphenol antioxidants assist in preventing ROS damage by scavenging free radicals. In contrast, the antioxidant ability of the extracellular space is less. Effects of ROS on cell metabolism have been well documented in a variety of species. These include not only roles in apoptosis (programmed cell death), but also positive effects such as the induction of host defense genes and mobilization of ion transport systems. This associates them more frequently with roles in redox signaling or oxidative signaling.

1.2.10 Ascorbate peroxidase

Ascorbate peroxidase (APX) is a class 1 peroxides that catalyses the conversion of H_2O_2 to H_2O and O_2 using ascorbate as the specific electron donor. This enzyme has a key function in scavenging ROS and the protection against the toxic effects of ROS in higher and lower plants. In rice, the APX gene family has eight members, which encode two cytosolic, two putative paroxysmal, and four chloroplast isoforms, respectively. APX has a fundamental role in photosynthetic organisms. Chloroplasts are the major sources of superoxide and H_2O_2. The genome databases of *A. thaliana* were also analysed using a methodology similar to that described for the rice sequences. Rice constitutes and excellent model to access the function of APX from different cell compartments (Teixeira et al., 2006)

The study of Bagis et al.,(2005) showed that free radical oxygen plays an important role in senescence and thereby in stress responses. Therefore the gene family

encoding H_2O_2 scavenging enzyme APX could be a key target transgene. There are three levels of mRNA quantified for cytosolic APX (APX1, APX2, APX6), two types of chloroplast (thylakoid tAPX, stromal sAPX) and three microsomal isoforms n(APX3, APX4, APX5) identified in the genome of *Arabidopsis*. The gene chloroplast thylakoid tAPX exhibits related decrease mRNA in leaves derived from plants in different ages while there is no remarkable change in mRNA levels with microsomal APX3, APX5 and cytosolic APX1, APX2, APX6.

A study by Koyro et al., (2012) investigated high temperature stress as one of the main factors constraining the growth and productivity of plants. It was shown that oxidative stress can be caused by heat shock and this encourages genes involved in the oxidative stress defence (Morgan et al., 1986). Under heat stress, severe Active Oxygen Species (AOS aka ROS) such as superoxide radicals, hydrogen peroxide, and hydroxyl radicals are created to effect oxidative injury of cell constituents (Shi et al., 2001).

APX activity has also been shown to rise in response to a number of stress conditions, such as drought (Mittler and Zilinskas, 1994), salt (Shalata and Tal, 1998) and a deficiency in microelements (Chao et al., 2012).

1.2.11 Hydrogen peroxide

H_2O_2 is a very pale blue liquid, a little more viscous than water that seems colorless in dilute solution. It is a weak base, has strong oxidizing properties, and is an excellent bleaching agent. It is used as a disinfectant, antiseptic and oxidizer. Hydrogen peroxide is naturally produced by organisms as a by-product of oxygen metabolism. More or less living things have enzymes known as peroxidases, which safely and catalytic decompose low concentrations of hydrogen peroxide to water and oxygen.

1.2.1 Breeding and selection for abiotic stress resistance

Breeding strategies for abiotic stress resistance have generally depended on screening techniques looking for tolerance mechanisms, genetic diversity, genetic mode and heritability. The breeding procedure for these stresses depend upon the pattern of inheritance, qualitative and quantitative genes, the number of genes with major effects, and the nature of the action of these genes. The association between heritability and stress in plants has attracted considerable attention because of the implication for plant breeding experiments aimed at obtaining rapid selection response. An understanding of the genetic basis and knowledge of the genetic control of abiotic tolerance in any species will be helpful for highly yielding and good plant type with tolerance to stress. The first recorded attempt to evaluate the inheritance of salt tolerance was made by Lyon, (1941). Carbonell et al.,(1992) considered that a genetic component was involved in salt tolerance with not only additive but also non-additive effects. Furthermore Zhu et al.,(1997) indicated that many genes that are regulated by salt stress are also responsive to drought or freezing stress. Heterosis was apparent under saline (NaCl) conditions in the elongation of stems in hybrids of *Lycopersicon esculentum* produced with three wild species (*L. cheesmanii, L.peruvianum*, and *L. pennellii*) (Tal and Shannon, 1983b). Total dry matter production of another F1 hybrid, between *L. esculentum and L. pennellii*, showed hybrid vigour under saline conditions (Saranga et al., 1991). Analysis of other species has also suggested that the genetics of slat tolerance is complex. There is also evidence of dominance in the salt tolerance of sorghum. Diallel analysis, based on assessing tolerance to NaCl as relative root length in salt-treated as compared with control plants, showed that there were both additive and dominance effects of NaCl (Azhar and McNeilly, 1988). These examples suggest

that while the assessment of tolerance is complicated by changes occurring during the ontogeny of plants and may be technically difficult under field conditions, there is evidence of a genetically complex trait (Shannon, 1985), showing heterosis, dominance and additive effects.

The evidence from the few studies that do exist, suggest that the salt tolerance is a heritable character (Allen et al., 1997, Ashraf et al., 1986). Several experiments have provided evidence that genetic variance decreases with increasing stress levels (Blum, 1988). Bernstein,(1976) found a single gene controls sodium and chloride uptake under salinity in grapevine. For tomato, Foolad,(1997) reported, from a parent–progeny comparison, that sodium accumulation under saline conditions was under genetic control, with more than 90% of the genetic variation attributable to additive effects and dominance had little influence. Analysis of sodium and potassium accumulation between F_n and F_{n+1} families in rice growing under saline conditions showed that net accumulation of both sodium and potassium to be heritable (with narrow sense heritabilities of between 0.4 and 0.5), although shoot sodium and potassium concentrations were unrelated, suggesting that the pathways for net accumulation of sodium and potassium in rice are separate (Garcia et al., 1997a).

Ramage,(1979) considered that salt tolerance is a complex character and its expression largely depends on genetic background but suggested recurrent selection as method of achieving salt tolerant lines. These changes could be controlled by genetic regulatory mechanisms which result in both the co-ordinate expression of several genes and multiple staple genetic changes by perhaps the simultaneous rearrangement of several genes. Mahmood,(1991) showed clear evidence that shoot dry weight and water potential are controlled by non-additive

gene effect while shoot fresh weight and Na, Cl and K are effects by additive gene under salinity stress.

Flowers and Yeo,(1995) indicated that breeding crops for salt tolerance has been a controversial issue for several reasons: 1 - salt resistance is a complex character controlled by a number of genes or group of genes and involves a number of component traits which are likely to be quantitative in the nature; 2 - salinity resistance is a complex trait resulting from the interaction of several morphological and physiological properties. However, Lutts et al.,(1996a) and Vera-Estrella et al.,(1999a) concluded that is possible to select salt resistant cell lines, which can integrated in breeding scheme.

Flowers and Yeo,(1995) indicated that although the physiological traits governing the inheritance of salt tolerance is not clear, screening for appropriate diversity in response to salinity among modern cultivars or related species have been identified as the first stage in breeding for salinity resistance in crop plants. The second stage identified was treatment with mutagens in order to produce mutants which show hypersensitive or reduced responses to salinity as compared with the wild type (Tsugane et al., 1999) (Shabala et al., 2005); the third stage was identified as engineering transgenic plant which express one or more foreign genes that are expected to increase cellular resistance to salinity (Bohnert and Jensen, 1996) , (Zhang et al., 2000). Jain et al., (1991) indicated that the advances in understanding the effectiveness of stress responses, and distinctions between pathology and advantage, are increasingly based on transgenic plants and mutant analysis.

1.3 Brassicas

Brassicas are important crops used around the world and this single genus provides high diversity which man has exploited. Brassicas are eaten fresh as leaf, flower bud or root vegetables. Furthermore Brassicas are used as fodder and forage which supply animals with protein and are a food source low in fat. Brassica are also a source of oils (edible and industrial lubricants), condiments (mustard, herbs) and some brassicas are used as soil conditioners as green manure and composting crops (Goldbohm, 2007). Brassica species are cultivated across the whole of the world where they contribute to (1) agricultural economy, (2) health since Brassica vegetables and oils contain vitamins, anti-carcinogenic compounds and polyunsaturated fatty acids and (3) the environment, for instance rapeseed oil is used as a biofuel renewable source for industrial applications (Saka and Kusdiana, 2001).

Generally, Brassica species are moderately sensitive to the environment:

- Internally, they are able to better adjust to the osmotic effect of high salt concentration than sensitive plants such as carrot, onion, bean and strawberry.
- They can be less resistant to the effect of some element such as sodium, chlorine and boron compared with resistant plants such as beet and squash.
- They have a limited ability to adjust and avoid injury at relatively high salt concentrations where expected loss in relative growth and a yield (%) will be 100% if the electrical conductivity of the soil is more than 16 dsm^{-1}(Table 1.1)

Table 1-1. Soil salinity level and yield potential of Brassica. Source: (Blaylock 1994).

Soil salinity level (EC,dsm^{-1})	Expected loss in relative growth and yield (%)
<3.110	0.0
2.7-6.3	25
4.2-9.5	50
<16.5	100

Irrigation with highly saline waters (more than 1500 ppm) has been practiced for date palm trees since 1977 and illustrates that some plants can tolerate high levels of salinity. The use of brackish groundwater is also reported for tomato irrigation in the south of Iraq (Natural Resources Management and Environment Department).

Recent estimates have shown that 4% of the irrigated areas of the world are severely saline, 50% medium saline and 20% slightly saline, i.e. a total of 74% of the irrigated areas suffers from some degree of salinity. More than 25% of irrigated land is saline in Egypt, Iran, Iraq, India, Pakistan and Syria

In Turkey approximately 4.3 million ha of agricultural land are degraded, out of which 1.5 million ha are arid and 2.8 million ha saline-alkaline. The saline areas in Turkey are smaller in size than in neighbouring countries like Syria (532,000 ha), Iraq (6,726,000 ha) and Iran (27,085,000 ha) (Guvensen et al., 2006). The area of degraded soils in the Mediterranean region of Turkey is around 635,197 ha and saline soils are 209,510 ha. Some of the factors responsible for the salinity alkalinity problems are accumulation of salts in plains due to heavy rains, a long standing high water table, and the impact of sea water on the coastal alluvial plains. These areas

are the true habitats for halophytic plant communities (Breckle, 1986). The number of halophytic taxa distributed in the world lies around 3000 (Guvensen et al., 2006). Nearly 700 species are distributed in the Mediterranean climatic zone (Öztürk et al., 2011). A total of 183 taxa (49 monocotyledons and 134 dicotyledons) belonging to 48 families and 136 genera were identified from the area.

1.3.1 Cytogenetics of Brassicas

The cytogenetic relationships between the six cultivated Brassica species are represented by U's triangle (figure 1.3) (Ostergaard and King, 2008) where chromosome numbers, nuclear genome types and mitotypes are shown inside or outside the circle for each species (Chang et al., 2011).

There are three basic diploid species of *Brassica (nigra, oleracea and rapa*) and three hybrid amphi-diploid species (*carinata, juncea* and *napus*) which are fertile due to allopolyploidization. These 6 species are considered as the ancestors of all brassica crops. *Brassica carinata* (n=17) was formed from *B. oleracea* (n=9) × *B. nigra* (n=8), *Brassica juncea* (n=18) is a hybrid between *B. rapa* (n=10) and *B. nigra* (n=8) (Fransden.1943). The third hybrid, *Brassica napus* (n=19) developed from *the hybridisation of B. rapa* (n=10) and *B. oleracea* (n=9). All brassicas belong to the Brassicaceae or Crucifer family characterised by the 4 petalled cross (or crucifix) flower. Cabbage, broccoli, cauliflower and forage kale are all varieties of the highly polymorphic species *Brassica oleracea.* Other vegetable crop members of the Brassicaceae include swedes (*Brassica napus var. napobrassica*) and turnips (*Brassica rapa*). Together, these plants are commonly known as brassicas. The family Cruciferae also includes the small weed species *Arabidopsis thaliana* which has been used as a model plant in molecular biology (Goldbohm, 2007). There is a

close genetic relationship between *Brassica napus* and Arabidopsis *thaliana* and the physiology and developmental biology of Brassicas and Arabidopsis are very similar. Brassica species genomes are more complex than *A. thaliana* and *B. napus* contains over 100,000 genes compared to 28,000 in *A. thaliana.* As a result this provides generous opportunity for divergence of gene function in Brassica compared to *A. thaliana*. (www.jic.ac.uk/centerary).

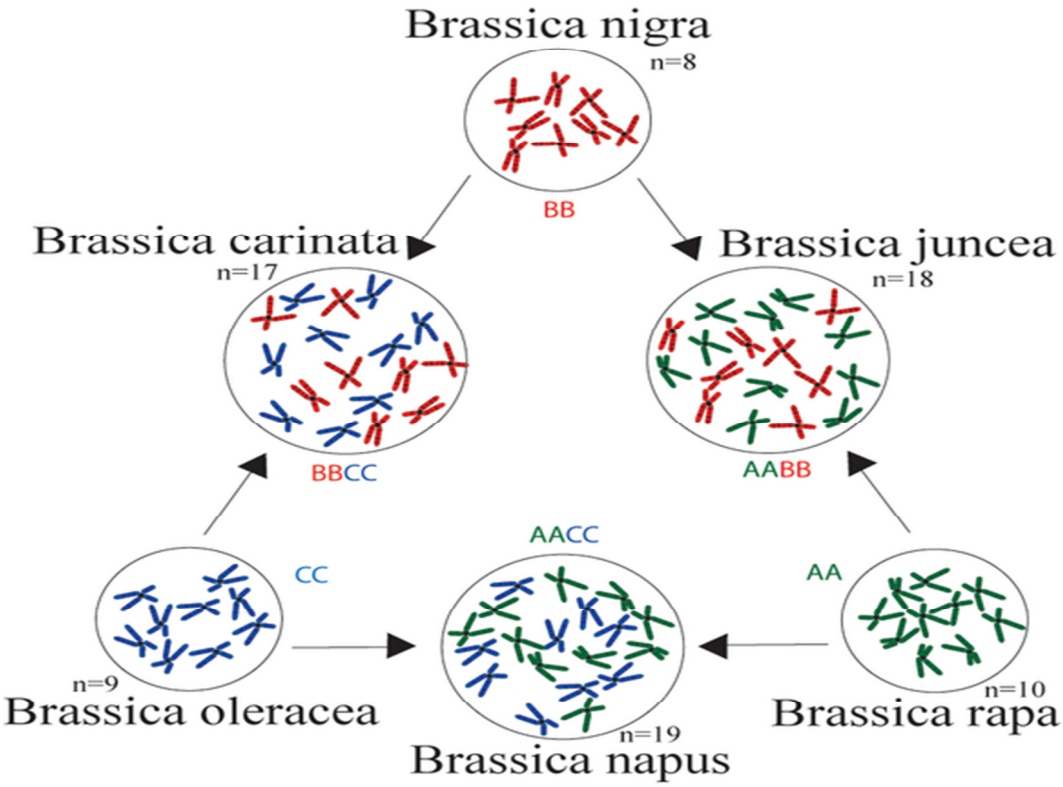


Figure 1-3. Illustration of the three basic species and three hybrid of brassica (Versailles& Thaliana 2006-2008). Known as the triangle of U (1935)(copyright clearance granted).

1.3.2 Characteristics of vegetable Brassicas

Vegetable Brassicas provide many sources of food to mankind. There are different types of Brassica vegetables belonging to the species *B. oleracea* including

cauliflower, cabbage, kale, broccoli and brussel sprouts which are consumed across the whole world (Podsedek 2007). These types are considered as sub-species or genetic varieties of *B. oleracea*. Within each of these sub-species there are many cultivars produced by plant breeders especially in the popular vegetables of cauliflower, broccoli and cabbage. Certain types of cancer, particularly bowel cancer, are thought to be reduced by the consumption of Brassica vegetables. This effect is thought to be due to the glucosinolates present in brassicas which are metabolised to isothiocynates which can be quantified by liquid chromatography with tandem mass spectrometric detection (LC-MS/MS) and have been shown to affect tumours and cancerous cells. Storage and culinary practices (cooking) with Brassica vegetables can affect the bioavailability of glucosinolates and isothiocyanates. Losses of glucosinolates can be reduced by steaming and microwave cooking since during boiling they are leached into the cooking water. Therefore avoiding boiling of vegetables to increase bioavailability of dietary isothiocyanates is desirable (Song and Thornalley, 2007).

Glucosinolates are a group of organic compound derived from glucose and containing nitrogen and sulphur groups. They occur as secondary metabolites in Brassicas plants especially in the family Brassicaceae. Glucosinolates are water-soluble anions and belong to the glucosides. Every glucosinolate contains a central carbon atom which is bonded via a sulphur atom in the glucose group, and via a nitrogen atom to a sulfonated oxime group. The central carbon is bonded to a side group and different glucosinolates have different side groups. These substances used as natural pesticides against herbivores and are responsible for the bitter taste of many common brassica vegetables. The plants contain myrosinase enzyme which cleave glucose from glucosinolate in the presence of water. The remaining molecule

converts to a thiocyanate which is the active substances for plant defence. Myrosinase and glucosinolates are stored in plants to prevent damage of the cell during stress conditions. Glucosinolates were evaluated in 5 groups and65 accessions of *Brassica oleracea* (50 broccoli, 4 Brussels sprouts, 6 cabbages, 3 cauliflowers, and 2 kales) grown under uniform cultural conditions. Consequently it leads to varies in concentration of Glucosinolates within groups (Kushad et al., 1999). Brassica Glucosinolate levels are affected by the growing location (Shelp, 1993), soil type (Josefsson, 1970).

1.3.3 Cauliflower (*Brassica oleracea* var*. botrytis L.)*

Cauliflower is of great economic importance worldwide. Plants are grown as annuals which are mainly consumed as vegetables or in salads, used raw, cooked or pickled.

Figure 1.4 shows that China, India, Italy, France and Spain are the leading producers worldwide, while the countries which consume most of this vegetable are the United Kingdom, Germany and Canada.

Cauliflower is one of the varieties of the highly polymorphic species *Brassica oleracea*, which includes broccoli, brussel sprouts, cabbage, kale and kohlrabi. All these vegetables contain high amounts of vitamins C, K, A (beta-carotene), folic acid, fibre and flavonoids which gives cauliflower antioxidant and anti-inflammatory properties (Toussaint-Samat, 2006). Having adequate amount of vitamin C in the diet has been shown to be beneficial in lessening the symptoms of asthma in children, and can help decrease the risk of multiple sclerosis (MS) in the young and old. Cauliflower can also help protect against arthrosclerosis, reduce bruising, and

offers a high degree of protection from strokes (Pattison et al., 2004). Eating adequate amounts of cruciferous vegetable such as cauliflower can lower the risk of cancer, particularly bowel, breast and other female cancers (Liu and Lv, 2012) and the flavonoids in this vegetable help support the structure of capillaries, and the vitamin A content can help reduce the risk of cataract formation (Herr and Buchler, 2010). Cauliflower is also claimed to be an important carbohydrate food that is an efficient fuel for energy production, which is useful for athletes involved in prolonged, strenuous exercise (Baranowski et al., 2011).

The evolution of cauliflower probably occurred in the eastern Mediterranean (Snogerup, 1980, Gustafsson, 1982) and it has been reported as being under cultivation in Europe since the 15th century and by the 16th century it was eaten thorough Western Europe. It is now grown throughout the world with an estimated world production in 2009 of over 20Mt (Table 1.2)

Table 1-2. World Cauliflower production

Country	Cauliflower production (Mt) 2009
United States of America	338,744
Italy	515,942
France	448,000
India	7,315,832
China	9,428,012
Others	4,180,427
Total	22,226,957

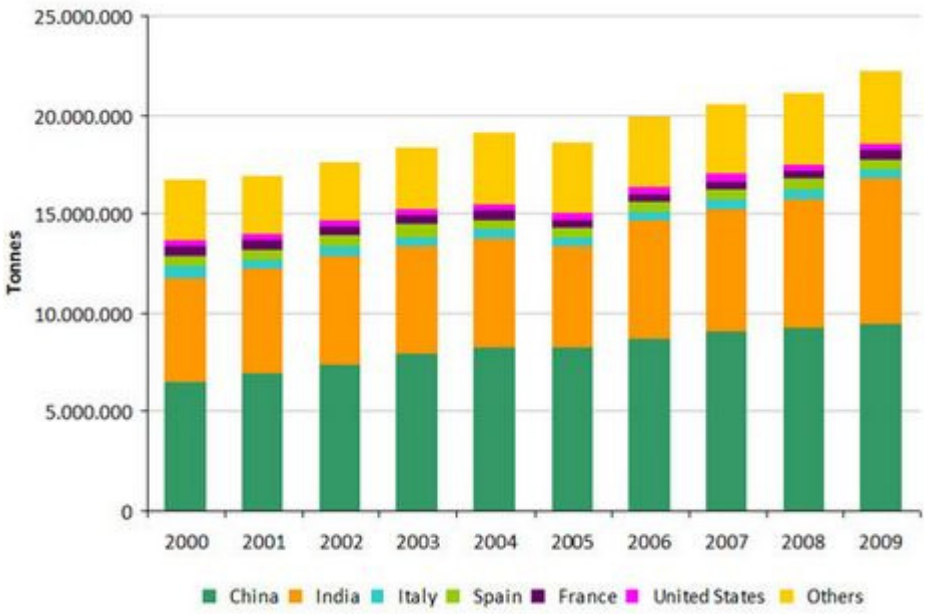


Figure 1-4. World production of cauliflower during 2000-2009.(United States Department of Agriculture - USDA) (copyright clearance granted).

Cauliflower is a cool season vegetable with a requirement for rich, fertile and well-drained soil, with good moisture holding capacity and a distinct temperature requirement for producing a marketable curd (the edible immature flower buds). In Britain, summer cauliflower is grown throughout the country but autumn and winter cauliflower is grown mainly in mild coastal areas. The crop is frost sensitive, with exposed curd being liable to frost damage, and unexposed curd also at risk from prolonged temperatures below zero. As such, the distribution of cauliflower as a profitable crop between the month of November and April is limited largely to maritime regions of the south and south west such as Cornwall where the relatively mild winters reduces the risk of frost damage. In winter large in severe winters when the production from Cornwall is restricted, quantities of cauliflower are sometimes imported into the UK from Spain, Brittany and occasionally Italy (Figure1.4).

1.4 Research Questions?

1.4.1 Why cauliflower?

- The laboratory at Plymouth University has a long history and experience of working with cauliflower and so there is expertise in the growing and handling of this plant species. This has arisen from an association with the local cauliflower production and breeding industry in Cornwall and Northwest France (Brittany) (Ellis et al., 2010).
- Cauliflower is amenable to tissue culture and micropropagation and is the main resource available for cloning techniques are used (Sharma et al., 2008).
- Cauliflower has a pre-inflorescence structure known as a curd which is covered in millions of apices and these are amenable to tissue culture (Kieffer and Fuller, 2013).
- Cauliflower is a vegetable full of potential for the science lab - and, certainly, easy and cheap to source all year round. To date cauliflower has been recalcitrant to genetic modification with only limited successes reported in the literature and most of these cannot be reproduced outside of the laboratories reporting them.

So the main research question applicable to this research investigation was: “can the recalcitrance of cauliflower to genetic transformation be overcome by the generation of a more productive and efficient plant tissue culture system, and if so is it possible to improve the abiotic stress resistance of the species by the insertion of an exogenously sourced antioxidant gene”

2 Chapter Two: Micro propagation of cauliflower

2.1 Introduction

Meristems are the basic unit used in plant micropropagation and cauliflower curd has unique *in-vitro* tissue culture potential because it has millions of meristems on its surface which are ideal for micro propagation. In plant tissue culture it is a common feature of plants that most meristems can provide shoots which are clones of the mother plant and this is the case in cauliflower. Several factors have been reported to affect regeneration *in-vitro* such as culture medium composition, growth regulators and the use of antibiotics. Growth regulators are normally used to support a basic level of growth but are equally important to direct the developmental response of the cultures. Early in tissue culture research it was established that a relatively high auxin: cytokinin ratio favoured shoot production (Skoog and Miller, 1957).

Meristems are the most genetically stable part of a plant and in consequence the most suitable for the production of true-to–type propagules or clones. Micropropagation using curd meristems has been used to maintain cauliflower parent lines (Crisp and Walkey, 1974) and for the early screening of curd quality (Crisp, 1979). More recently this tissue has been used as a source of protoplasts (Yang et al., 1994) and for chemical mediated mutagenesis (Dean et al., 1996) and transgenic cauliflower plants (Kieffer et al., 1996). The technique of curd meristem micropropagation for cauliflower clonal propagation is now well established (Rancillac et al., 1982, Kumar A, 1993). Kieffer,(2001) has more recently described a technique for rapid mass production of cauliflower propagules involving the homogenisation of curd tissues and they termed the protocol “microshoots”. The technique produced tens of thousands of meristematic pieces in liquid culture in relatively small volumes (e.g. 1 - 2000 propagules in 20 mL) and the technique offers

unique and exciting possibilities for mutagenesis and genetic transformation by facilitating rapid screening of numerous propagules in culture.

To initiate growth of explants it is important to provide basic nutrients within the medium: this usually consists of a mixture of salts which provide the essential macro and micro elements as well as a carbon source (usually sucrose). The most widely used of the formulations available is that of Murashige and Skoog (MS) (Murashige & Skoog, 1962). Culture medium effects on regeneration have been demonstrated by several studies (Rangaswamy, 1986) and a survey of embryogenesis in crop plants found that 70% of explants were cultured on MS medium. MS medium satisfies the nitrogen requirement in the case of carrot embryos because of the presence of high concentrations of inorganic nitrate (Reinert. et al., 1967).

Cauliflower Micropropagation using curd meristems

The meristem is the most genetically stable part of a plant and in consequence the most suitable for production of true-to-type propagules. Clonal multiplication is of paramount importance in modem cauliflower breeding programmes and is most needed for the maintenance of elite lines such as the parents of F1 hybrids which are usually strongly self-incompatible or male sterile (Sharma et al., 2005). Maintenance of these lines using seed requires time consuming manual self-pollination of immature flower buds. Micropropagation using curd meristems has been used for some time to maintain parent lines and bulk up plants prior to seed production (Crisp and Walkey, 1974, Zhao et al., 2012). It has also been used for the production of virus-free cauliflowers (Walkey, 1981), and for the early screening of curd quality (Crisp, 1984). The technique of curd meristem micropropagation for cauliflower clonal propagation is now well established, but despite a few extensive

studies (Torres, 1980b, Kumar A, 1993), fundamental information concerning this culture system is limited. The aim of this chapter is to investigate the factors limiting the efficiency of micropropagation systems, to improve the understanding of shoot regeneration from curd meristem and to establish a protocol which better utilises the full potential of cauliflower curd tissue.

Factors limiting shoot regeneration

There are many factors that affect shoot regeneration, some authors clearly regenerated shoots from proliferating (callusing) tissue of the curd explant (Shah et al., 1993) but the reported physical properties of the explant used are very variable. Explant size (diameter) also varies considerably, which has consequences with respect to tissue age, complexity, and number of pre-existing meristems carried. Successful micropropagation must depend on the physiological state of the mother curd, and curd physiology and quality can vary significantly with age and climatic conditions (Smith and King, 2000). The location on the curd from where the explants were collected has rarely been reported, and it is possible that a physiological gradient within the curd exists as reported in curd stem branches (Pati et al., 2004).

Plant preservative mixture (PPM) Apollo Scientific Limited, UK) is widely used with tissue culture in micropropagation of cauliflower (Rihan et al., 2012). PPM is heat stable and is thus able to be autoclaved with the culture medium (Fuller and Pizzey, 2001). However it is phyto-toxic and as with any anti-microbial compound there is a risk during *in-vitro* culture or in the following *in-vivo* formation.

The following experiments try to clarify these aspects of the culture system with the aim of determining the factors limiting the scale of multiplication.

Micro-propagation and sub-culturing of clones

The plant micro-propagation process is used for propagation of clones of genotypes. The process usually consists of the following steps; selection of the suitable plants, surface sterilization, initiation of explants, establishment of explants, subculture for multiplication of explants, shooting and rooting induction, and the weaning/hardening step to produce complete *in-vivo* plant clones (Ahloowalia et al., 2002). Plant micro-propagation is initiated from small pieces, known as explants. The explant is isolated from surface sterilized part of a plant. The widely used explants are shoot tips or meristem-tips which are the most popular explant source (Medford, 1992). Other explant sources such as floral meristems and buds, nodal or axillary buds, anthers, pollen and microspores have also been used as explants.

The cultured tissues generate a complete plant through regeneration process either by callus production or by direct shoot induction. Callus is a mass of unorganized cells which have the potential to produce complete plant upon transfer to suitable media and widely used for plant clonal generation (Pierik, 1988). The explants are stimulated to differentiate shoots and roots using specific types of media and grow into a complete plant – a process of regeneration and organogenesis. Media having comparatively high auxin (2,4-D - 2, 4-dichlorophenoxyacetic acid) form callus which can be further sub-cultured and multiplied for clonal production. In some cases, explants e.g. leaf-discs and epidermal tissue can also generate plants by direct organogenesis and somatic embryogenesis without callus formation (Hanning and Conger, 1986).

The regeneration and growth of plants in *in-vitro* condition depends on the composition of media. The major components of culture media are mineral salts,

sugar and water although growth regulators, organic supplements and gelling agent are other important components included in media (Gamborg et al., 1968) (Gamborg. and Phillips, 1995). The quantity of the components differ depending on plant species and stage of growth, but the most widely used media for plant tissue culture are the basic MS (Murashige and Skoog, 1962) media. Many plant tissues grow better on solid media whereas others benefit from more liquid media; the solid, liquid and semi liquid status depends on the absence or presence of a gelling agent, with Agar being the most commonly used agent (Debergh, 1983).

Growth regulators such as auxins and cytokinins play important role and their ratio in media controls the morphogenic response for root or shoot formation; a relative high cytokinin : auxin ratio induces shoot production while high auxin : cytokinin ratio induces root formation (Skoog and Miller, 1957). Commonly high concentration of auxin is favoured for root induction but in some cases exogenous auxin shows an inhibitory effect on roots (Hou et al., 2004, Thomas and Street, 1970). Another important supplement in media is silver nitrate which plays a role in maintaining the callus as well as improving regeneration (Sethi et al., 1990), although at high concentration this can cause necrosis; however concentrations as high as 15 mgL^{-1} silver nitrate do not produce any negative effect on *Brassica rapa* culture (Kuvshinov et al., 1999) .

2.1 Aim

To establish an efficient cauliflower *in-vitro* technique as a Standard Operating Procedure (SOP) to be used for plant transformation.

2.2 Objective

- To optimise a microshoot technique of micropropagation of cauliflower by using different size of sieves, different volume and different durations of blending in order provide sufficient numbers of explants for plant transformation.
- To investigate the potential of a seedling explant technique for the micropropagation of cauliflower for plant transformation.

2.3 Material and methods

Risk assessment

Risk assessment forms and relevant Control of Substances Hazardous to Health (COSHH) forms were completed prior to any work being carried out (Appendix 1).

Chemical material source

Chemicals, equipment and instruments used in the laboratory were supplied by Sigma, Aldrich and Fisher Company. In making solutions, chemicals were weighed either on an ATIAA 31955 or a Sartorius, 4 decimal place balance. Distilled or deionised water was used for preparing all stock solutions. Before autoclaving, the pH of all solution was adjusted with a Corning pH meter using a stock solution of NaOH and HCl (Appendix 7.a & b) with concentrations ranging from 1 to 2 mM. Before adjusting the pH of solutions, the pH meter was always calibrated with standards (pH = 4. 0 and 7.0).

2.3.1 Tissue culture and micropropagation

Plant material

Five varieties of F1 hybrid winter heading cauliflower - Clemen (February heading), Dionis (January heading), Redoutable (February heading), Mascaret (March heading) and Medallion (February heading) were grown in the field in a raised bed at the University of Plymouth, Devon, UK according to good commercial practice (Whitwell et al., 1982). Harvested curds were taken to the laboratory and either used immediately or stored at 4^0C for up to 14 days until used. For seedling derived explants the varieties Medallion and Dionis were used.

Preparation of explants

Curd Explants

Large pieces of curd (1 - 5 cm) were cut from the curd and surface sterilised firstly by immersing for 15m in 10% unthickened commercial bleach (sodium hypochlorite 0.06% active chlorine), followed by 3 washings in sterile distilled water. Following surface sterilisation, explants were produced manually. The first step eliminated the mass of non-responsive tissue (stem branches) by shaving off the upper meristematic layer using a scalpel under sterile conditions in a laminar flow hood. The second step was a mechanical partial homogenisation of the selected meristematic tissue using a commercial blender (Waring model 800) at approximately 17000 rev. min^{-1} followed by the use of precision sieves (600, 300, 212 µm aperture size) to rank the explants into the size classes 212-300 µm and 300-600 µm (Figure 2.1). Explant culture density was controlled by using a constant volume of homogenised explants per container containing 20 mL of culture medium.

Seedling explants

Seeds of cauliflower were surface sterilised for 20 second in 70% (v/v) ethanol followed by shaking for 5 minutes in 10% (v/v) commercial unthickened bleach solution in a laminar flow cabinet and washed three times in sterilised distilled water. The germination medium was M&S (Murashige and Skoog 1962) solidified with 7.0 g L^{-1} Agar in sterilised plastic pots (9.0 cm inner diameter at the top and 5.0 cm at the bottom). Seeds were sown aseptically in a laminar flow cabinet, and the pots were maintained in a growth chamber at 23 ℃, 16h photoperiod with a radiant light intensity of 50 µmol $m^{-2}s^{-1}$ for 8 days.

Hypocotyls and cotyledons

Seedling were removed from the germination medium and cut into explants in a laminar flow cabinet. Cotyledons were placed upright with cut ends embedded in the medium. Hypocotyl explants were arranged horizontally and gently pressed into the surface of the medium. Ten to twelve explants were cultured per Petri dish. The culture plates were sealed using 3M surgical tape and were incubated at 23 ^{0}C using a 16 h photoperiod producing 50 µmol m^{-2} s^{-1} at the Petri dish level.

Callus induction from seedling explants was achieved by adding the phytohormones 2, 4 D and BAP to the medium. After one week, explants were transferred onto fresh medium and shoot initiation was observed after two weeks. Explants bearing shoot initials were transferred onto propagation medium, consisting of basal medium (S23).

Plate 2.1. Mass production of cauliflower propagules from fractioning and graded cured (Kieffer et al, 2001).

Hormone stock solutions

Stock solutions of indole-3-butyric acid (IBA) 2 mg mL^{-1} and kinetin 1 mg mL^{-1} were prepared. Kinetin was dissolved in few drops of 2M HCl and IBA in 2M NaOH prior to making stock solutions. The volume was then increased to 50 mL with distilled water. From stock solutions 1 ml of IBA and 2 mL of kinetin per litre was transferred to each tube of shoot induction medium.

Maintenance of *in-vitro* shoots

All cauliflower clonal material (transformants (TA) and control (C) were reproduced *in-vitro* through tissue culture. A shoot induction medium was prepared for both propagation and growth rate experiments. Propagation medium S23M (Kieffer et al., 1995a) was prepared by dissolving the following ingredients in distilled water. MS (Murashige and Skoog, 1962) (Appendix2) basal salts 4.4 g L^{-1}, Thiamine 0.4 mg L^{-1}, Adenine sulphate 80 mg L^{-1}, sodium dihydrogen orthophosphate 170 mg L^{-1}, sucrose 30 g L^{-1}, Kinetin 2 mg L^{-1}, indole-3-butyric acid (IBA) 1 mg L^{-1} ,PPM (Plant Preservative Mixture)1 mg L^{-1} and agar was added at 7 g L^{-1} aid autoclaved. Twenty mL pot^{-1} of medium was poured into sterile plastic pots (5 cm x 4 cm) under aseptic conditions in a laminar flow cabinet; then a lid was placed on each pot and they were allowed to cool overnight at room temperature.

Rooting media and sub-culturing

After a 4 week growth period, young shoots were transferred to S23M with 7 mg L^{-1} of agar. Pots were kept at 23^{o}C with a 16 h photoperiod in an incubator and cultured until completely rooted plantlets were produced. Sub-culturing of *in-vitro* clones on S23M (Appendix4) was carried out regularly after each 3 to 4 months period to maintain plant materials for continued experimentation during the project work.

Culture growth conditions

In-vitro cultures were maintained in one of two growing environments. For the large scale curd experiments the shaker(s) were kept on a laboratory bench at 20^{o}C with supplementary fluorescent lighting to achieve a photoperiod of 16 h at 50 μmol m^{-2} s^{-1}. To grow cultures on pots and petri-dishes were transferred to a Sanyo plant growth cabinet (model MLR-350) at 23^{o}C with a 16h photoperiod and a light intensity of 150 umol m^{-2} s^{-1} (Plate 2.3). The growth cabinet containing the co-cultivation experiments and transformed plants was a locked cabinet in order to maintain security.

Phytohormone combinations

Different phytohormone balances were tested with the same basal medium previously reported for shoot induction: combinations of Kinetin (0.0-3 mgL^{-1}) and IBA (0.0-0. 2 mgL^{-1}) were tested (Table 2.1). Liquid and semi-solid (agar 0.7%) culture media were tested on microshoots. The frequencies of rooted shoots were determined as well as the number of roots produced per shoot for each treatment of 50 micro-shoots, three to five replications were recorded.

Twelve different combinations of Naphthalene acetic acid (NAA) and kinetin were applied in cauliflower tissue culture media, as indicated in Table 2.1 using 5 pots (replications) per treatment. Pots were randomly arranged on a shaker (Plate 2.2). Observations of shoot and root development were taken after 2 weeks for each hormone combination and repeated every 5 days until 5 readings were taken. By this time the shoots had become large and were transferred to new media.

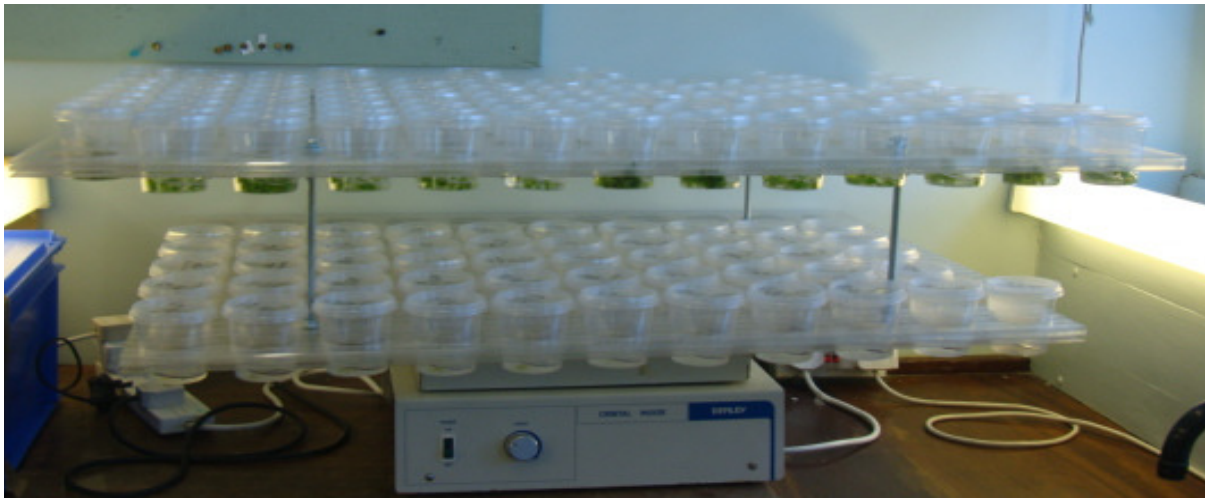


Plate 2.2. Cauliflower micropropagation on the shaker. Each layer is one experiment. Each pot contains about 100-700 microshoots.

2.3.2 Optimization of conditions for cauliflower curd explant production.

A series of iterative experiments were carried out to optimise the explant harvesting technique from cauliflower curd. The optimization experiments were repeated many times with curds of the same genotypes and with different genotypes.

The effect of blending duration

Different blending durations (15, 30, 60, 90 and 120 seconds) were used to determine the optimal duration for specific sieving size classes and culture volumes. The total number of samples used for the analysis was 180 pots - 5 time durations x 3 replicates x 12 treatments (4 different volumes x 3 size classes), as follows;

- 15 sec., sieve Size class (212, 300 and 600 µm), culture volumes (23, 34, 74 and 240 µL).
- 30 sec., sieve size class (212, 300 and 600 µm), culture volumes (23, 34, 74 and 240 µL).

- 60 sec., sieve size class (212, 300 and 600 µm), culture volumes (23, 34, 74 and 240 µL).
- 90 sec., sieve size class (212, 300 and 600 µm), culture volumes (23, 34, 74 and 240 µL).
- 120 sec., sieve size class (212, 300 and 600 µm), culture volumes (23, 34, 74 and 240 µL).

The effect of sieve size

Different sieve sizes (212, 300 and 600 µm) were used to evaluate the optimum sieve size for explants production with specific volume (Al-Swedi, 2012). A total of 36 pots were used (1 time duration x 3 replicates x 12 treatments (4 volumes x 3 size classes), as follows;

- 212 µm, culture volume (23, 34, 74 and 240 µL) and 30 sec.
- 300 µm culture volume (23, 34, 74 and 240 µL) and 30 sec.
- 600 µm culture volume (23, 34, 74 and 240 µL) and 30 sec.

The effect of culture volume on explants production

Different culture volumes (23, 34, 74 and 240 µL) with duration time (30 sec.) were used to evaluate the optimum volume for explant production. From one head of cauliflower different sieve size samples were collected. In total 36 pots were used for the analysis - 1 time duration x 3 replicates x 12 treatments (4 volume x 3 size class), as follows;

- Culture volume 23 µL, sieve size class (212,300 and 600 µm) and 30 sec.
- Culture volume 34 µL, sieve size class (212,300 and 600 µm) and 30 sec.

- Culture volume 74 µL, sieve size class (212,300 and 600 µm) and 30 sec.
- Culture volume 240 µL, sieve size class (212,300 and 600 µm) and 30 sec.

All experiments were laid out in completely randomized designs.

Plate 2.3. Photo to illustrate the growth cabinet (Sanyo plant growth chamber (model MLR-350)) for maintaining the plants after co-cultivation with a bacterial strain. The plants are being grown in shaken liquid media in the upper part of the growth cabinet and in solid media in the lower part.

Plant Preservative Mixture (PPM)

Many of the initial experiments failed due to bacterial contamination which was difficult to control in the liquid media used with the curd explants. Thus only a selection of the results obtained are presented. It proved necessary to develop a safe antibiotic addition to the media in order to progress the optimization of this technique. Since there was a need to use a range of antibiotics in the transformation selection section of the work, it was important to find an antibiotic treatment which did not compromise this part of the investigation. A broad spectrum antimicrobial material was investigated.

Plant Preservative Mixture (PPM) is a combination of two broad-spectrum industrial isothiazolone biocides, chloromethylisothiazolone and methylisothiazolone. PPM is heat stable and is thus able to be autoclaved with the culture medium (Lunghusen, 1998). It targets specific multiple enzyme sites in the Krebs cycle and the electron transport chain of micro-organisms (Chapman and Diehl, 1995). As with any anti-microbial compound there is a risk that it is phyto-toxic and checks need to be made to ensure that they do not inhibit or alter plant growth during *in-vitro* culture or in subsequent in-vivo establishment. Fuller and Pizzey (2001) indicated an important role for PPM to control the contamination in brassica culture media. Three concentrations of PPM (0, 0.1 and 1.0 mL L^{-1}) (Apollo Scientific Limited, UK) were used with both, plant growth regulator (PGR) free S23 liquid medium and S23 supplemented with 2 mg L^{-1} kinetin and 1 mg L^{-1} IBA. Five culture vessels were used with each treatment. The aim of this experiment was to investigate the effect of concentrations of PPM on the growth of microshoots and to determine if PPM has an effect on the growth of spoilage micro-organisms in the curd explants protocol.

Weaning and field culture

Fully developed plantlets were transferred into the glasshouse mix comprising 1 bag of multipurpose compost (Mole Valley Ltd. UK) with 2 bags of John Innes No2 Compost in expandable peat pellets (type 7, Jiffy® products Ltd., UK). They were then maintained for 10 days under high temperature and humidity in a propagator then placed on a mist bench with base heating (23℃) for 10 days, before being transferred to a normal glasshouse environment. Plantlets were fed with a nutrient solution (N/P/K, 10: 10: 27, Phostrogen Ltd. UK) and treated against pests when required. Plants were stored (if necessary) at 4℃ until field planting time. Plants raised from seed of the same genotypes were also transplanted to the field plot when possible as controls. Plant quality and defects were recorded all through the production process. Plants were grown according to good commercial practice (MAFF., 1982, Champion et al., 2003). Curd quality as the ultimate goal was observed for genotype Dionis and Medallion in the season 2009-2010.

2.4 Statistical analysis

Data were statistically analysed by analysis of variance (ANOVA) or using the derived general linear model (GLM) for analysis where unbalanced data sets were used, using the statistical programme Minitab16. For the determination of significance, the probability of the F test (p) to reject the null hypothesis was examined at the significance levels of 5, 1 and 0.1 %; where the F-test showed significance further analysis was carried out in order to determine which means were significantly different from each other, using the Tukey test or LSD test. In addition where appropriate Sys Stat Sigma Plot 12analysis was used using simple sequential curve analysis.

2.5 Results;

2.5.1 Explant physical parameters

Effect of blending durations on meristem and debris production

Observation of explants (0.2 mm) under a zoom binocular microscope over time revealed that shoots arose from existing meristems or from meristems deriving from them. Most of the meristems developed to shoot and then to a new plant whilst debris did not develop (Plate 2.4). The number of meristems carried per explant varied widely between the size classes and most debris was useless because it did not produce microshoots. (Plate 2.5)

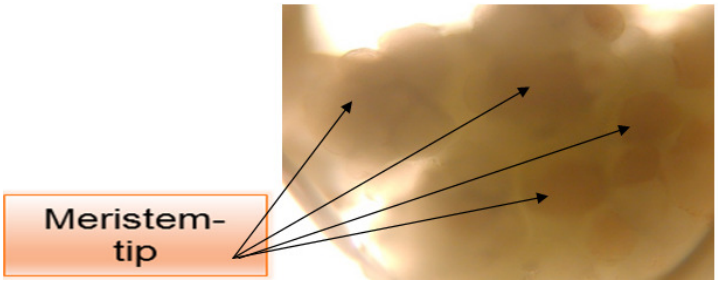


Plate 2.4. Meristem-tip cultures of cauliflower by light microscope (magnification 103 xs)

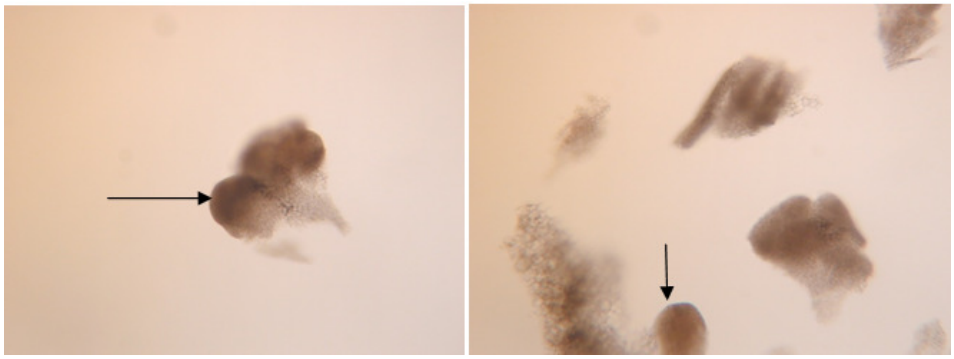


Plate 2.5. Meristems dome and debris (magnification 103 xs)

Effect of different blending durations of explants in size 212 *µm.*

This experiment was carried out to study the effect of the blending duration on explant development reflecting the number of meristems and amount of debris that were produced. Blending duration for 60 seconds decreased the number of explants bearing meristems and increased those classified as debris. Since only meristem bearing explants develop into shoots then the 30 second blending duration proved better (Figure 2.1).

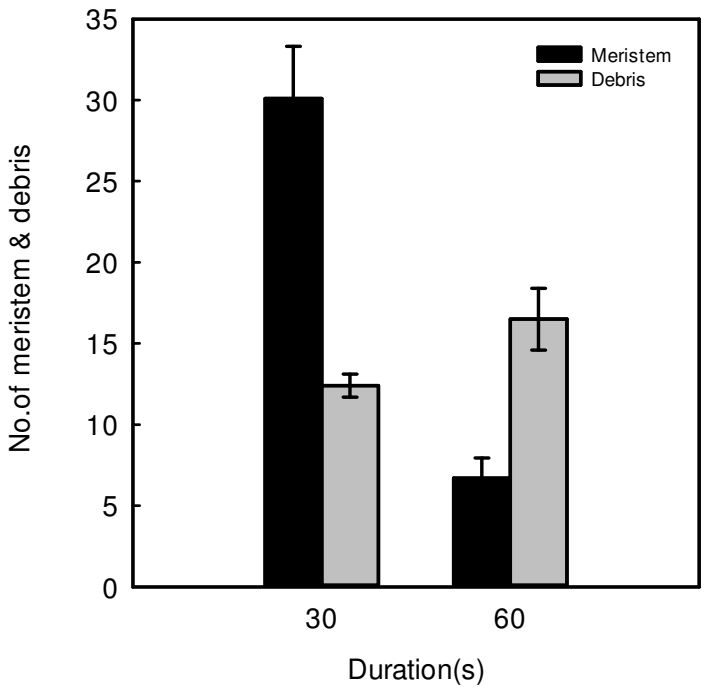


Figure 2-1. Effect of duration of treatment on numbers of meristem and debris production (sieve size 212 µm, volume 34 µl). Standard error were used. Bars represent the standard error of the mean.

Effect of different explant culture volumes of explant development in size class 212 µm.

In this experiment was shown the effect of different volumes of meristematic tissues on the number of explants production after 212µm separation. Effectively this experiment doubled the size of the explant culture volume and it can be seen from Figure 2.2 that the number of meristem bearing explants was doubled but the number of debris pieces were more than doubled.

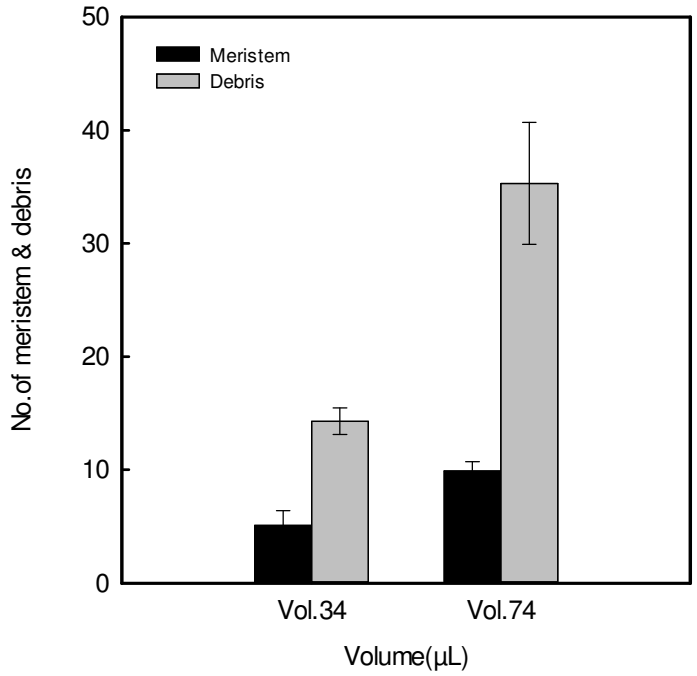


Figure 2-2. Effect of different culture volumes (34, 74 µl), at sieve size 212 µm with a blending duration of 30s on number of meristem& debris production. Bars represent the standard error of the mean.

Effect of different culture volumes of explants in size 300 µm.

The study in this experiment showed the effect of different culture volumes of meristematic tissues (74, 34 µl) from sieve size class 300 µm. There was a significant difference between the debris and meristem, the amount of debris particles being more than meristems resulting in reduced shoot production. The results were similar to the previous experiment (Figure 2.3) and in addition the amount of debris.

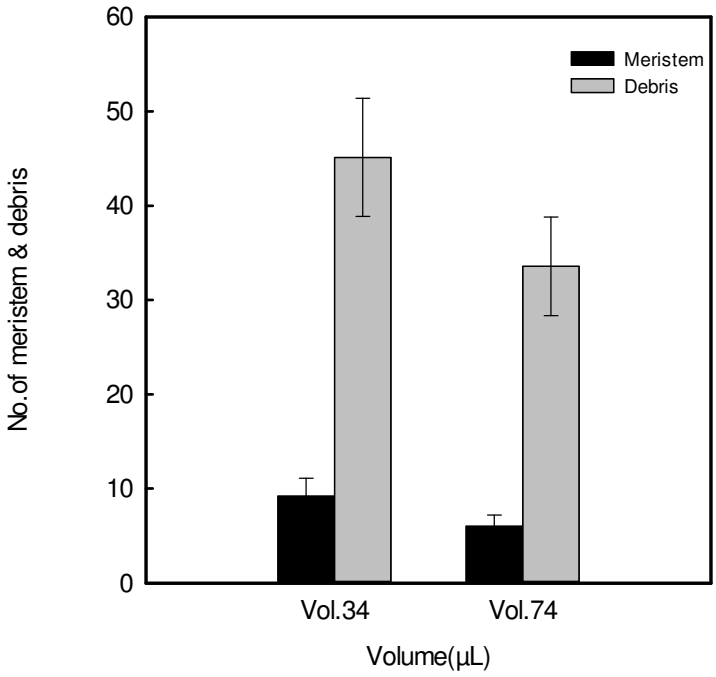


Figure 2-3. Effect of different volumes (34, 74 µL) on production of meristems and debris, sieve size 300µm, duration 60s. Bars represent the standard error of the mean.

Effect of different volumes on explants in size 600 µm.

The study in this experiment showed the effect of different volumes of meristematic tissues (74, 34 µL) on preparations from size class 600 µm. There was a significant difference between the debris and meristem, the amount of debris particles being more than meristems in spite of different treatment in size 600µm, volume 34, 74µL, duration 60 seconds Resulting in reduced shoot production, the same results with the above experiment with size class 300 µm (Figure 2.3) in addition to the number of debris increased with increase the time of duration (Figure2.4).

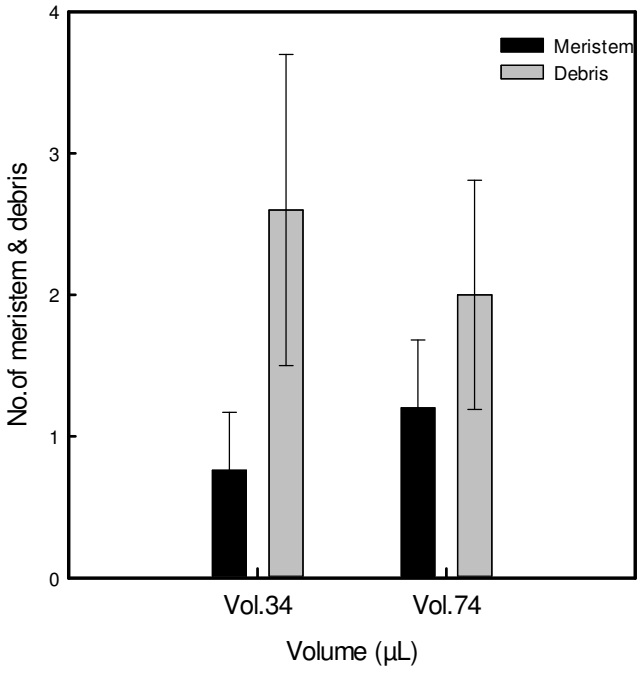


Figure 2-4. Effect of different volumes (34, 74 µl) on production of meristems and debris, sieve size 600µm, duration 60s. Bars represent the standard error of the mean.

2.5.1.1 Effect of different sieving size class on meristem and debris production.

The study in this experiment was shown the effect of different sieving size class (212,300 and 600 µm) on meristem and debris production. There was a significant difference between the debris and meristem, the number of debris particles being less than meristem in spite of different treatment of size sieving size classes with duration 30 seconds resulting in increased shoot production, in addition to the number of debris increased with increase the time of duration (Figure 2.5).

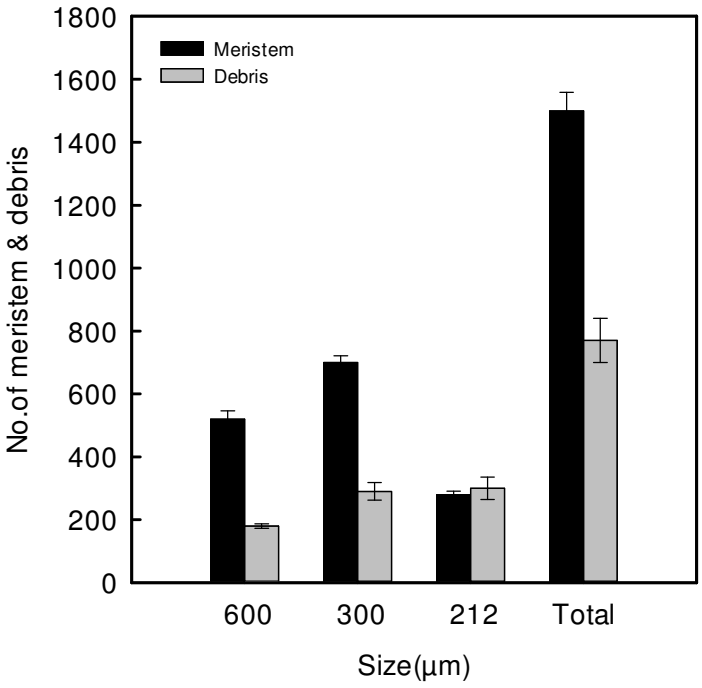


Figure 2-5. Effect of different sieve size class (212,300 and 600µm) on production of meristems and debris, Bars represent the standard error of the mean.

2.5.2 The effect of PPM on microshoots production

A range of concentrations of PPM (plant preservative mixture) were used in this study and these have an effect on meristematic cell development, microshoots production and culture media as shown in (plate 2.6). It is very clear that the media was clear of bacterial contamination and that the presence of PPM resulted in the production of a large number of microshoots.

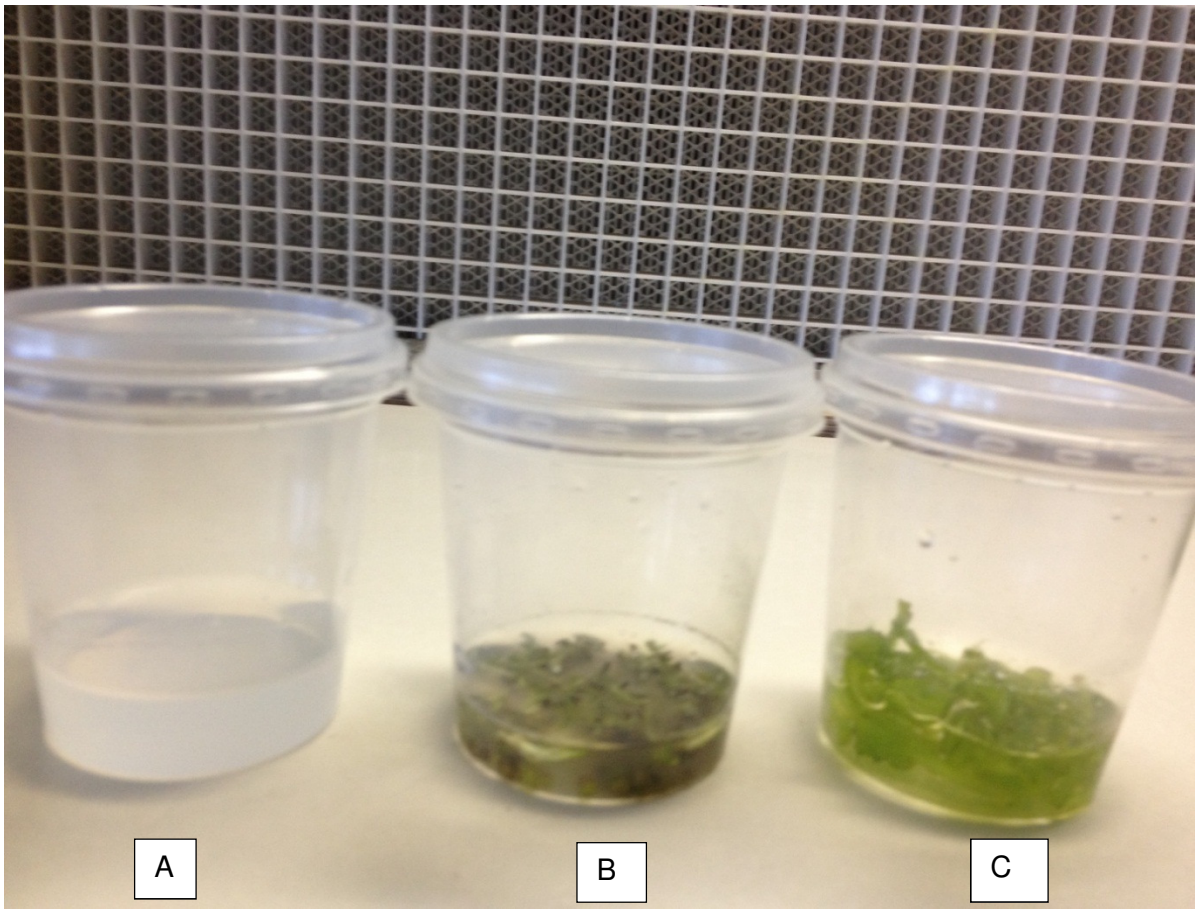


Plate 2.6. Different concentrations of PPM in culture medium A) No PPM. B) 0.001ml L-1 C) 1.0 ml L^{-1}.

2.5.3 Effect of blending duration on shoots and root production

The variety Dionis was used in this experiment. The figure was illustrated different size classes (212,300 and 600 µm) were used. After one month, the reading was taken which obviously the higher production of shoot and root in 5 mm length in comparison with the other measurements in all sieving size classes. As shown in Figure 2.6, shoot growth varies between three size classes. Generally there was good shoot growth but a very little growth of roots in all cases. Many samples were lost as a result of bacterial contamination.

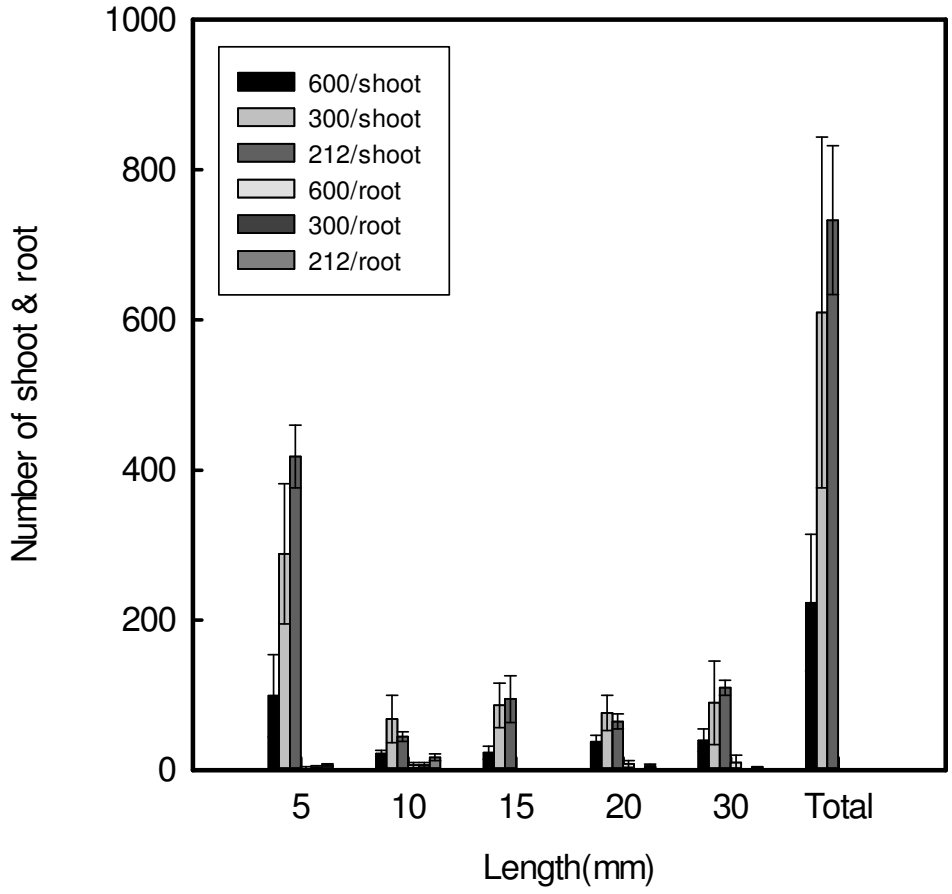


Figure 2-6. Effect of three sieving sizes (212,300 and 600 µm) on production of shoot/ root in different length. For all treatments, duration (30s) and volume (34 µL), Bars represent the standard error of the mean.

2.5.4 The effect of blending duration 30 s. and volume 34 µm on explant production.

It was very clear that the micro explant was very uniform in length and shape (Plate 2.7). When this exercise was repeated with variety Redoubtable. Shoot growth was seen to vary with treatment. Again very little growth of roots was evident in all treatments and also many samples were lost to bacterial contamination.

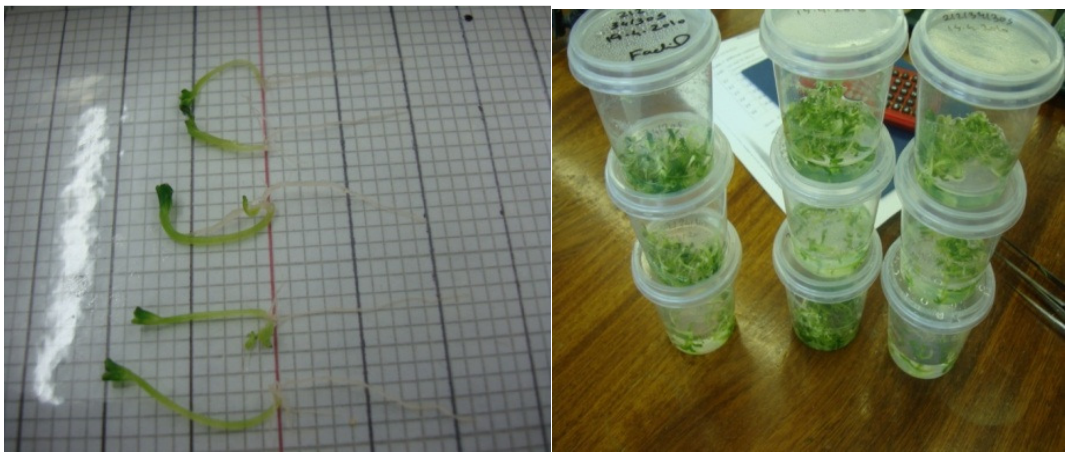


Plate 2.7. Illustration of the explant production at volume 34 µL and blending duration 30s.

The effect of blending duration on shoot and root production for both size class (212 & 300 µm) with blending duration (30s) in variety Clemen was used in this experiment and the reading was taken after 4 weeks. Generally for this treatment, the shoots produced were very short in length while the root production was very dense (Plate 2.8). It was clear that the there was no root production with length 15, 25 and 35 mm in comparison with the other root length 5, 10, 20 and 30 mm (Figure 2.7). In comparison between 2 treatments for both varieties, the first treatment more shoot was produced and less dense of root was produced while the second treatments there was denser root developed with short shoot growth.

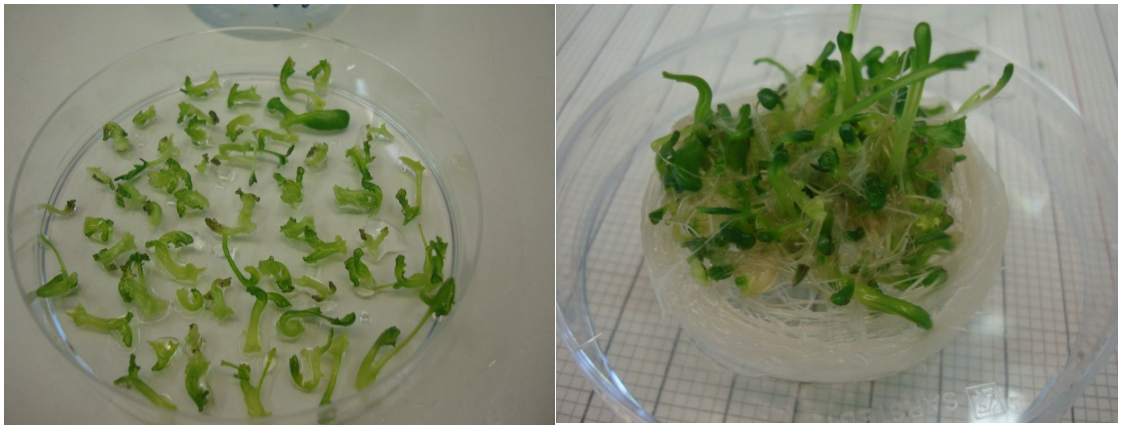


Plate 2.8. Effect of blending duration on shoot & root production with volume 74 µL and sieving size class300 µm at30s.

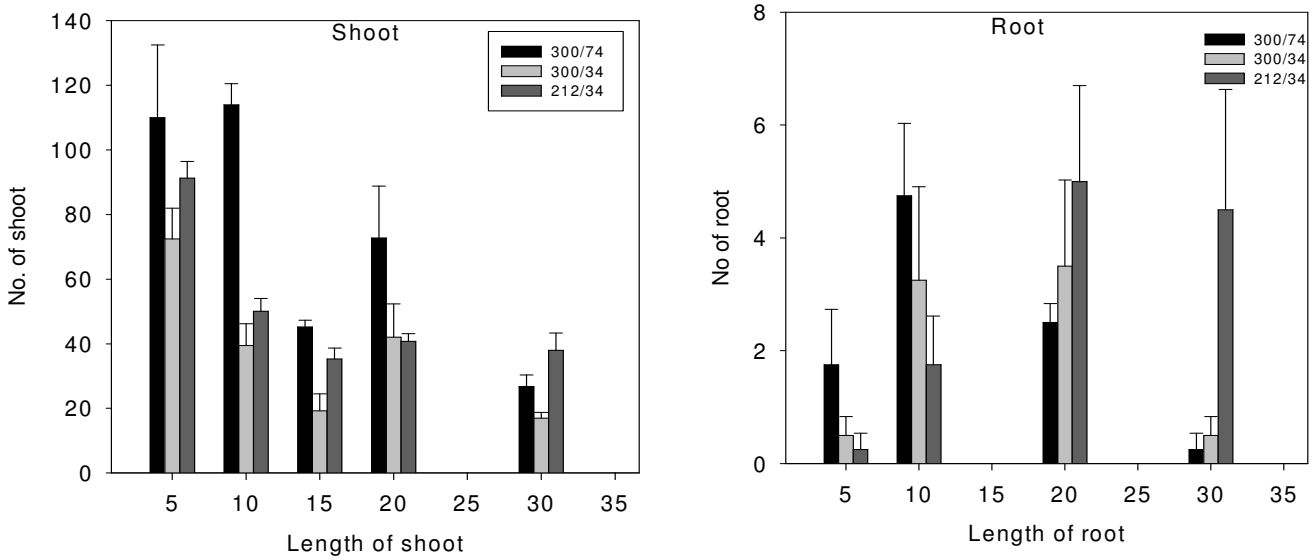


Figure 2-1. Illustrate the effect of blending duration on shoot production for both size class (212&300) µm for 30 s (length unit; cm). Bars represent the standard error of the mean.

2.5.5 Weaning

Successfully acclimated explants were transferred from semisolid media under fully controlled condition to the soil following full root development. Plant growing in the soil containing compost mixture and at this stage the plant ready to transfer to the field.

All the following steps (Plate 2.9) have therefore been successfully achieved

a) Microshoots growing in semisolid media.

b) Plants weaned.

c) Plants growing very well in big pots.

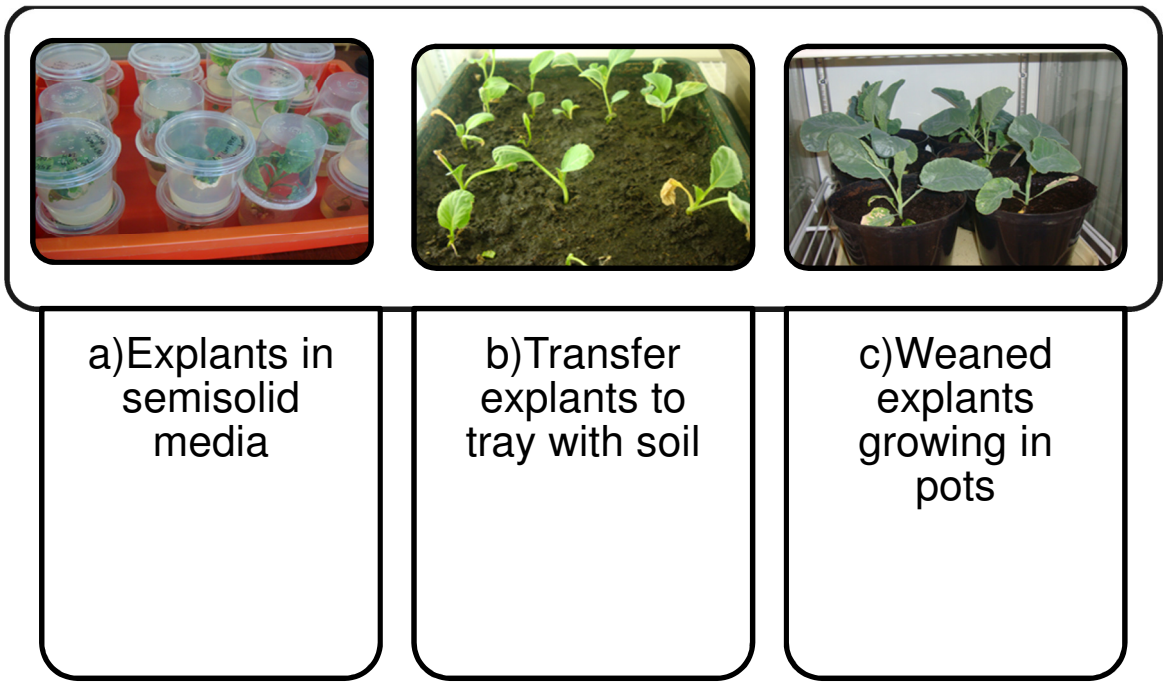


Plate 2.9. Steps of acclimated explants from semisolid media to the soil then to the field.

2.5.1 Callus cultures from seedling explants

A callus is a mass of unorganized cells, which upon transfer to suitable medium is capable of giving rise to shoot-buds and somatic embryos, which then form complete

plants. Such calli in solid or/and liquid media on shakers are used for initiating cell suspensions under controlled conditions (Ahloowalia, 2002). Figure 2.8 shows the higher rate of callus formation in hypocotyls compared with formation of callus from roots; very few calli form from cotyledons Plate 2.10.

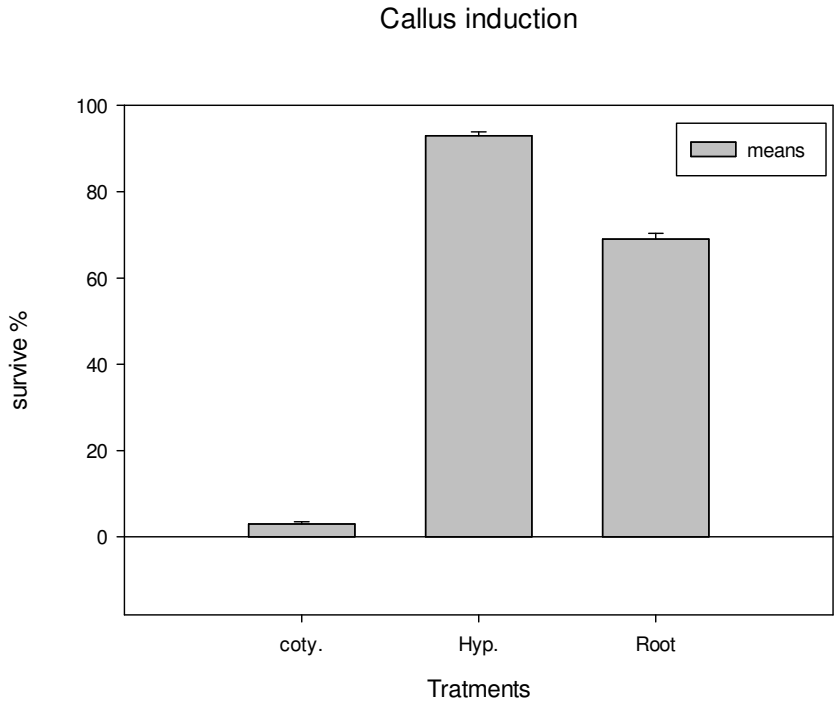


Figure 2-8. Frequency of Callus formation from hypocotyl, cotyledon and root in cauliflower.

Plate 2.10. Callus derived from hypocotyl explants

2.5.2 The Effect of different concentrations of hormones on plant growth

Twelve combinations of NAA and kinetin were tested to optimize the best combination. There were big variations between all treatments (Table 2.1) and the

best combination treatment between NAA and kinetin was selected for subsequent experiments.

Table 2-1. Effect of different concentrations of hormones (NAA and kinetin)

Treatments	NAA mg L^{-1}	Kinetin mg L^{-1}	Descriptions
T1	0.0	0.0	The shoots were very short and plant in general 5mm shoot and 10% was rooted in length.
T2	0.05	0.0	The excessive root was developed like network in the base of pot as one piece (matrix), it was difficult to separate from each other
T3	0.1	0.0	The root growth (matrix) was very dense as a network with thin and short shoot development.
T4	0.2	0.0	The growth was not good for both shoot and root in comparison with other treatments but it did show dense root development.
T5	0.0	1.0	Shoot regeneration was good and a little bit of root growth, no dense root growth but very short and thin root development.
T6	0.05	1.0	The shoot development is good and a large number of leaves are developed but less root development, not dense and thin.
T7	0.1	1.0	There are no dense growths of shoot or root.
T8	0.2	1.0	There are good growth of shoots (more than 3 leaves per shoot) and very dense root development.
T9	0.0	3.0	The shoot development is good (more than 2 leaves per shoot). Less dense and little root growth.
T10	0.05	3.0	The shoot development is good (more than 2 leaves per shoot). Less dense and little root growth.
T11	0.1	3.0	The shoot development is good (more than 2 leaves per shoot). Less dense and little root growth.
T12	0.2	3.0	The shoot development is good (more than 2 leaves per shoot and small size). Less dense, thin and little root growth.

There were highly significant differences between treatments, days and the interaction between them for shoot length (Table 2.2 & appendix 2.5). The longest length of the shoot was found in treatment; T8 (33.4 cm), T11 (30 cm) and T5 (27 cm) and also these treatments clearly differed from the rest (Table 2.2). Therefore, these treatments can be used for the next experiment.

Table 2-2. The effect of hormones and time on the shoot length (cm). (*= very high significant,**=high significant and *=significant).**

Treats.	15 days	20 days	25 days	30 days	Mean
T1	0.26	1	2.2	2.4	1.465
T2	0.8	2.2	1.4	12.4	4.20
T3	1.4	1.4	4	17	5.95
T4	1.4	0.74	2	6.4	2.63
T5	3.2	22	27*	20	18.05
T6	3.2	12.2	13.4	11	9.95
T7	3.2	8	6.4	10.2	6.95
T8	2.2	14	33.4***	23	18.15
T9	2.8	20	13.6	16	13.10
T10	1.6	9	8.8	8.4	6.95
T11	1.6	17	30**	22	17.65
T12	2.6	9	13.6	15.4	10.15
Mean	2.02	9.71	12.98	13.68	

There were highly significant differences between treatment, days and the interaction between them for root length (Table 2.3 & appendix 2.5). The longest length of root was found in T11 (42 cm), T3 (39 cm), T11 (34 cm), T10 (33 cm), T2 (33 cm), T6 (31.40 cm), T11 (29 cm) and T2 (28 cm), and also these treatments clearly differed from the rest (Table 2.3). Therefore, any of these treatments can be used for the next experiments.

Table 2-3. The effect of hormones and time on the root length (cm). (*= very high significant, **=high significant and *=significant).**

Treats.	15 days	20 days	25 days	30 days	Mean
T1	0.40	0.00	9.00	4.00	3.35
T2	0.00	0.00	28.00*	33.00**	15.25
T3	0.20	0.00	20.00	39.00***	14.80
T4	0.00	0.40	9.40	20.20	7.50
T5	0.00	0.00	1.00	0.00	0.25
T6	0.00	5.00	31.40**	7.00	10.85
T7	0.00	3.00	25.00	15.40	10.85
T8	0.00	14.00	22.00	14.00	12.50
T9	0.00	0.00	0.00	0.00	0.00
T10	0.00	20.00	33.00**	26.00	19.75
T11	1.20	34.00**	42.00***	29.00*	26.55
T12	0.00	19.00	12.60	17.40	12.25
Mean	0.15	7.95	19.45	17.08	

There were highly significant differences between treatment, days and the interaction between them for shoot weight. The greatest weight was found in treatments; T11 (0.166 g), T8 (0.147 g), T5 (0.126 g), T11 (0.121 g), T5 (0.114 g) and T6 (0.102 g), and these treatments clearly differed from the rest (Table 2.4). Therefore, these treatments can be used for the next experiment.

Table 2-4. The effect of hormones and time on weight (g). (*= very high significant, **=high significant and *=significant).**

Treats.	15 days	20 days	25 days	30 days	Mean
T1	0.001	0.001	0.009	0.015	0.007
T2	0.001	0.008	0.008	0.067	0.021
T3	0.003	0.007	0.031	0.061	0.026
T4	0.003	0.002	0.014	0.035	0.014
T5	0.052	0.114*	0.071	0.126**	0.091
T6	0.017	0.090	0.102*	0.080	0.072
T7	0.016	0.042	0.061	0.069	0.047
T8	0.011	0.083	0.147***	0.060	0.075
T9	0.015	0.085	0.078	0.046	0.056
T10	0.009	0.049	0.053	0.030	0.035
T11	0.003	0.088	0.166***	0.121**	0.095
T12	0.014	0.055	0.092	0.081	0.061
Mean	0.012	0.052	0.069	0.066	

2.6 Discussion

2.6.1 Optimization protocols for curd regeneration

Cauliflower tissue culture has been carried out for long time with plants regenerated from tissue such as curd (Kieffer, 2001 , Al-Swedi, 2012), hypocotyls (Lv Lingling et al., 2005, Chakrabarty et al., 2002), cotyledons (Prem, 1998), roots (Grout, 1980), and stem discs (Eimert K, 1992, Bagga et al., 1985). The regeneration of shoot from these different tissues is affected by several variable factors. These variables include the type and character of explants and the medium composition (Greco et al., 1984, Finer, 1987, Burrus et al., 1991, Sun et al., 1998, Müller et al., 2001, Dhaka and Kothari, 2002, Lv Lingling et al., 2005, Trevisan and Mendes, 2005).

2.6.2 Effect of growth regulator on shoot regeneration and root formation

The cauliflower explants were cultured on media containing 12 combinations of plant growth regulators NAA and kinetin. Depending on the type and concentration of the growth regulators in culture media, callus formation, shoot regeneration and root formation was induced in both hypocotyls and cotyledons. In general terms, a high cytokinin ratio to auxin was found to stimulate shoot production.

In this research the maximum regeneration response (95%) was observed on S23 media with a high concentration of cytokinin (3 mg L^{-1} kinetin) and auxin (0.1 mg L^{-1} NAA). Similar results were found by (Omer and Nuray, 2000), who reported that the average number of buds formed on excised epicotyl explants of linden *(Tilia platyphyllos)* on media with BAP, was higher than that with kinetin. (Jain et al., 1991) likewise found that in *Brassica* all callus and cell suspension failed to regenerate shoots on MS media containing kinetin and IAA whereas (Murata and Orton, 1987),

also using MS media supplemented with kinetin and IAA, obtained regeneration from callus in *Brassica* species. In addition limited shoot regeneration was obtained on medium without growth regulators. Similar results were obtained in *Vigna radiate* (Gulati and Jaiwal, 1990) and in linden (Omer and Nuray, 2000). This response could be related to the endogenous hormone balance in the explants.

Analysis of the response of different explant types to different shoot induction media indicated that the interaction was highly significant. Also, (Punia and Bohorova, 1992) reported that regenerating whole plants from sunflower depended on the regeneration ability of the genotype, the nature of the explant and the hormone content of the medium. Most of the adventitious shoots rooted on medium supplemented with 0.1 NAAmgl^{-1}. This auxin (NAA) has been reported to stimulate a high frequency of root formation on regenerated shoots in various plant species such as *Arabidopsis* (Malamy and Ryan, 2001); Orchidaceae (Kerbauy, 1984); Tobacco; (Campanoni and Nick, 2005) and *Posidonia oceanica* (Balestri and Lardicci, 2006). This possibly shows that cells in the highly regenerative area of meristematic cells have the potential to grow into roots depending on the level of auxin, as suggested by (Mante et al., 1989) and (Mackay, 1996). Previous work with *Brassica napus, Brassica oleracea* (Biddington and Robinson, 1991); *Brassica rapa* (Kuvshinov et al., 1999) and *Brassica campestris ssp.* Chinensis (Chi and Pua, 1989) demonstrated a significant improvement in regeneration rates in a variety of explant types with inhibitors of ethylene action. By increasing shoot elongation the competition between leaves to catch the light will be less and this can lead to increased leaf area and also increased photosynthesis.

Explant size-class

The explant size-class significantly influenced the number of shoots produced per explant, with the smaller the explant the lower the number of shoots produced per explant, but the mean number of shoots per explant was not statistically different for the largest size-class. The large shoots initially appeared to be the ones that were the most interesting for micropropagation. However in fact the fully developed meristems present on a cauliflower curd were in size class 212 µm - the smallest one. As a consequence, small explants shoots develop more homogeneously. Explant size class affects the number of shoots produced and the homogeneity of explants. The size class 300 µm produced more micro shoots but these were less homogenous than those from the 212 µm class.

Explant culture conditions

Transfer after one month of culture to fresh culture medium resulted in a statistically significant increase of the mean number of shoot recovered per explant. In fact without transfer, the number of shoots recovered was very low and most of the shoots remained at the bud stage. Further experiments tested the effect of a transfer to new medium after one, two or three weeks on culture density of explants per container. Over this time period the effect on mean number of shoots produced per explant was not statistically significant when one explant was used per container but was significant for higher densities. The difference in terms of shoot size was also statistically significant with larger shoots recovered after transfer for all culture densities, with a stronger effect with the late transfer (the large amount of microshoots per container effect explant growth in the same amount of media).

These experiments highlighted the fact that when there is more than one explant per container, the culture medium available is limiting the number of shoots recovered

per explant. Increasing the nutrient supply allows regeneration of more shoots per explant. Increasing the nutrient supply by reducing the culture density yielded larger and better quality shoots.

Culture system

The nutrient supply appeared to be of paramount importance. Since at the beginning of culture many meristems start to develop without dominance, the medium requirement must increase exponentially with time. It is obvious that under such conditions the available nutrients must quickly become limiting to growth of all of the initiated shoots. This explains why any transfer to fresh medium induced more shoots to develop and led to the recovery of better quality shoots. It also explains the beneficial effect of using small explants which carry fewer pre-existing meristems and therefore more nutrients become available for each developing bud. From the literature it is clear that many other parameters of the culture system can influence the shoot regeneration efficiency such as light intensity (Kumar A, 1993), agar concentration (Kieffer et al., 1995a) and phytohormone balance((David and ET Margara, 1979, Bognar et al., 1984, Yanmaz et al., 1986, David and J., 1979).

Protocol for mass production of cauliflower propagules

All previous protocols were designed on the principle that a piece of curd was expected, even if of relatively small size, to carry large numbers of meristems many of which were able to produce shoots. The growth of these explants requires an exponentially increasing nutrient supply. Furthermore when such explants show shoot development they produce numerous shoots requiring time consuming manipulation in shoot separation. The aim of the work reported in this section is to develop a simple and efficient multiplication system using the whole

micropropagation potential of curd. The final goal of the production of one propagule per explant.

Shoot regeneration

There was no callus produced and the occurrence of shoot meristems was very low as explants carry a very small amount of stem (pedicel) tissue. The culture of explants without a meristem (i. e. stem debris) did not regenerate any shoots whatever the size-class used. Bract pieces did develop in culture but were easily identified and discarded. The percentage of explants developing in culture and the number of shoots produced per explant were highly dependent ($p<0.01$) on the explant size-class and then on the number of pre-existing meristems carried. For a constant volume of explant in culture, in non-limiting conditions, the percentage of explants developing were maximal for explants larger than 0.6 mm in diameter and decreased in proportion to the reduction in sieve size-class. The number of shoots produced per explant was highest for the highest size-class (0.6 mm) and decreased with the smaller size-class. Explants of the smaller size-classes gave bigger shoots than larger explants, probably due to a better nutrient supply. It is interesting to note that the two smallest size-classes have a different mean number of regenerated shoots per explant. For the explants of size-class 0.2-0.3 mm and 0.3-0.6 mm over 9000 micro-shoots were routinely produced from each curd for the winter heading types (Figure 2.10). It is important to recall that these figures are for the two smaller size-classes and that a large volume of explants of larger size-classes is also available.

2.7 A Standard Operation Procedure for curd micropropagation (S.O.P.$_{CM}$)

The outcome of the investigations reported in this chapter was to produce a standard operating procedure for curd micropropagation to be used for subsequent transformation experiments. The following describes this SOP$_{CM}$

1. Harvest cauliflower from the field or glasshouse at the normal stage of commercial marketability.
2. Cut pieces from the curd 1 – 5 cm in size and surface sterilize these in 10% unthickened bleach for 15 mints, followed by 3 rinses in sterile distilled water.
3. Transfer the material to a laminar flow hood.
4. Using aseptic techniques shave the 1 to 3 mm meristematic layer from the surface of the curd pieces using a scalpel and collect the shavings into a petri dish. Discard the stem branches.
5. Place the meristematic shavings into a Commercial blender (Waring or similar) fitted with a metal homogenising blade together with liquid S23 medium.
6. Blend at approx. 17,000 rev min^{-1} for 30 seconds.
7. Take the blended meristematic "soup" and pass it through a series of precision sieves arranged in descending order of size – 600, 300 and 212 mm. Wash through with S23 medium. Collect the sieve size fractions 212 to 300 mm and 300 to 600 μm. Discard the wash through solution.
8. Place a 34 μL aliquot of explants into 20 mL of liquid S23 medium supplemented with Kinetin (2 mg L^{-1}) and IBA (indol-3-butyric acid (1 mg L^{-1}) and 1 mL L^{-1} in culture vessels and place on a shaker (50 revs. min^{-1}) in a

culture room or laboratory at 23°C with supplementary lighting to provide a 16h photoperiod and a minimum PAR of 50 μmol m^{-2} s^{-1}.

9. Grow cultures for 7 to 14 days.

2.8 Conclusion

The study presented here provides further understanding of the meristem of the cauliflower curd. The number of meristems carried suggests a high micropropagation potentiality of this tissue since each of these meristems has, theoretically, the potential to produce a shoot.

The main difficulty in this experimentation was sterilisation because many experiments failed because of contamination and because of the absence of antibiotics. The rate of contamination increased with decreasing the amount of PPM used and initially 95% of the experiment failed to grow into microshoots but this altered to 95% success with 1 mL L^{-1} PPM.

Different precision sieves were used - 212,300 and 600 µm - but the optimum size for shoot production was 300 µm, producing uniform explants and shoot production, in addition to a large amount of explants being produced for further experimentation.

Different durations have been used - 15, 30, 45, 60, 90 and 120 seconds - with an optimal duration of 30 second for shoot production; the blending duration effected the production of meristem and debris fractions in addition to the production of uniform samples. Meristem loss increased with increasing blending duration, while less than 30 seconds failed to produce uniform samples

In this experiment one curd produced large numbers of explants (about 27,060 to 42000 shoots per head), with the largest amount produced in 300 µm preparations with 30 s duration.

The understanding of curd development in cauliflower will hopefully contribute to the understanding of the genetic control of floral development in general.

3 Chapter Three: Effect of antibiotics on *Agrobacterium tumefaciens* & cauliflower shoot growth

3.1 Introduction

Resistance is the ability of an organism to survive in the presence of concentrations of a chemical which are normally lethal to organisms of that species. When bacteria are exposed to an antibiotic various things can occur: they can be killed, they can be weakened (disabled), or they can remain unchanged (resistant). The antibiotic of the correct type to work against a particular bacterial type also depends on whether the antibiotic is present in a sufficiently high concentration, suggesting that bacteria can detoxify the antibiotic at low concentrations. Not all antibiotics will work against all bacteria and some bacteria require significantly higher concentrations of antibiotic to kill them. Antibiotic resistance is a natural phenomenon and was present in bacteria before antibiotics came into use (Shackelford and Chlan, 1996), but indiscriminate use of antibiotic in medicine has led to an increased incidence of antibiotic resistance.

Most antibiotics are chemical substances created by one species of micro-organism that contain or influence the growth of other micro-organisms. The term antibiotic has been extended to include both chemically modified natural antibiotics and completely man-made substances which would more technically be referred to as semi-synthetic or synthetic antimicrobial agents. Competition among microbial species most probably resulted in the occurrence of natural antibiotics, and micro-organisms responded accordingly by developing multiple mechanisms of antibiotic resistance. The most important antibiotic resistance in bacteria is the result of the gaining of antibiotic resistance plasmids or other exchangeable genetic elements which carry multiple genes for resistance to different antibiotic classes (Pieterse et al., 1996).

There are many reasons for consideration of the effect of antibiotics in higher plants; a) some antibiotics may be produced naturally in the soil in the rhizosphere; accordingly the root may be naturally exposed to this organic matter; b) many antibiotics have been isolated from tissues of higher plants; c) some of them are a growth inhibitor of plants which involve a dormancy phenomenon; d) some antibiotics can be used to control bacterial and fungal disease infections of plants; e) the biological activity and unique structure of antibiotics has naturally led to remarkable instances of stimulation and inhibition of growth (Brian, 1957).

Antibiotic resistance traits for plant biotechnology involve the production of proteins, and antibiotic resistance markers (ARMs) are used in plant biotechnology for two purposes. ARMs are frequently used in the production of plasmids or vectors used to achieve recombinant DNA technology and plant transformation, such as the propagation (multiplication) of genetic material in *Escherichia coli* or the production of genetically modified *Agrobacterium tumefaciens*. The ARM must follow a bacterial promoter sequence and in some cases the finished plant product may contain an ARM gene that bears a bacterial control sequence (Marchant and Marchant, 1999, Laxminarayan, 2003) and consequently will not function efficiently in the nuclear genome of plants (Tepfer et al., 2003).

Plants do not always carry an ARM gene and in a few cases development of plant transformation has proceeded without antibiotic markers through the investment of additional time and resources necessary to screen many different cells or plants for the desired trait with a gene insert.

Antibiotic selection markers are not essential for the insertion of herbicide resistance traits in plant cells, as the herbicide itself can be used to select for transforming cells

(Padgette et al., 1995, Malik and Saroha, 1999). For other traits such as insect resistance a selectable marker such as antibiotic resistance is useful, if not essential, for the efficient selection of transformed cells (He et al., 2006).

The most commonly used ARM for plant cell selection is nptII (neomycin phosphotransferase II), also referred to as APH-3′-II or aminoglycoside phosphotransferase 3′- II) (Goldstein et al., 2005). This ARM inactivates the aminoglycoside antibiotics neomycin and kanamycin. Other ARMs such as rifampicin are commonly used for bacterial selection during the preparation of plasmids and bacterial DNA prior to their use in plant transformation. The markers conferring resistance to Hygromycin or other similar antibiotics (Tetracycline, Chloramphenicol) have been used extensively in plant research (Liau et al., 2003), but do not presently appear in commercial genetically modified plants (Miki and McHugh, 2004).

The development of an effective system for gene transfer in cauliflower will depend largely on the availability of tissue culture methods that permit capable DNA delivery, selection of transformants and recovery of transgenic plants. Increasing regeneration efficiency was critical for the development of a transformation system in apple using *A. tumefaciens* vectors (Klee and Rogers, 1989), and in soybeans (Liu et al., 2004) and/or the biolistic process (Yepes and Aldwinekle, 1994). In many perennial plants, the absence of an efficient regeneration system is the major limiting factor preventing the development of efficient gene transfer technologies (James et al., 1992).

One of the main interests in our laboratory has been to develop genetic transformation techniques for commercially important cauliflower cultivars with the goal of using genetic engineering for cauliflower improvement. The objective of this work was to optimize regeneration frequency from cauliflower micropropagation for

several commercial genotypes with the final goal of enhancing recovery of transformed cauliflower plants. The effect of some antibiotics that are used through transformation either to eliminate *Agrobacterium tumefaciens* from the culture medium (Ogawa and Mii, 2005) or to select transgenic plants was studied because previous reports suggested that some of these antibiotics may be phytotoxic and may inhibit regeneration (James et al., 1984, Leblay et al., 1991).

Shoot regeneration is ultimately necessary for plantlet formation from cells transformed by *Agrobacterium.* The antibiotic Kanamycin has been used extensively to screen for transgenic plants, as it can effectively inhibit root and shoot organogenesis from non-transformed susceptible tissue, and a gene for resistance to Kanamycin has been included in many *Agrobacterium* vectors (Klee et al., 1987). Transgenic cells resistant to kanamycin have been selected on media typically containing 50 - 100 mg L^{-1} Kanamycin (Klee et al., 1987).

3.2 Material and methods

Five antibiotics were used in the present study; Carbenicillin disodium salt (Sigma–Aldrich, St. Louis, MO, USA), Cefotaxime sodium salt (Claforan, Aventis Pharma, Frankfurt, Germany), Kanamycin sulphate (Sigma–Aldrich, St. Louis, MO, USA) Gentamycin sulphate (DUCHEFA, Haarlem the Netherlands) and Tetracycline (DUCHEFA, Haarlem the Netherlands). Further details can be found in Appendix 5. The concentrations used were based on their anti-agrobacterial activities (Chilton et al., 1974) when applied to transformation experiments. All explants were theoretically susceptible to Kanamycin (Kan), Gentamycin (Gent), Tetracycline (Tetr), Cefotaxime (Cef) and Carbenicillin (Carb). The experiments were designed to determine the critical level of antibiotic required to inhibit regeneration during subsequent transformation screening experiments.

3.2.1 Experiments to develop a Standard Operation procedure for Antibiotics (SOP-A)

In order to study the formation and growth of callus and shoots of cauliflower explants exposed to antibiotics, the following experiments were carried out. The experiments were designed to determine the critical level of antibiotic required to inhibit regeneration during subsequent transformation screening experiments.

Experiment 1. Effect of different combinations of antibiotics on plant regeneration.

Manipulations were performed in a horizontal laminar air flow cabinet. This was swabbed with 70% ethanol prior to use. Instruments such as spatulas, forceps and scalpels were immersed in 100% ethanol and flamed prior to use. Sieves, filter papers, forceps and pipette tips were autoclaved at 121 ºC for 20 minutes. Media

were sterilised in an autoclave, with a standard sterilisation cycle of 20 min at 121 ºC. Since growth regulators and antibiotic solutions can be heat labile they were sterilised by passing them through a Millex-Gv 0.22 µm filter unit and they were then added to the media after autoclaving.

For shoot regeneration, the culture medium employed was S23 medium supplemented with a combination of antibiotic (Table 3.1). Stock solutions of antibiotics (Appendix 5) were added to cooled (but still liquid) sterile regeneration media before being poured into pots; then the pots were incubated in growth chamber for 4 weeks. The experiment was laid out as a completely randomized design and each treatment was composed of 16 pots (4 replicates).

The explants were incubated for 4 weeks prior to recording regeneration frequency as the mean percentage of explants with shoot induction (number of regenerated explants/total number of explants * 100) in comparison to the control (treatment 1).

Table 3-1. Different combinations of antibiotics (Each 1 mgL^{-1}, equivalent to 100 µl of antibiotic per 25 ml media aliquot).

Treatments	Kanamycin (Kan)+ Gentamycin (Gent) mg L^{-1}	Kanamycin (Kan)+ Tetracycline (Tetr.) mg L^{-1}	Tetracycline (Tetr.) + Gentamycin (Gent) mgL^{-1}
T1	0.0 (Kan) + 0. 0 (Gent)	0.0 (Kan) + 0.0 (Tetr.)	0.0 (Tetr.) +0. 0 (Gent)
T2	2.0 (Kan) +1. 0 (Gent)	2.0 (Kan) +1. 0 (Tetr.)	1.0 (Tetr.) +1. 0 (Gent)
T3	6.0 (Kan) +2. 0 (Gent)	6.0 (Kan) +4. 0 (Tetr.)	4.0 (Tetr.) +2. 0 (Gent)
T4	20 (Kan) +6. 0 (Gent)	20 (Kan) +8. 0 (Tetr.)	8.0 (Tetr.) +6. 0 (Gent)
T5	30 (Kan) +8.0(Gent)	30 (Kan) +10 (Tetr.)	10 (Tetr.) +8 (Gent)
T6	40 (Kan) +10 (Gent)	40 (Kan) +15 (Tetr.)	15 (Tetr.) +10 (Gent)

Experiment 2. Effect of different concentrations combinations of Cefotaxime and Carbenicillin on plant regeneration and Agrobacterium elimination on explants.

Cefotaxime and Carbenicillin antibiotics are routinely used to eliminate *A. tumefaciens a*fter co-cultivation mainly due to their low plant toxicity. This experiment was conducted to determine the effect of Cefotaxime and Carbenicillin on cauliflower plant regeneration and *Agrobacterium* disinfection. Non-transformed (control) explants were cultured, as described before on S23 media supplemented with different concentrations of Cefotaxime and Carbenicillin (0, 250, 500, 750 and 1000 mgL^{-1}). The pots were incubated in a growth chamber for 4 weeks and then scored for plant regeneration as in experiment 1, while elimination was then scored as follows: +ve = no elimination (visible bacteria growth and culture death) -ve = elimination (no sign of bacteria growth, explants green and growing). This experiment was conducted on 5 replicates of 25 pots for each treatment.

The experimental design was 10 explants per treatment, and each treatment was repeated at least 4 times with various concentrations of Carbenicillin or Cefotaxime (0, 250, 500, 750 and 1000 mgL^{-1}) added to the shoot regeneration medium

Experiment 3; Media preparations for bacterial growth with antibiotic solutions

Media for bacterial growth studies was prepared in the laboratory under aseptic conditions next to a gas flame to provide an up draught to avoid airborne contamination, wearing a lab coat and gloves. Liquid and solid media were prepared with and without antibiotics as follows:

For liquid; 5 g. Luria Broth media (Appendix 6.a) without agar was added to 200 mL distilled water. 10 ml of liquid from LB media was poured into each universal bottle, then 20 universal bottles autoclaved. For solid; 25 g. of Luria Broth media was added into one litre of distilled water then 10 g of agar was added to the media to prepare three bottles with 200 mL in each and one bottle with 400 g. Finally all four bottles were autoclaved at 121^0C for 15 minutes.

Three small sterile plastic tubes were prepared with antibiotics for stock solution as follows; firstly, 0.5 g of Gentamycin was added to 10 ML water. Secondly, 0.5 g of Kanamycin was added to 10 ml of water. Thirdly, 0.5 g Tetracycline was added to 10 ml of water. Then 200 µL of Kanamycin and 200 µL of Gentamycin were added to 200 mL liquid media (first bottle). 200 µL of Tetracycline and 200 µL of Gentamycin were added to 200 mL liquid media (second bottle). 200 µL of Tetracycline and 200 µL of Kanamycin were added to 200 mL liquid media (third bottle).Luria Broth media with 7g agar containing Kanamycin and Gentamycin was poured into 15 Petri dishes. LB with Tetracycline and Gentamycin was poured into 15 Petri dishes. LB with Tetracycline and Kanamycin was poured into 15 Petri dishes. Finally LB without antibiotic was poured into 30 Petri dishes.

Second step; under aseptic conditions (gas flame, gloves and lab coat) tubes containing liquid media without antibiotic were inoculated with three strains of *A.*

tumefaciens bacteria APX, TA and SOD (APX and TA were cloned on pRTL2 and pCGN1578 vector, respectively while SA was on pBiN+ARS vector). Then, the tubes were incubated at 28º C for 48 hours. Finally 20 µL of each three strains of bacteria were added to LB Media containing antibiotics.

3.3 Results

3.3.1 Experiment 1;

I. Effect of Kanamycin, Gentamycin, Tetracycline, Cefotaxime and Carbenicillin on shoot generation.

A selection of antibiotics - Kanamycin, Gentamycin, Tetracycline, Cefotaxime and Carbenicillin were used to select between non-transformed cell lines and those carrying the antibiotic resistance gene introduced by transformation. The effectiveness of the antibiotic to interfere with shoot regeneration was determined prior to transformation experiments. Shoots were induced on S23 medium supplemented with 2 mg L^{-1} Kinetin and 1 mg L^{-1} IBA.

After 10 days of treatment the effect of antibiotic concentration on the colour and length of regenerated shoots on explants was clearly obvious (Plate3.1). The 10 mgL^{-1} Kanamycin + 8 mg L^{-1} Gentamycin combination was subsequently chosen to select between transformed and non-transformed plants.

Plate 3.1. Effect of different combinations (T1=0. 0; T2=100; T3=300; T4=500; T5=750) µl L^{-1} of antibiotics (Kanamycin, Gentamycin, Tetracycline, Cefotaxime and Carbenicillin) on shoot where KG (Kanamycin & Gentamycin), KT (Kanamycin & Tetracycline), TG (Tetracycline & Gentamycin) and CC (Carbenicillin & Cefotaxime).

Table 3-2. Different concentrations (T1=0. 0; T2=100; T3=300; T4=500; T5=750) µl for all antibiotics (Kanamycin, Gentamycin, Tetracycline, Cefotaxime and Carbenicillin) respectively.

Size	Volume	Antibiotics	% Shoot Survival				
			0.0 µL	100 µL	300 µL	500 µL	750 µL
300	74	Kan.	100	90	90	90	50
		Gent.	100	90	90	90	50
		Tetr.	100	90	90	80	80
		Cef	100	100	100	100	100
		Carb.	100	100	100	100	100
212	34	Kan.	100	90	90	90	50
		Gent.	100	90	90	90	50
		Tetr.	100	90	90	80	80
		Cef.	100	100	100	100	100
		Carb.	100	100	100	100	100

Cefotaxime proved an effective antibiotic against *A. tumefaciens*. A concentration of 250 mg L^{-1} was high enough to eliminate *A. tumefaciens* strain EHA105 on agar plates with inoculated explants. There was no difference in the efficiency of the elimination of *Agrobacterium* between Carbenicillin (150 mg L^{-1}) and Cefotaxime (500 mg L^{-1}). Shoot regeneration was not significantly inhibited on medium containing Carbenicillin (Table 3.2 and Plate 3.1).

To screen transformants, regenerated shoots were first selected in the presence of Kanamycin on rooting medium because untransformed shoots were not able to form roots on this medium. All rooted shoots were considered putative transformants and

analysed by PCR to screen for the presence of T-DNA. About 75% of rooted shoots showed a positive PCR reaction.

II. Sensitivity to Kanamycin, Gentamycin and Tetracycline

Effect on shoot regeneration

The antibiotics Kanamycin, Gentamycin and Tetracycline were used to investigate the efficacy of selection between non-transformed cell lines and those carrying the antibiotic resistance gene introduced by transformation. The effectiveness of the antibiotic to interfere with callus growth and shoot induction was determined prior to the transformation experiments. Callus tissue developed in the medium containing 2 mg L^{-1} 2, 4-D (Plate containing callus, shoot and root formation) was produced on S23 medium supplemented with 0.2 NAA mg L^{-1} + 3.00 mg L^{-1} BAP. In all concentration treatments of Kanamycin and Gentamycin; T1 (0.0), T2 (2, 1) mg L^{-1}, T3 (6, 2) mg L^{-1},T4 (20, 8) mg L^{-1}, T5 (40,10) mg L^{-1} during period of time (5,10,15,and 20) day recorded, there is no significant differences between any treatments (Figure 3.1).

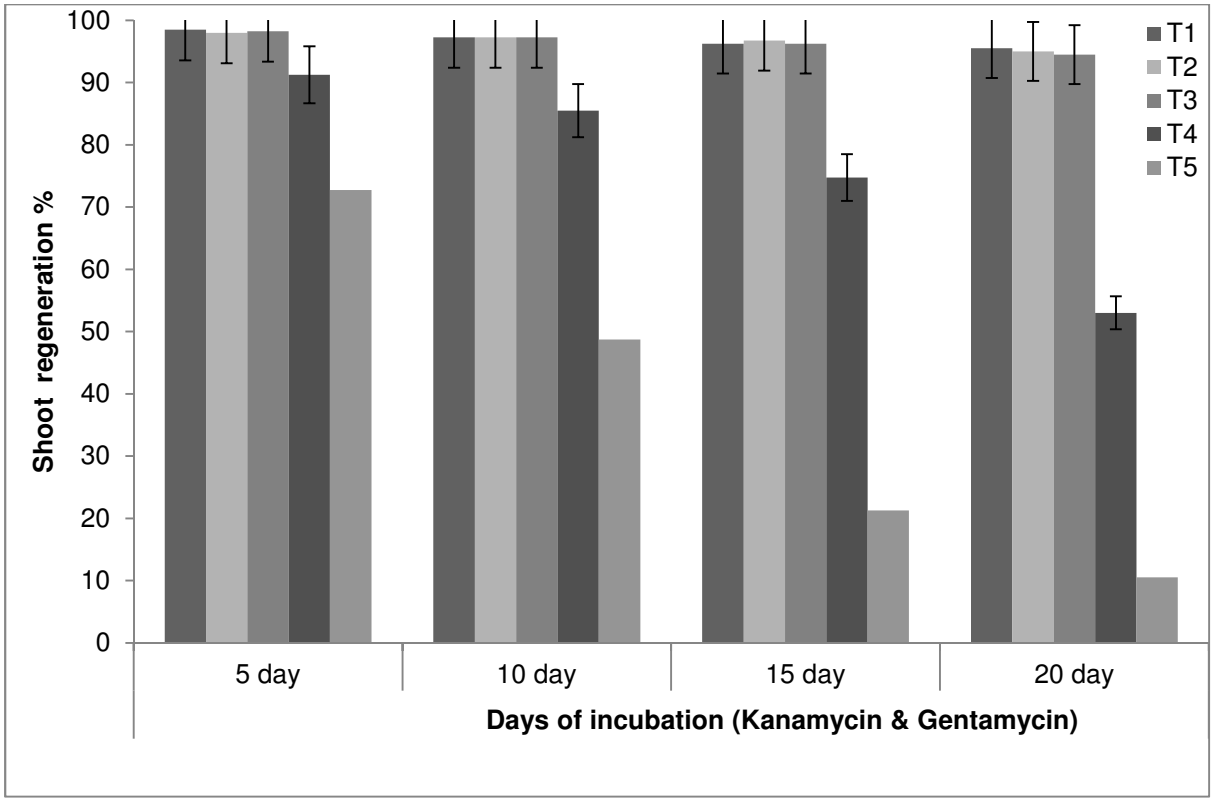


Figure3-1. Effect of Kanamycin or Gentamycin on shoot regeneration. Treatments as follows: T1 (0.0), T2 (2, 1) mg L^{-1}, T3 (6, 2) mg L^{-1}, T4 (20, 8) mg L^{-1}, T5 (40, 10) mg L^{-1}. Bars represent the standard error of the mean.

III. Effect of Kanamycin and Tetracycline

Various concentrations of Kanamycin and Tetracycline (Table 3.2), in addition to 250 mg L^{-1} Cefotaxime were added to callus induction and shoot regeneration medium. The results showed significant inhibition of shoot regeneration for explants at concentrations higher than Kanamycin 20 mg L^{-1} + Tetracycline 8 mg L^{-1} as in Figure 3.2 and Plate 3.2. The result showed that the increase in concentration of antibiotics led to an increase in the survival percentage of shoots. As a result the Kanamycin 20 mg L^{-1} + Tetracycline 8 mg L^{-1} was selected as an effective concentration for selection between transgenic and non-transgenic plants. In all 5 treatments; T1 (0.0), T2 (2, 1) mg L^{-1}, T3 (6, 4) mg L^{-1}, T4 (20, 8) mg L^{-1}. T5 (30, 10) mg L^{-1}, the reading

for all the days (5, 10, 15 and 20) showed high effects of antibiotic (Kanamycin and Tetracycline) on shoot regenerations in compared with control(no antibiotics).

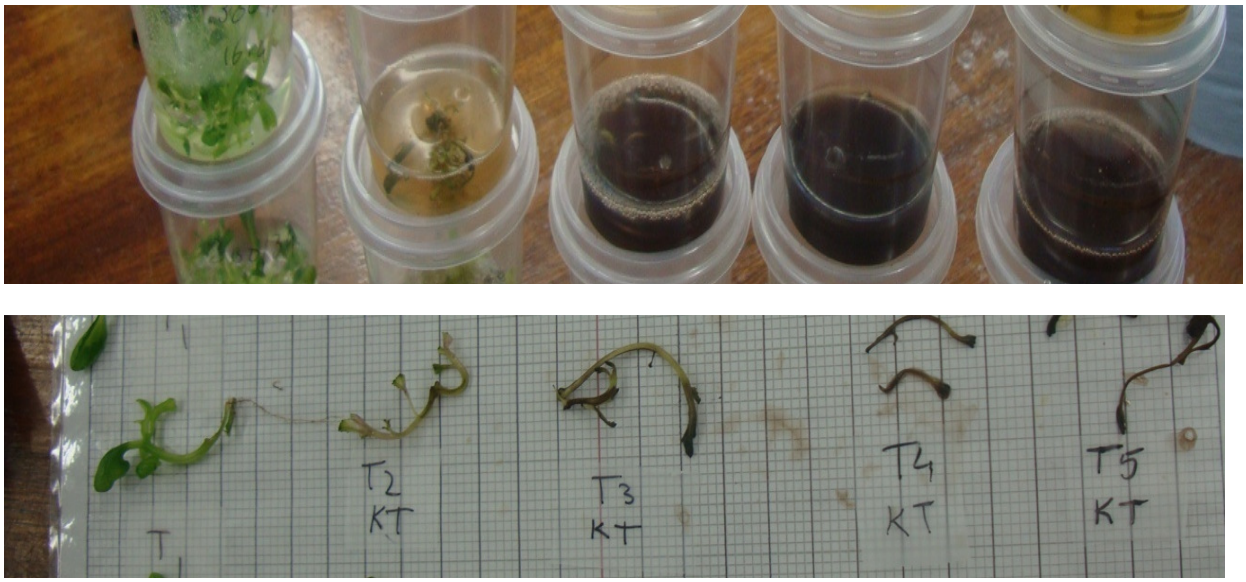


Plate 3.2. Illustrations to show the influence of different combinations of Kanamycin and Tetracycline on shoot differentiation of explants of cauliflower before inoculation with *A. tumefaciens* and cultivated on medium S23. The picture was taken after 30 days.

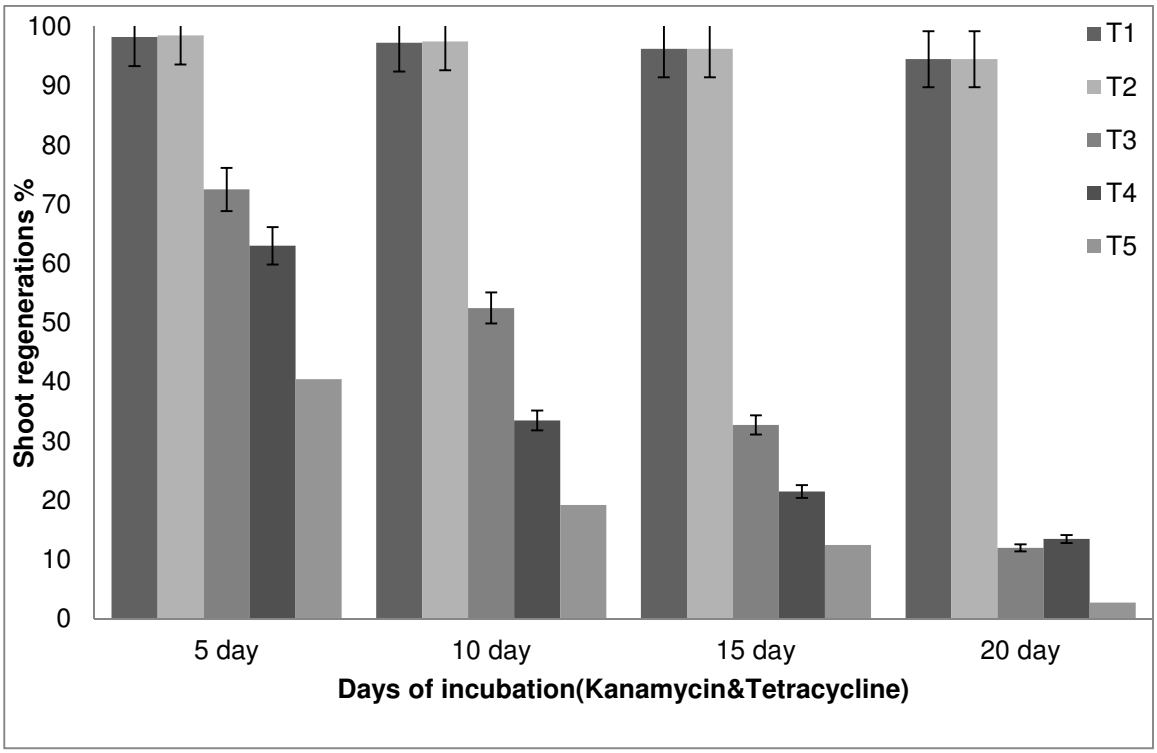


Figure 3-2. Effect of Kanamycin and Tetracycline on shoot regeneration. Treatments as follows: T1 (0.0), T2 (2, 1) mg L^{-1}, T3 (6, 4) mg L^{-1}, T4 (20, 8) mg L^{-1} and T5 (30, 10) mg L^{-1}. Bars represent the standard error of the mean.

IV. Effect of Tetracycline and Gentamycin

A combination of different concentrations of Kanamycin and Gentamycin was applied in this experiment to select an effective selection concentration between transgenic and non-transgenic plants (Table 3.1). The results showed that there was an inhibitory effect and a gradual decrease in green colour as antibiotic concentrations increased.

Tetracycline, at 4 mg L^{-1} combined with Gentamycin at 2 mg L^{-1}, significantly reduced shoot regeneration (Figure 3.3 and Plate 3.3). Very small numbers of explants produced shoots and most of these shoots went brown by the end of three weeks incubation. Shoot regeneration was completely inhibited and there was no growth at a concentration of Tetracycline of 10 mg L^{-1} + Gentamycin 8 mg L^{-1} or greater.

It was clear that of antibiotics had negative effect on shoot regenerations at high concentration and the effect decrease at low concentrations.

Plate 3.3. Illustrations to show the influence of different combinations of Gentamycin and Tetracycline on shoot differentiation of explants of cauliflower before inoculation with *A. tumefaciens* and cultivated on medium S23. The picture was taken after 30 days.

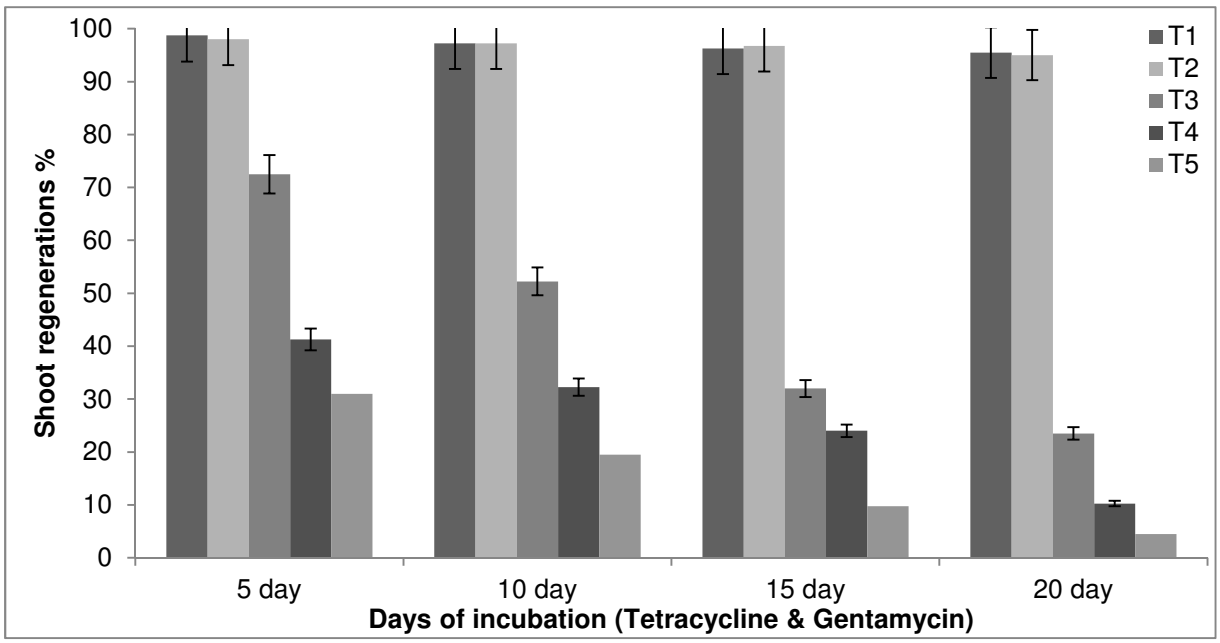


Figure 3-3. Demonstration the effect of Tetracycline and Gentamycin on shoot regeneration (T1=0.0 Tetracycline + 0.0 Gentamycin; T2=1.0 Tetracycline + 1.0 Gentamycin T3=4.0 Tetracycline + 2.0 Gentamycin; T4=8.0 Tetracycline+6.0 Gentamycin; T5=10 Tetracycline+8.0 Gentamycin (mg L^{-1}). Bars represent the standard error of the mean.

3.3.2 Experiment 2

1. Effect of Carbenicillin and Cefotaxime on shoot regeneration

The data presented in Figure 3.1 and 3.2 shows that there was no significant inhibition of shoot regeneration by Carbenicillin and Cefotaxime.

2. Effect of Carbenicillin and Cefotaxime on elimination of *Agrobacterium* after co-cultivation

The efficiency of the elimination of *Agrobacterium* increased linearly with increased Carbenicillin and Cefotaxime concentration. The highest efficiency (98.5% & 98%) was recorded for explants treated with higher concentration of Carbenicillin and Cefotaxime (Figure 3. 4 and plate 3.4).

Carbenicillin was slightly more efficacious than Cefotaxime but neither antibiotic was able to eliminate *Agrobacterium* completely at the concentrations tested. When the results of this experiment are combined with the previous experiment, it is clear that a compromise between *Agrobacterium* elimination and shoot regeneration must be made; it was decided to use a concentration 250 mg L^{-1} Cefotaxime so as not inhibit shoot regeneration unduly.

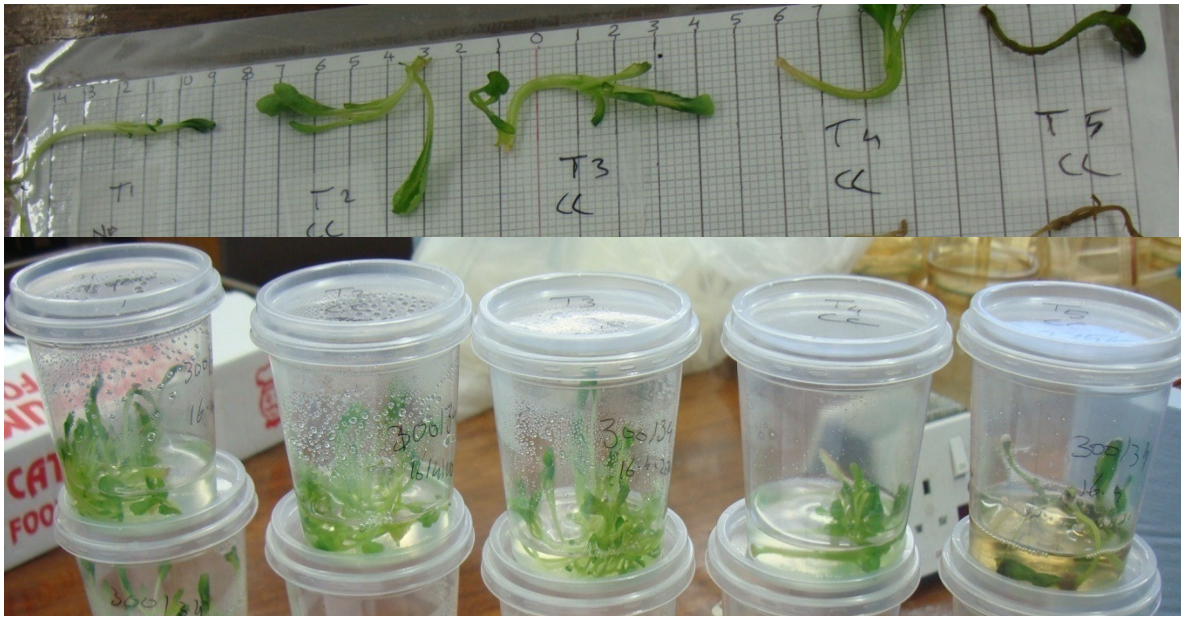


Plate 3.4. Illustration of the influence of different combinations of Cefotaxime and Carbenicillin on shoot differentiation of explants of cauliflower before inoculated with *A. tumefaciens* and cultivated on medium S23. Treatments as follows'; T1 (0.0), T 2 (1, 1) mg L^{-1}, T 3 (4, 2) mg L^{-1}, T4 (8, 6) mg L^{-1}, T5 (10, 8) mg L^{-1}. The picture was taken after 30 day.

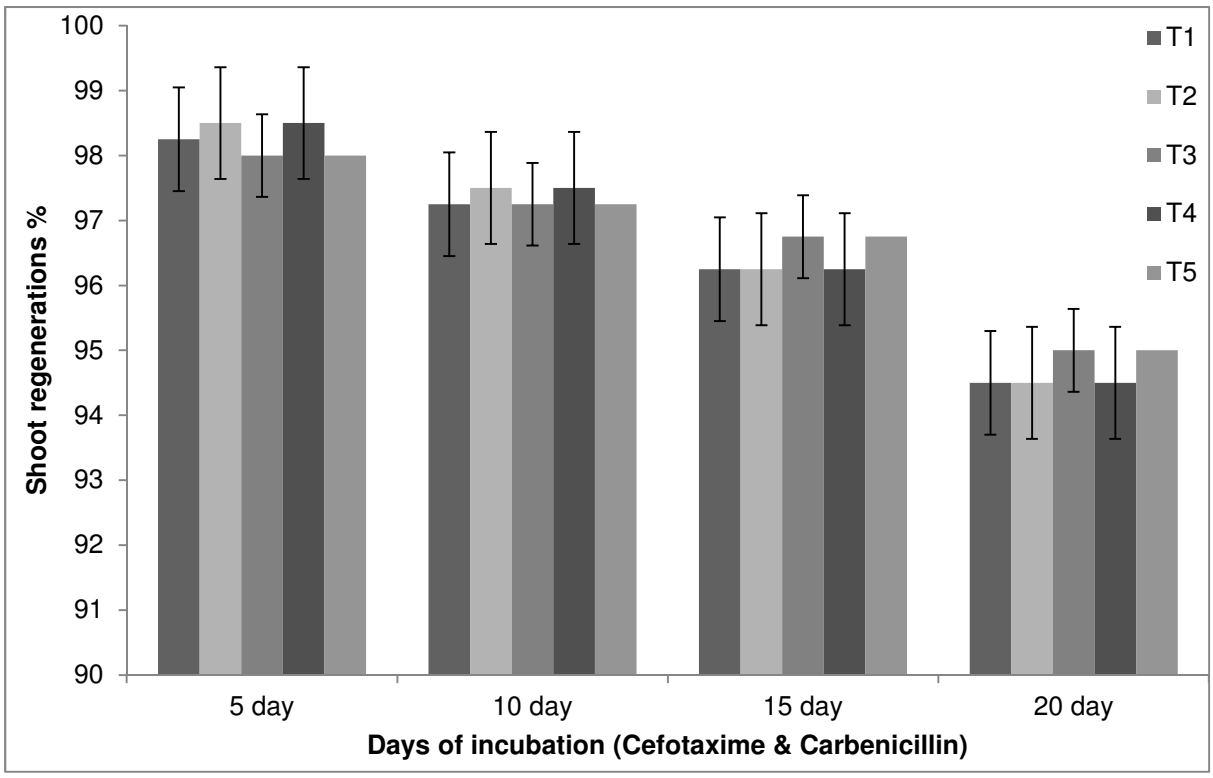


Figure 3-4. Illustration of the effect of Carbenicillin and Cefotaxime on shoot regeneration. Treatments as follows; T1 (0.0), T 2 (1, 1), T 3 (4, 2), T4 (8, 6), T5 (10, 8) mg L^{-1}. Bars represent the standard error of the mean.

3. Bactericidal effect (*Agrobacterium*):

Both Carbenicillin & Cefotaxime effectively controlled *Agrobacterium* strains and explant growth (Table 3.2). The growth of *A. tumefaciens* strain APX was more obvious on LB medium as opposed to YEB medium and was greater under dark conditions than in the presence of light. When Cefotaxime and Carbenicillin were combined, the antibactericidal effect was greater in the short-term (4 d after treatment), but growth on LB or YE media was just as high as when the equivalent concentration of either antibiotic was used singly. The use of Carbenicillin & Cefotaxime together proved to be highly effective in the elimination of *Agrobacterium*, even when each was used at a low concentration (<100 mg L^{-1}).

3.3.3 Experiment 3

a) Effect of antibiotics on shoot regeneration in liquid media

Regeneration was completely inhibited at concentrations of 50 mg L^{-l} or greater (Kanamycin). Kanamycin prevented shoot greening but frequently the top sides of cotyledons were green on 10 to 25 mg L^{-l} Kanamycin. In addition, roots sometimes formed on the top of I cm long shoot explants on medium containing 10 mgL^{-1} Kanamycin. The same effect was recorded with Gentamycin and Tetracycline on shoot regeneration in liquid medium at the same concentrations (Plate 3.5).

Plate 3.5. Illustration of shoot development in liquid media containing different concentration; 0, 100, 300, 500 and 750 µL of antibiotics (Kanamycin, Gentamycin and Tetracycline) separately.

b) Effect of antibiotics on shoot regeneration on solid media

A combination of antibiotics (Kanamycin & Gentamycin), (Kanamycin & Tetracycline) and (Gentamycin & Tetracycline) at different concentrations (0.0, 100 and 300 µL) were applied to explants growing on solid medium S23 with 7 g L^{-1} agar. As shown in Plate 3.6 the effect of all treatments on colour of shoots was very marked and in addition root development was much reduced compared with the control (no antibiotics). Table 3.3 showes the effect of antibiotics combinations on bacterial growth.

Plate 3.6. Illustration of shoot and root development on solid media supplied with different combinations of antibiotics (Kanamycin & Gentamycin), Kanamycin & Tetracycline) and (Gentamycin & Tetracycline).

Table 3-3. Table to show the effect of different treatments of antibiotics (Kanamycin, Gentamycin, Tetracycline, Kanamycin & Gentamycin, Kanamycin& Tetracycline and Kanamycin & Tetracycline) on A. tumefaciens APX strain.

Treat.	Kan	Gent	Tetr.	Kan & Gent	Kan & Tetr	Gent & Tetr
APX	+ve	+ve	-ve	+ve	-ve	-ve
	+ve	+ve	-ve	+ve	-ve	-ve
	+ve	+ve	-ve	+ve	-ve	-ve

c) Effect of antibiotics on *A. tumefaciens* strain APX growth in LB media.

A range of antibiotics were applied on APX bacterial strain separately or in combination to assess effects on bacterial growth in LB medium. The table (3.4) illustrate the effect of different combinations of antibiotic on APX strain growth. There was a positive effect of Kanamycin, Gentamycin separately and in combinations on growth of *Agrobacterium*, while there was negative effect of antibiotic (Tetracycline alone and in combinations of Tetracycline & Kanamycin and Tetracycline& Gentamycin) on *Agrobacterium*

Table 3-4. *A. tumefaciens* strain APX inoculated with different concentrations of antibiotics in LB agar media with varying concentrations of antibiotics µL antibiotics/100 mL media to (25, 50, 75, 100) mg L^{-1} antibiotics/50 mL media

Antibiotics	Reps.	Concentrations of antibiotics (LB media)				
		0	25 mg L^{-1}	50 mg L^{-1}	75 mg L^{-1}	100 mg L^{-1}
Carbenicillin (Carb.)	1	+ve	-ve	-ve	-ve	-ve
	2	+ve	-ve	-ve	-ve	-ve
	3	+ve	-ve	-ve	-ve	-ve
	4	+ve	-ve	-ve	-ve	-ve
	5	+ve	-ve	-ve	-ve	-ve
Cefotaxime (Cef.)	1	+ve	-ve	-ve	-ve	-ve
	2	+ve	-ve	-ve	-ve	-ve
	3	+ve	-ve	-ve	-ve	-ve
	4	+ve	-ve	-ve	-ve	-ve
	5	+ve	-ve	-ve	-ve	-ve
Tetracycline (Tetr.)	1	+ve	-ve	-ve	-ve	-ve
	2	+ve	-ve	-ve	-ve	-ve
	3	+ve	-ve	-ve	-ve	-ve
	4	+ve	-ve	-ve	-ve	-ve
	5	+ve	-ve	-ve	-ve	-ve
Kanamycin (Kan)	1	+ve	+ve	+ve	+ve	+ve
	2	+ve	+ve	+ve	+ve	+ve
	3	+ve	+ve	+ve	+ve	+ve
	4	+ve	+ve	+ve	+ve	+ve
	5	+ve	+ve	+ve	+ve	+ve
Gentamycin (Gent.)	1	+ve	+ve	+ve	+ve	+ve
	2	+ve	+ve	+ve	+ve	+ve
	3	+ve	+ve	+ve	+ve	+ve
	4	+ve	+ve	+ve	+ve	+ve
	5	+ve	+ve	+ve	+ve	+ve

+ve = no elimination (visible bacteria growth and culture death)

-ve = elimination (no sign of bacteria growth, explants green and growing).

3.3.4 Overview the effects of antibiotics on shoot regeneration.

Ant.	Conc. µL	Number& length of shoot/root (cm)	Shoot colour
Kan.	750	6 leaves/shoot,30 length of shoot& root	70% white,20% green,10% pink
	500	6 leaves/shoot,40 length of shoot& 30 root	50% white,50% green (hard leaves)
	300	9 leaves/shoot,70 length of shoot& 30 root	80% white,20% green
	100	12 leaves/shoot,40 length of shoot& no root	10% white,10% green,80% pink(50%hard leaves)
Gent.	750	2 leaves/shoot,30 length of shoot&10 root	50% green,50% light green
	500	3 leaves/shoot,35 length of shoot& no root	50% green,50% light green(hard leaves)
	300	13 leaves/shoot,50 length of shoot& no root	20% green,80% white
	100	10 leaves/shoot,50 length of shoot& no root	80% light green,80% white,10% pink
Tetr	750	8 leaves/shoot,30 length of shoot& root	80% light green,20% green(50% hard leaves
	500	1 leaves/shoot,,20 length of shoot& 5 root	green to brown and weak leaves
	300	2 leaves/shoot,60 length of shoot& 5 root	50% green,50% light green
	100	4 leaves/shoot,60 length of shoot& no root	50% green,40% white,10% pink(long thin leave)

3.4 Discussion

3.4.1 Effect of Kanamycin, Gentamycin and Tetracycline on Shoot regeneration

The use of antibiotics in plant tissue culture is not usually mentioned because plant cells are commonly known to be sensitive to antibiotics such as Kanamycin, Tetracycline and Gentamycin (Dix et al., 1977) and this means that these antibiotics can be used as selectable markers for identification of rare transformants. One of the objectives in this study was to obtain plant transformation using selection with Kanamycin, Tetracycline and Gentamycin resistance genes. It was thought that these selective agents might also effect shoot regeneration. Commonly, the regeneration frequency of explants decreased as the concentration of Kanamycin or Tetracycline or Gentamycin increased and, at high concentrations, the regenerated shoots were weak, had white leaves and died after a short time. The same response was described in *H. annuus L.* (Pugliesi et al., 1993), rootstocks of Malus cultivars by (Yepes and Aldwinckle, 1994) and plum plants by (Gonzalez Padilla et al., 2003). The last mentioned writers described that once the explants had been exposed to kanamycin; initiated shoots began a slow bleaching development which improved over several subcultures. This phenomenon was also detected in both transformed and non-transformed shoots in the preliminary transformation experiments described here. Furthermore, with 6 mgL^{-1} Tetracycline + 2 mgL^{-1} Gentamycin or 6 mgL^{-1} Kanamycin + 4 mgL^{-1} Tetracycline or 20 mgL^{-1} Kanamycin + 6 mgL^{-1} Gentamycin the necrosis seemed to occur on the regenerated shoot whereas with 8 mgL^{-1} Tetracycline + 6 mgL^{-1} Gentamycin or 20 mgL^{-1} Kanamycin + 8 mgL^{-1} Tetracycline or 30 mgL^{-1} Kanamycin + 8 mgL^{-1} Gentamycin shoot development was completely

prevented. This showed that both antibiotics have detrimental effects on shoot regeneration.

There are many reports referring to the relations between selective agents and subsequent regeneration ability (Schopke et al., 1996). From the current results, 8 mgL^{-1} Tetracycline + 6 mgL^{-1} Gentamycin or 20 mgL^{-1} Kanamycin + 8 mgL^{-1} Tetracycline or 30 mgL^{-1} Kanamycin + 8 mgL^{-1} Gentamycin are all effective for an irreversible suppression of growth for non-transformed cauliflower; these concentrations are, therefore, suitable for use in experiments involving selection of Kanamycin or Tetracycline or Gentamycin resistant transformed cauliflower cultures.

3.4.2 Effect of Carbenicillin and Cefotaxime on *Agrobacterium* elimination and shoot regeneration

For effective *Agrobacterium* mediated transformation, the antibiotic system should control *Agrobacterium* overgrowth without inhibiting the regeneration capacity of the plant cells. For this purpose different concentrations of Carbenicillin and Cefotaxime were assessed to study the effect on shoot regeneration and *Agrobacterium* elimination and to regulate which antibiotic will be more effective and at what concentration.

The presence of Carbenicillin and Cefotaxime in the culture medium had significant effects on regeneration but the effect of Cefotaxime was less than that of Carbenicillin. Increasing the concentration dramatically inhibited shoot regeneration but at the same time led to better elimination of the *Agrobacterium.* Similar results were also described by (Mathias and Mukasa, 1987b)and by (Lv et al., 2005.). The results also showed the different sensitivity of *Brassica oleracea* explant tissues to the two commonly used antibiotics for *Agrobacterium* elimination. The results

indicated that Cefotaxime at the concentration 250 mgL^{-1} was the best agent to eliminate *Agrobacterium* after co-cultivation explants without causing toxicity to the *in-vitro* cauliflower cultures. These results confirmed these of (Borrelli et al., 1992) who found that Cefotaxime strongly promoted plant regeneration in two durum wheat cultivars. Moreover, (Mihaljević et al., 2001) compared the effect of Carbenicillin and Cefotaxime at 500 mgL^{-1} on embryonic tissue growth and revealed that Cefotaxime was less toxic to the embryonic tissue and more suitable for *Agrobacterium* elimination.(Chakrabarty. et al., 2002.) have reported that bacterial growth around cauliflower explants could be controlled with 250 mg L^{-1} Cefotaxime.

Some antibiotics have a detrimental effect on plant tissue cultures (Pollock et al., 1983, Holford and Newbury, 1992, Lin et al., 1995). Some investigators choose to use Cefotaxime (Eimert, 1992) while others used Carbenicillin (Chakrabarty et al., 2002, Prem, 1998) to eliminate *Agrobacterium.* The differences here could be results from the interaction between the genotypes and antibiotics or the mechanisms of action of these antibiotics which is closely connected to the physiological reactions of the cultured tissues (Escandon and Hahne, 1991). In this experiment 250 mgL^{-1} Cefotaxime was added to cauliflower cultures to enhance the regeneration frequency and efficiency and at the same time suppress *Agrobacterium* growth.

3.5 Conclusion

As efficient regeneration is essential for successful transformation and because of some antibiotics have either a positive or negative effect on plant regeneration depending on concentration of antibiotics.

It can be concluded from the results of this investigation that for the elimination of *Agrobacterium tumefaciens* and efficient shoot regeneration, Cefotaxime and Carbenicillin at 250 mg L^{-1} were the best concentrations to use.

4 Chapter Four: Transformation of cauliflower explants with *Agrobacterium tumefaciens*

4.1 Aim

The aim of this study was to establish an efficient and reproducible transformation protocol for production of transgenic cauliflower plants using *A. tumefaciens*. The transformants were produced using microshoots treated with the antibiotics (Kanamycin) and then selected for resistance. Kanamycin selection was used to obtain transgenic lines as a strategy for improving abiotic stress tolerance with APX.

4.2 Objective

Development of reliable transformation protocol for cauliflower is dependent on;

- A high transformation rate.
- Effective selection of transformed plants.
- The establishment of efficient shoot regeneration procedures.
- To addition of a functional gene of interested (APX).

4.3 Introduction

Cauliflower is known as one of the most responsive species for tissue culture but one of the most recalcitrant species for genetic transformation (Passelègue and Kerlan, 1996, Puddephat et al., 1996). Therefore the target of the present investigation was to optimize an efficient protocol for tissue culture. A second goal was to integrate the APX gene into cauliflower and test the transgenic plants under abiotic stress.

Agrobacterium tumefaciens is the main tool for plant genetic engineering. Nevertheless, the low transformation efficiency of many commercial crops is the main factor limiting its use. Among several factors, ethylene produced by plants is one that inhibits *A. tumefaciens* mediated transformation efficiency. For instance, it has been reported that reducing the ethylene level increased the expression of the vir genes of *A. tumefaciens*, thereby increasing gene delivery efficiency (Nonaka et al., 2008). Moreover, application of ethylene inhibitors such as silver ions in the tissue culture medium has been reported to improve the transformation efficiency of many plant species, such as bottle gourd, cauliflower, apricot and apple trees (Chakrabarty et al., 2002, Burgos and Alburquerque, 2003, Han et al., 2005, Petri et al., 2005, Seong et al., 2005) .

The most common method used for the transformation of *B. oleracea* is the use of *Agrobacterium* species. Both *A. tumefaciens* and *A. rhizogenes* have been used to transform a number of other *B. oleracea* crops (Puddephat et al., 1996).

A. tumefaciens-mediated transformation of cauliflower has been attempted with a variety of explants such as hypocotyl, leaf, seed and seedling stem. Oncogenic *Agrobacterium* was used with leaf discs by (Srivastava et al., 1988b) and (Block,

1993) but their procedures could not be reproduced in other laboratories (Kazan et al., 1997, Metz et al., 1995). Eimert,(1992) experimented with the use of *Agrobacterium* with leaf discs and protoplasts by electroporation, and direct DNA uptake. Irrespective of the method used, stable transformation at very low frequency has always been reported. *A. tumefaciens* was used to infect seedling explants (cotyledon and hypocotyl) using a modified procedure by (Block, 1993) who made the following comments:

"The present protocol modified from (Clough and Bent, 1998) is extremely simple. We have found that the MS salts, hormones, etc. make no difference, that the OD of bacteria doesn't make much of a difference, and that the vacuum doesn't even make much of a difference as long as you have a decent amount of bacteria present. Plant health is still a major factor - healthy fecund plants make a big difference. With this method you should be able to achieve transformation rates above 1% (one transformants for every 100 seed harvested from *Agrobacterium*-treated plants".

A more efficient and simpler method for genetic transformation and regeneration of cauliflower plants involves the use of explants from 8-day old seedlings or mass propagation inoculated and co-cultivated with *Agrobacterium tumefaciens* strain EHA105 harbouring vector containing APX gene.

4.3.1 Explant source

In-vitro derived materials are generally preferred over field-grown, greenhouse, or growth room materials as explants for co-cultivation because they need no surface sterilization, are often younger and therefore have higher regeneration capacity, and are less lignified (Civínová and Sladký, 1990). However, *in vitro* grown explants usually exhibit less hardiness and resistance to desiccation during transformation.

4.3.2 Transformation protocol

The transformation protocol developed for routine use includes some of the factors (explant source, bacterial stock and explant pre-culture) and other factors known to be important in other species (Han et al., 2000). Most of the cauliflower clones shown have also been transformed to the point of rooting in Kanamycin containing media. This includes the strains EHA105 and the plasmid pCGN178. The common transformation system is based on indirect organogenesis, where shoots regeneration follows a period of callus induction and involves several weeks or months of unorganized growth prior to the appearance of shoots. It differs only modestly from the transformation protocol by (Leple et al., 1992), primarily in some of the phytohormones used and some media modifications.

4.3.3 Selectable marker genes

Reporter genes are essential to plant genetic engineering and for the development of transgenic crops. These are almost always present in engineered DNA plasmids used for genetic transformation of plant tissue (Lee and Gelvin, 2008). Untransformed cells and tissues are killed while the cells carrying the desired gene grow and regenerate into plants. A visible marker gene will show a colour characteristic when the transformed tissue is exposed in certain assays. Selectable markers and visible marker reporter genes rarely affect the studied trait of interest, but provide a powerful tool in determining the success of the transformation events or identification of transformation events before the gene of interest can be recognized in the culture (Sheen et al., 1995).Two main aspects of the marker gene have to be considered. Firstly, its structure (nucleic acid sequence) and secondly, that the gene product is responsible for the dominant expression of a suitable selective phenotype.

4.4 Materials and Methods

4.4.1 Agrobacterium tumefaciens

Three *A. tumefaciens* strains were kindly supplied by the United States Department of Agriculture. The pCGN1578 has three 35S promoter elements and the construct has a dual CaMV 35S promoter as well as a TEV leader and CMV terminator. All bacteria strains contained β-glucuronidase (GUS) operator gene under the transcriptional control of cauliflower mosaic virus 35S promoter. APX contain of the APX gene (Figure 4.1) and TA, SA the SOD stress gene, respectively. Both TA and SA contained Tetracycline resistance genes. Three *Agrobacterium* strains - APX, TA and SA - were able to grow in media including (Kanamycin + Gentamycin), (Tetracycline + Gentamycin) and (Kanamycin + Tetracycline) respectively.

The APX gene was ligated into a TEV (tobacco etch virus) portion of the pRTL2 vector, which contains a dual 35S promoter; the 35S dual promoter - APX - TEV segment of that construct was digested out with Hind III and ligated into pCGN1578. TA was SOD in place of APX, while SA had the dual 35S - SOD - TEV into pBIN+ARS rather than pCGN1578.

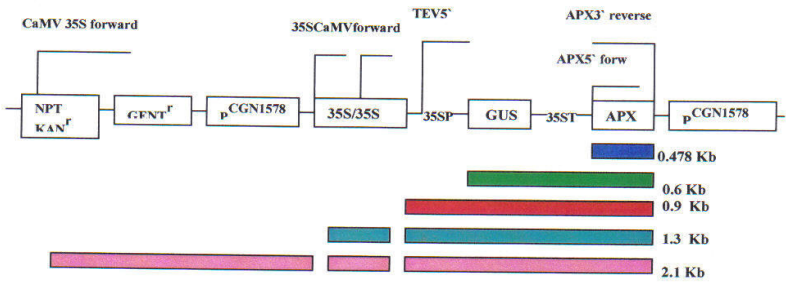


Figure 4-1. Schematic presentation of APX strain obtained from *Pisum sativum* (Pea). Bacterial strain supplied by the United States Department of Agriculture.

4.4.2 Bacterial growth curve – background information

Bacteria were cultured in sterile nutrient medium and incubated at the optimum temperature for growth. Samples were removed at intervals and the number of viable bacteria counted. A logarithmic growth curve was plotted, which shows various phases (Figure 4.10). In the lag phase there is only a small increase in numbers of bacteria. The length of this phase depends on which medium was used to culture the bacteria before the investigation and which phase the cells are already in. As the life span (generation time) of the cells decreases, they enter the *log* (or *exponential*) *phase*, in which the cells reach a maximum rate of reproduction and the number of bacteria increases directly with time, giving a straight slope on a logarithmic scale(as in exponential phase). Growth rate can be estimated in this phase. With time, as the population grows, it enters the *stationary phase*, when the nutrients are exhausted. As the cell's energy stores are exhausted the rate of cell division decreases. The death (or final) phase occurs when the rate at which the bacteria die exceeds the rate at which they are produced; the population declines as the levels of nutrients fall and toxin levels increase.

4.4.3 Safety routines

As a safety precaution, during the work in tissue culture and transformation experiments neoprene gloves, a dust mask, and a special lab coat were used routinely. All waste material was removed in hazard bags then autoclaved at 121^0C for 15 minutes. For transformation experiments, the disinfection agent Virkon (10 g L^{-1}) was used to clean the working area and to disinfect all the transformation waste material before autoclaving.

4.4.4 Sterile technique and autoclaving

Manipulations were performed in a horizontal laminar air flow cabinet. This was always swabbed with 70% ethanol prior to use. Instruments such as spatulas, forceps and scalpels were immersed in 100% ethanol and flamed prior to use. Sieves, filter papers, forceps and pipette tips were autoclaved at 121^0C for 20 minutes. Media were sterilized in an autoclave, with a standard sterilisation cycle of 20 m at 121^0C. Growth regulators, antibiotics were sterilised using a Millex-Gv 0.22 µM filter unit and added after media autoclaving.

Agrobacterium strain maintenance

Building on the results of the previous experiment, for all transformation experiments the strains were maintained by sub culturing single colonies on LB solid or liquid medium using a flow aseptic technique supplemented with different combinations of antibiotic (20 mg L^{-1} Kanamycin+ 6. 0 mg L^{-1} Gentamycin) and 80 µM acetosyringone using a flow aseptic technique. The antibiotics used selected for bacteria containing the transformed plasmid and prevented the cultures reverting to wild type non-plasmid bearing strains.

- Solid culture; A single colony was streaked onto a solid plate and incubated at 28 ºC in the dark for 24 to 48 hours (plate 4.1). After colonies had grown sufficiently to be visible in the agar medium, the plates were sealed with paraffin and stored at 4ºC to act as a working plate.

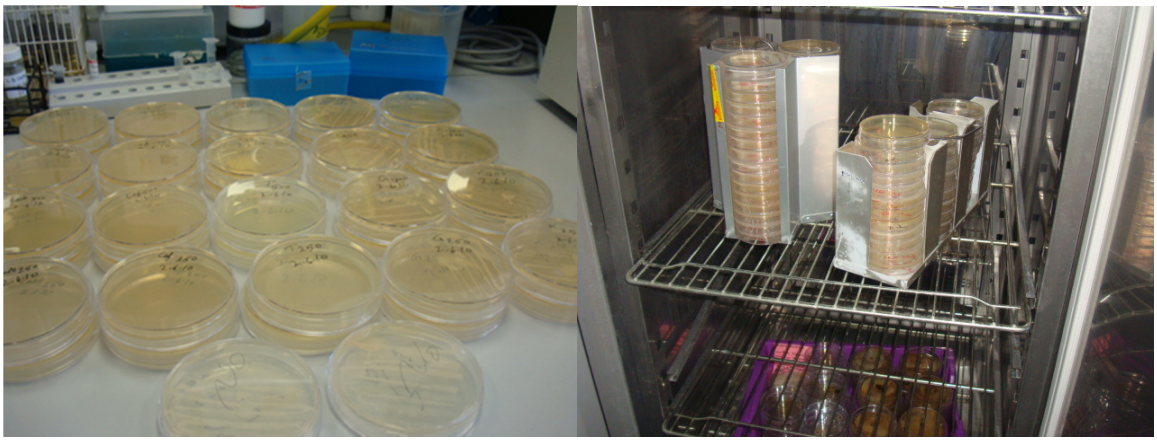


Plate 4.1. Bacteria growing in solid medium in growth incubator at 28ºC (left; analysis of the bacterial growth on the working bench, right; bacteria growing inside the incubator).

Liquid culture (Plate 4.2) Strains were grown in liquid culture using a shake bench at 100 rpm in growth room at 28ºC; suitable culture vessels included conical flasks and 10 mL sterile plastic pots. Growth of the strains was generally good after 48 hours unless they had been inoculated directly from a cold stored culture, when the lag phase was extended.

Plate 4.2. Bacterial growth in liquid media at 28ºC (left; bacteria growing in liquid medium inside the incubator and right; bacterial growth in the conical flask inside the shaker at 100 rpm).

Preservation of *Agrobacterium tumefaciens* strain

- Short term storage colonies of APX strain of *A. tumefaciens* strains were maintained for periods of a week on the surface of agar media with the plates tightly wrapped in Parafilm and stored inverted at 4 ºC.
- Long term storage. Long term storage of the *A. tumefaciens* strain was carried out by first pipetting 0.85 mL of bacteria on overnight growth culture into a sterile vial containing 0.15 mL of sterile glycerol, then the cap was placed on the vial and the contents were mixed by vortexing. The glycerinated cultures were stored at -20 or -70 ºC

Experiments;

Experiment 1.Tissue culture media and shoot regenerations

The standard operation procedure of curd micropropagation (SOP_{CM}) described in chapter 2 was used as a starting point for co-cultivation experiments.

Experiment 2. Determination of a growth curve for *A. tumefaciens* strain APX.

For transformation experiments it is generally recommended that the cultures are in the log growth phase with an optical density at 600 nm of about 0.6 (Sezonov et al., 2007). The following experiment was conducted to identify the log phase stage for APX strain.

The growth curve was obtained from cultures inoculated from actively growing liquid culture (1 mL culture in 100 mL LB media) supplemented with appropriate combination of antibiotic (e.g. Kanamycin and Gentamycin). The OD was recorded after 8, 16, 24, 32, 40 and 48 hours. The absorbance was monitored using a Helios Epsilon spectrophotometer (UNICAM) at 600 nm .

Experiment 3. Developing an expedient and reliable method to identify APX gene construct in *Agrobacterium tumefaciens* vectors using PCR

The experiment was carried out to develop a procedure for detecting the presence of an insert of DNA in individual *A. tumefaciens* colonies without the necessity of separate procedures for DNA isolation and purification.

4.4.5 Procedure for DNA extraction (bacterial plasmid)

Plasmids were isolated using a Kit from Sigma.com/oligos Company as follows;

A 3 mL pellet was prepared from an overnight (incubate bacterial culture at 28℃ for 24hr) recombinant *Agrobacterium* culture by centrifugation. The optimal volume of culture at shaking incubator to use for this depends upon the plasmid and culture density (less volume with high density and high volume with low density). The appropriate volume (3-5 mL) of the recombinant *Agrobacterium* culture was transferred to a micro centrifuge tube and pellet cells at > 12,000 rpm for 1 minute. The supernatant was discarded.

The bacterial pellet was resuspended completely with 200 µL of the suspension solution, pipetting up and down to thoroughly re-suspension the cells until homogeneous. The lysed cells were re-suspended by adding 200 µL of the lysis solution. Immediately, the contents were mixed by gentle inversion (6-8 times) until the mixture becomes clear and viscous. 350 µL of the neutralization/binder solution was added to precipitate the cell debris. The tube was gently inverted 4-6 times. The cell debris was then pelleted by centrifuging at >12,000 rpm for 10 minutes.

A Gen Elute Minipred Binding column was inserted into a micro centrifuge at 12,000 rpm for 30 seconds to 1 minute. The flow-through liquid was discarded. The cleared lysate was transferred from the precipitated cell debris from the previous step in neutralizing/binding solution to the prepared column before, and centrifuged at >12, 000 rpm for 30 second to 1 minute. The flow-through liquid was discarded. 500 µl optional wash solution was added to the column and centrifuged at 12,000 rpm for 30 second to 1 minute. The flow-through liquid was discarded. 750 µL of the dilute wash solution was added to the column, centrifuged at 12,000 rpm for 30 second to

1 minute. The column was transferred to a fresh collection tube, 100 µL of elution solution or molecular biology reagent water was added and the sample again centrifuged at >12,000 rpm for 1 minute. The DNA was now present in the elute and ready for immediate use or storage at -20ºC.

Step 1: **Bacterial colony template preparation**

Agrobacterium tumefaciens (strain APX) was grown in solid or liquid LB medium supplemented with different combinations of antibiotic selection, (20 mg L^{-1} Kanamycin +6.0 mg L^{-1} Gentamycin). The culture was incubated at 28 ºC in the dark for 24 to 48 hours. After *Agrobacterium* colonies had grown suitably to be visible in the agar medium, a sample from each colony was obtained using a sterile wooden toothpick.

A single colony was re-suspended in sterile double-deionised water (20 µL) until no clumps were visible. The *Agrobacterium* suspension was heated at 95ºC for 20 minutes then centrifuged briefly (13000 rpm for 10 seconds) to pellet debris. The majority of the sample was then directly placed into 25 µL microfuge tube.

Step 2. **PCR Reaction**

Since PCR amplification is very sensitive, it is imperative that sources of cross contaminating DNA, including extraneous microbes, be avoided. All solutions supplied for PCR were autoclave sterilized. The following three primers were used for the PCR reaction of the APX strain:

5̀-CACGTCTTCAAAGCAAGTGG-3` (35SCaMV 5 frw),

5̀-TTTCGGAACAATTAAGCACCAA-3 (APX5) frw

5`-AAGAGGGCGGAATACAGAGTCAGT-3` (APX 5 rev)

Each PCR reaction mixture was 50 µl PCR mixture, 2 µl DNA, 1 µL of each primer forward and 1 µL of reverse,1 µL DNTPS, 5 µL Taq buffer,2 µL Taq and 38 µL deionized autoclaved water. Before starting the first PCR cycle, the thermal cycle (Gene Amp® PCR system 9700) was heated to 95^0C for 30 min, and then the sample was heated to 94^0C for 2 min. This was followed by 30 cycles of 55^0C for 1 min, 75^0C for 1 min. In the final cycle, the reaction period at 75^0C was extended to 10 min.

Step 3. Preparation of agarose gel

8% agarose (0.8g) and 100 mL of 1x TAE buffer were thoroughly mixed and boiled in a microwave oven for 3-4 minutes, then 2.5 µL ethidium bromide was added and the entire mixture poured into a 50ml agarose gel tray. The gel was left for 10-15 minutes until solid, then the tray was put into position in the gel tank and enough electrophoresis buffer (1x TBE) added to cover the gel to a depth of 1-2 mm. The gel comb was carefully removed so as not to damage the sample wells.

Step 4. Preparation sample and loading

After the PCR processes finished, 5 µL samples were taken from each PCR tube and transferred to a new microfuge tube and after adding 1 µL of loading buffer the resulting 6 µL samples were loaded into the sample wells slowly.

Step 5. **Running and visualization**

After loading the lid was placed carefully on the gel tank the gel was run at 100 volts for approximately 45 minutes and then viewed under UV light using the Trans illuminator/gel documentation system (UVItec).

4.4.6 Experiment 4. Optimization of parameters enhancing transformation efficiency

The high frequency of shoot regeneration from cauliflower curd micropropagation explants appeared to be ideal for *Agrobacterium*-mediated gene transfer. The following experiment was designed to develop an efficient transformation system from these explants using *A. tumefaciens* (strain APX). Parameters were tested, one at a time, in sequential order: density bacterial culture (0.6 OD_{600} and at a range of dilutions (1:10 and 1:20); explant types; explants age (0,10 and 20 days old); the virulence inducer (0, 40, 80,100 and 120 µM acetosyringone); pre-culture (0, 2 and 4 days); and co-cultivation (0, 5,10,15 and 20 days). These parameters were evaluated on the basis of fluorometric GUS activity coupled with regeneration efficiency.

Effect of acetosyringone

Acetosyringone was supplied by Sigma-ALDRICH (UK), cat number: D134406; D13, 440-6, CAS-No. : 2478-38-8. This experiment was designed to determine whether the addition of acetosyringone (3, 5-dimethyoxy 4- hydroxide acetophenone) to the incubation medium would increase the transformation efficiency of *Agrobacterium tumefaciens* strain APX. Different concentrations of acetosyringone were applied (0, 40, 80,100 and 120 µM during the transformation steps.

Effect of pre-culture period of explants prior to transformation

In this experiment explants were first pre-cultured (0, 2 and 4) days on S23 medium prior inoculation with *Agrobacterium*. The transformation procedure was as described previously.

Effect of co-cultivation period

The importance of the duration of the co-cultivation period of plant materials with *A. tumefaciens* was recorded using five different co-cultivation period (0, 5, 10, 15 and 20 days).

Selection of plant transformation

Explants co-incubated with *A. tumefaciens* were washed for 30 second with 250 mgL^{-1} Cefotaxime to inhibit *A. tumefaciens* growth, followed by three washes with sterilized water. After 5 days of growth in S23 medium with 250 mgL^{-1} Cefotaxime, the transformation was assessed using GUS assay. Explants were then transferred to S23 medium with different concentrations of the appropriate antibiotic dependant (20 mg L^{-1} Kanamycin +6.0 mg L^{-1} Gentamycin).

RNA extraction and purification (leaf)

- **RNA extraction**

Four replicate plants of each transformed line plant and 4 control plants were transferred from *in-vitro* conditions to small plastic pots containing compost in a growth chamber (Sanyo Fitotron) at 23℃ under a long day photoperiod (16 h) with a light intensity of 150 µmol m^{-2} s^{-1} and 51 % humidity.

To investigate the presence of the inserted APX gene in cauliflower, the total RNA was isolated from transformed and control plants. A leaf was detached from each plant and frozen immediately in liquid nitrogen and then ground in liquid nitrogen in a pestle and mortar. The RNA extracted because to extract the mRNA of APX so that a cDNA clone could be produced and sequenced to confirm it was the APX gene.

RNA isolation and purification

Total RNA was isolated by following the method of (Farrell, 2006, Green and Sambrook, 2012). Samples (100 mg powder of leaves) which had been stored at -80 ^{o}C was transferred to 500 µL of lysis solution (Sigma cat # L8167), vortexed immediately and shaken vigorously for at least 30 seconds and then incubated at 56^{o}C for 5 minutes (Sigma cat # STRN50). Samples were centrifuged at 13000 xg for 5 minutes to pellet the cell debris. The supernatant was pipetted into a filtration column (Sigma cat # C6866) in a 2-mL collection tube and then centrifuged at 13000 xg for 3 minutes and the flow through lysate collected. 500 µL of binding solution (Sigma cat # L8042) was pipetted into the lysate and mixed immediately and thoroughly by pipetting at least 5 times and vortexing briefly. 700 µL of the mixture was pipetted into a binding column (Sigma cat # C6991) in a 2 ML collection tube and centrifuged at 13000 xg for 1 minute to bind the RNA. The flowthrough liquid in the collection tube was decanted. The tube was cleaned with absorbent paper and returned to the column and the process repeated for the remaining mixture.

DNase1 (Sigma cat # DNASE10) was used for elimination of trace amounts of DNA. 80 µL of the DNase1 mixture was added directly onto the centre of the filter inside the binding column, the cap closed and incubated at room temperature for 15 minutes. 500 µL of wash solution1 was pipetted into the binding column and centrifuged at 13000xg for 1 minute. The flowthrough liquid was transferred and the tube cleaned with absorbent paper followed by a second column wash with ethanol diluted wash solution 2 (Sigma cat # W3261) and centrifuged at 13000 xg for 30 seconds and repeated the third column wash with wash solution 2 and centrifuged similarly. The tube was cleaned by absorbent paper and returned to the column into the clean tube, centrifuged at 13000 xg for 1 minute to dry the column. The column

was carefully removed and transferred into a new 2 ml collection tube and 50 µL of elution solution (Sigma cat # E8024) pipetted directly onto the centre of binding matrix inside the column and the cap closed and stood undisturbed for 1 minute before centrifuging at 13000 xg for 1 minute. The purified RNA in the flow-through elute was distributed in small aliquots and some were stored at -20^{o}C for short time and at -80^{o}C for long term storage.

APX gene identification PCR

Two step PCR was used for cDNA synthesis and amplification. Total RNA was used as the template for the synthesis of the first strand cDNA using ImProm-II™ Reverse Transcription System (Promega cat # A3800). The reverse transcription reaction mixture was prepared in sterile and nuclease free 1.5 mL microcentrifuge tubes (Ambion) on ice. The total mixture was distributed in aliquots of 15 µL for each cDNA synthesis reaction following the instructions of kit manufacturer. RNA was diluted to equilibrate all the samples as 0.8 µg in nuclease free water (Sigma cat # w1754) and each sample was combined with primer oligo (dT)15 (0.5 µg/reaction), giving a final volume of 5 µL reaction in 0.2 ml nuclease free PCR tubes (Ambion). Tubes were treated at 70^{o}C for 5 minutes and immediately chilled in ice for 5 minutes and centrifuged for 10 seconds to separate condensate and maintain the original volume. Tubes were retained on ice until subsequent use.

First strand cDNA synthesis was carried out by placing the tubes in a thermal cycler (Perkin Elmer 9700) under the following thermal cycle; Annealing: 25^{o}C for 5 minutes, Extension: 42^{o}C for 60 minutes, Inactivation of Reverse Transcriptase: 70^{o}C for 15 minutes. The first strand cDNA was directly amplified in 50 µL of reaction mixture. The mixture was prepared by adding components in the following ratio following the instructions of PCR master mix (Promega cat # M7502). PCR master mix, 2X (25 µL),

forward primer, 10 µM (5.0 µL), reverse primer, 10 µM (5.0 µL), cDNA (5 µL), nuclease-free water (Sigma cat # w1754) were added to make a 50 µL final reaction mixture for each sample. The following primers were used for amplification of cDNA strands:

Forward 5`-CACGTCTTCAAAGCAAGTGG-3` (35SCaMV 5 frw),

Forward 5`-TTTCGGAACAATTAAGCACCAA-3` (APX 5) frw

Reverse 5`-AAGAGGGCGGAATACAGAGTCAGT-3` (APX 5 rev).

The 50 µL reaction mixture for each sample was run under the following thermal cycle. For the first PCR cycle, the thermal cycler (Gene Amp® PCR system 9700) was heated to 95^0C for 30 min, and then the sample was heated to 94^0C for 2 min. This was followed by 30 cycles of 55^0C for 1 min, 75^0C for 1 min. In the final cycle, the reaction period at 75^0C was extended to 10 min., and then 4^oC ∞. The PCR products were analysed using 0.8 % high melting agarose (Sigma) gel with added Ethidium Bromide(EtBr) to a final concentration of 0.5 µg mL^{-1}, and compared with a PCR marker catalogue number (Bp2581-200 Fisher bio reagent) consisting of eleven DNA fragments with sizes of 50, 100, 200, 300, 400, 500, 750,1000,1400,1550 and 2,000 bp. Then band intensity was measured using Quantity One 4.6.3 Bio-Rad software.

Sequence analysis of PCR product (cDNA)

The sequencing reaction added to each sample was as follows: 4µl Big Dye Reaction Mix, 3.5 µL of 5x Sequencing Buffer, 1µL (5pmol) of 5 µM Primer, 10 µl H_2O, 1.5 µL (5-20 ng) of PCR product (DNA template) and the total reaction volume was 20 µL.

Run reactions on the thermal cycler program were as follows; 1 cycle at 96^{o}C for 1min, 35 cycles at 96^{o}C for 10 sec, 50^{o}C for 5 sec, 60^{o}C for 4 min, Final step 60^{o}C for 5min and then held at 10^{o}C.

The reaction products were precipitated with 5 µL 125mM EDTA and 60 µL Molecular Ethanol on ice for 15min then centrifuged at 4^{o}C at top speed in microcentrifuge for 20min. The supernatant was removed and the pellet rinsed with 100 µl 70% ethanol. After air drying the pellet was resuspended in 15 µL HiDi Formamide and loaded onto a sequencing plate.

The specific PCR products were isolated and purified from the gel slice using the Wizard® SV Gel and PCR Clean-Up System (Promega A9281) and the purified products were stored at 4^{o}C for a short time before being subjected to sequence analyses (Eurofins MWG Operon, Germany). The sequences were analysed using Clustal W and Basic Local Alignment Search Tool (BLAST) and then compared with nucleotide and deduced amino acids sequences of APX gene from other *Brassica* species by iterative multiple alignments.

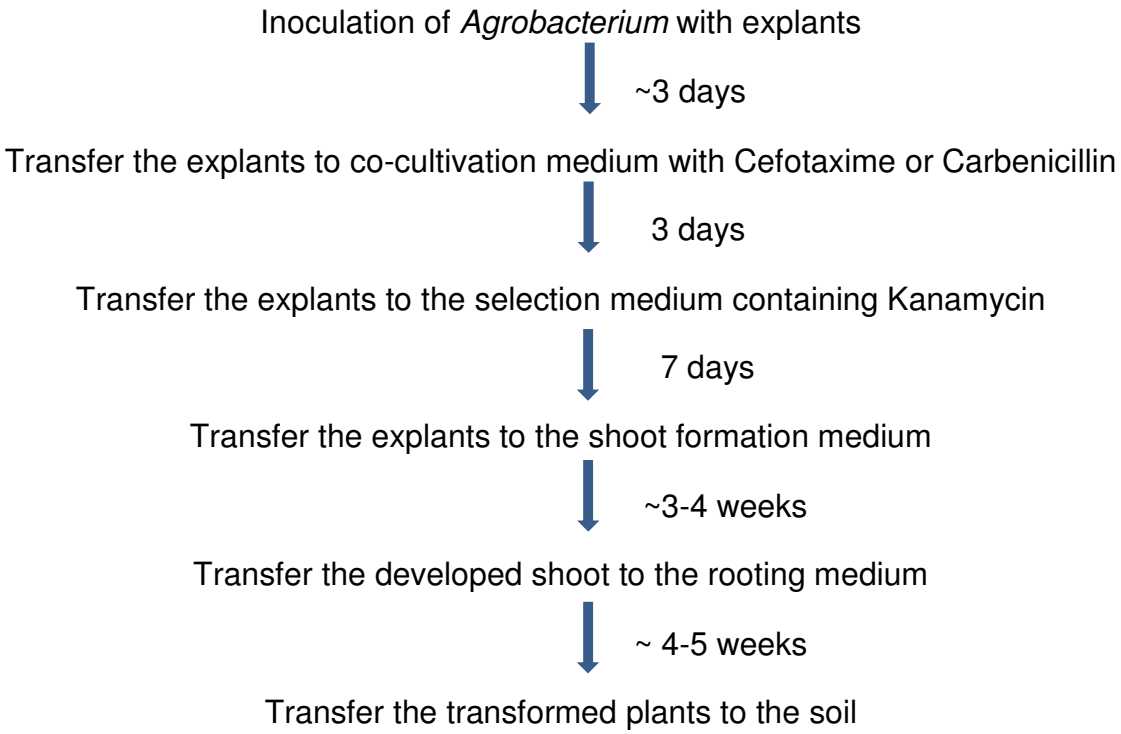
- **Purity and analysis of RNA and DNA**

The purity and concentration of DNA and RNA was determined by spectrophotometric analysis. The RNA was diluted 10-50 fold in TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 8) and absorbance measured using the A260/A280 ratio procedure (Holt and Wold, 1961). Nucleic acids have a higher absorbance at 260 nm than at 280 nm and pure DNA and RNA samples have expected A260/A280 ratios of >1.8 and >2.0 respectively (Maniatis et al., 1982).

- **Confirmation of quality of DNA**

A gel of 0.8% agarose was prepared in a 250 mL flask by melting 0.4 g of agarose in 50 mL of 1xTBE buffer using a microwave. The solution was allowed to cool for a couple of minutes to approximately 50 ºC. Ethidium Bromide (EtBr) was added to the agarose solution to a final concentration of 0.5 µg mL^{-1} and mixed before pouring the gel. Using a supplied gel tray and comb, the gel was cast and allowed to solidify for a minimum of 20 min at room temperature. 1xTBE buffer was added to submerge the gel. Samples were prepared in PCR tubes (0.2 mL) by adding 4 µL loading dye to 10 µL of sample, mixed and then 10 µL total volume of each sample was loaded into each well. 5 µL of the molecular weight markers was loaded in a well as a reference ladder. The gel was run at 100 V until the bromophenol blue dye front was about 3/4 through the gel (approx. 1 h). The tray with the agarose gel was carefully removed and taken to the UV trans-illuminator and the gel examined under UV light to confirm the DNA quality. Presence of a highly resolved high molecular weight band indicated good quality DNA. A photograph was made using a gel documentation system. 1 litre of 1xTBE buffer was prepared by diluting 10xTBE (Tris base 10.8 g, Boric acid 5.5 g, EDTA 4 ml from 0.5 M stock solution, and volume raised to 100 ml with dH2O).

4.5 Outline of the transformation-selection procedure using Micropropagation of cauliflower;



4.6 Lethal dose selection

Kanamycin was used as a selectable marker to differentiate between transformed and non-transformed explants. Different concentrations of kanamycin (20, 25, 50, and 100 mgL^{-1}) were tested to determine the lethal dose. For this purpose, the explants were transferred to selection medium (Plate 4.4).

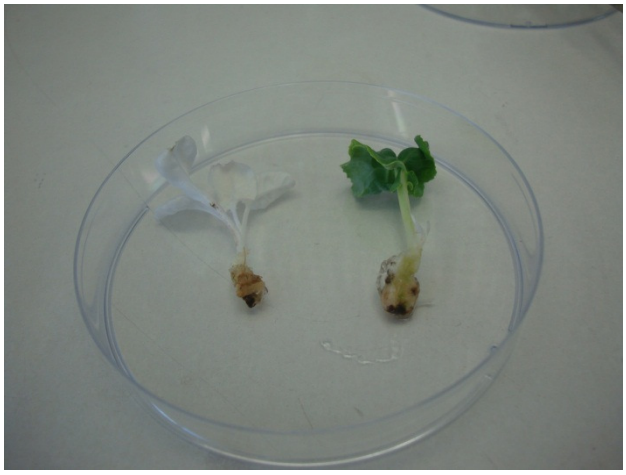


Plate 4.3. Effect of Kanamycin on shoot regenerations (left explant treated with kanamycin, right explant without Kanamycin).

4.7 Plant regeneration and weaning

After a 2 week selection period transformed explants were transferred to regeneration medium for shoot regeneration and root development. Transformation efficiencies (TEP) were calculated based on plant number.

TEP (%) = No. of Plants obtained/ No. of explants inoculated x 100.

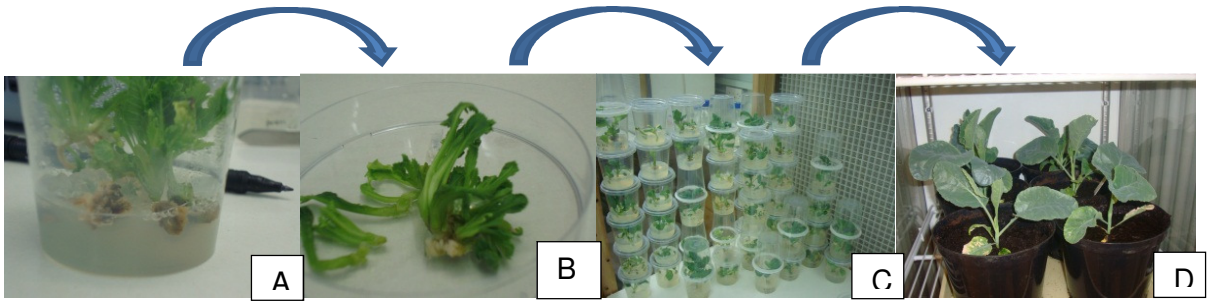


Plate 4.4. The developmental pathway of cauliflower microshoots *in vivo* and *in vitro*. Plant physical appearances maintained in standard growth conditions. (A) Microshoots in semisolid media after shoot regenerations; (B) Under very high aseptic technique microshoots were separated in small partition. (C) *in vitro* culture explants in semisolid media supplemented with growth regulator hormone(GRH) for root developments (D) After root development plants were transferred to pots containing fertile soil.

4.8 GUS assay

GUS assay was carried out as described by (Jefferson, 1987). Plant leaves and explants were placed in X - Gluc solution (1 mg L^{-1} 5-Bromo 4-Chloro 3-indoyl-B-D-glucuronide, 0.5% triton X - 100, 20% methanol, 50 mM phosphate buffer) overnight at 37℃ and then examined under a microscope for blue staining of tissues (Plate 4.7).

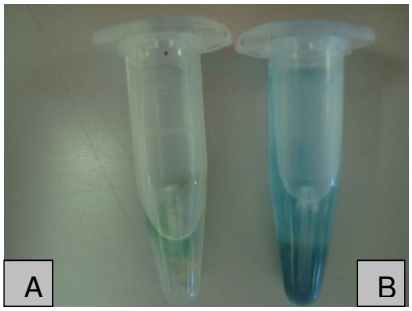


Plate 4.5. Illustration the blue colour of Gus assay examination A; non-transformed and B; explant transformed.

4.9 Confirmation of transgenic plants by PCR analysis

Genomic DNA was extracted from leaf tissues of transgenic and control plants according to CTAB method (Chen and Ronald, 1999); Plasmid (pRLT2) was isolated from *A. tumefaciens* strain EHA105 by the Miniprep method (Chowdhury, 1991).

4.10 Plasmid and bacterial strains used for transformation

Bacterial cells were harvested by centrifugation at 3000 rpm for 10 min in a 50 mL sterile centrifuge tube (Corning, USA) and then resuspended in 30 mL of liquid inoculation medium [Half-MS medium amplified with 1.5% sucrose and 80 µM of acetosyringone (3', 5'-dimethoxy-4'-hydroxy-acetophenone; Sigma-Aldrich, St Louis, MO, USA)] in 50 mL tube.

4.11 Sample preparation in Scanning Electron Microscopy (SEM)

There are many methods to prepare the sample examination depend on the type of Electron Microscopy which is used and the type of the sample which is prepared. The common method was illustrated from Bozzoela and Russell, (1999). Because SEM utilizes vacuum conditions and uses electrons to form an image, special preparations must be carried out to the sample to prevent destruction from the electron beam. All water must be removed from the samples because water would vaporize in the vacuum. All metals are conductive and require no preparation before being used. All non-metals need to be made conductive by covering the sample with a thin layer of conductive material. This is done by using a device called a "sputter coater."

The sputter coater uses an electric field and argon gas. The sample is placed in a small chamber that is at a vacuum. Argon gas and an electric field cause an electron to be removed from the argon, making the atoms positively charged. The argon ions then become attracted to a negatively charged gold foil. The argon ions knock gold atoms from the surface of the gold foil. These gold atoms fall and settle onto the surface of the sample producing a thin gold coating.

4.11.1 Surface cleaning or Rinsing

In SEM it is the outer surface of the sample that is of interest area for examination. Removing any matter from the surface area of the sample that may affect the shape and characteristic of the sample is necessary. Biological samples particularly must be cleaned before the chemical fixation stage, if not it will be fixed with the sample. There are many methods to clean the sample surface depending on the sample

type, mild detergent, surfactant or enzyme solution are suitable for any persistent surface matter, also a soft brush is suitable for hard sample like seeds.

4.11.2 Fixation

Glutaraldehyde then osmium tetroxide washes are commonly used for SEM sample Preparation(Table 4.1)

Table 4-1. General Tissue preparation Scheme for Electron Microscopy

Activity	Chemical	Time Involved
Primary fixation	Tissue is fixed with 2-4% glutaaldehyde in buffer	1-2 h
Washing	Buffer(three changes at 4ºC, one of which may be overnight)	1-12 h
Secondary Fixation	Osmosium tetroxide (1-2% ; usually buffered)	1-2 h
Dehydration	30 % ethanol	5 min
	50% ethanol	5-15 min
	70% ethanol	5-15 min
	95% ethanol(2 changes)	5-15 min
	Absolute ethanol (2 changes)	20 min
Transitional solvent	Propylene oxide (3 changes)	10 min
Infiltration of Resin	Propylene oxide; resin mixtures; gradually increasing concentration of resin	Overnight -3 d
Embedding	Pure resin mixture	2-4 h
Curing(at 60ºC)		1-3 d

4.11.3 Drying

SEM works under the high vacuum so water cannot be placed inside the sample chamber the sample has to be dried.

The largest problem for biological sample preparation is high amount of water and this should remove with least damage to the sample. Hard samples are easily dried by air (Air Drying). Other types of drying are freeze dried or critical point drying method, which prevent the passage of receding air or water interface through the sample that would cause surface tension forces to distort or flatten most samples.

4.11.4 Critical point drying

Most biological samples use this method for drying. After steps of dehydration using ethanol, the sample transferred to gas container which completely removes the remaining ethanol from the surface via pressurised fluid and usually Carbone dioxide (CO_2) or Freon. Heat is used to slowly raise the temperature of the fluid, increase the pressure until the fluid reaches the critical point. At this point the transition occurs which is the liquid and vapour density (concentration) are equal. This is a quick reliable method with minimal effect of damaging a liquid/air interface. The vapour is released from the chamber reducing it back to atmospheric pressure allowing the dried sample to be mounted on a sample stub.

4.11.5 Coating Sample

When the sample has been fixed on the stub, they are coated with a thin layer of about 10 nm of conductive material such as gold, platinum or gold-palladium alloy. The coating is applied by sputter coating or by thermal evaporation. Their purpose is to inhibit a build-up of high voltage charge on the sample, by conducting the charge to the grounded sample stub; this has the dual purpose of acting as an excellent

source of secondary electrons and prevents potentially damaging heat from the sample. Samples of *Agrobacterium* co-cultivated explants were examined by SEM.

4.12 Statistical analysis

Total shoot differentiation, production of green tissue and explant formation among the all cauliflower cultivars with different treatments were statistically analysed using the software package Minitab 16. Treatment means were compared using factor CRD (Completely Randomized Design). A standard analysis of variance (ANOVA) was used and Sigma plot 12 used to identify any significant differences between the treatments. Correlation among the different parameters was also investigated using Excel curve fitting and values of the correlation coefficient for different levels of significance investigated according to (Fisher and Frank, 1948).

4.13 Results

4.13.1 Determination of optimum culture conditions for *Agrobacterium tumefaciens* strain APX

The total means CFU (colony forming unit mL^{-1}) was recorded for *A. tumefaciens* average of high concentration suspension *Agrobacterium tumefaciens* strain (APX) growing on LB&YE medium. Significant differences were recorded between the media at ($P<0.001$) (Appendix 2.1).

Agrobacterium tumefaciens strain APX carrying the plasmid pRTL2 and pBIN+ARS as a vector grew faster and was more homogeneous in LB medium. Cultures grown in LB medium had higher CFU/ml) while YE culture resulted in lower concentrations of cell suspension, which was mainly grouped in clumps (Figure 4.2). The results showed that the LB medium was more suitable for the growth of APX strain. The LB medium was used thereafter in all transformation experiments.

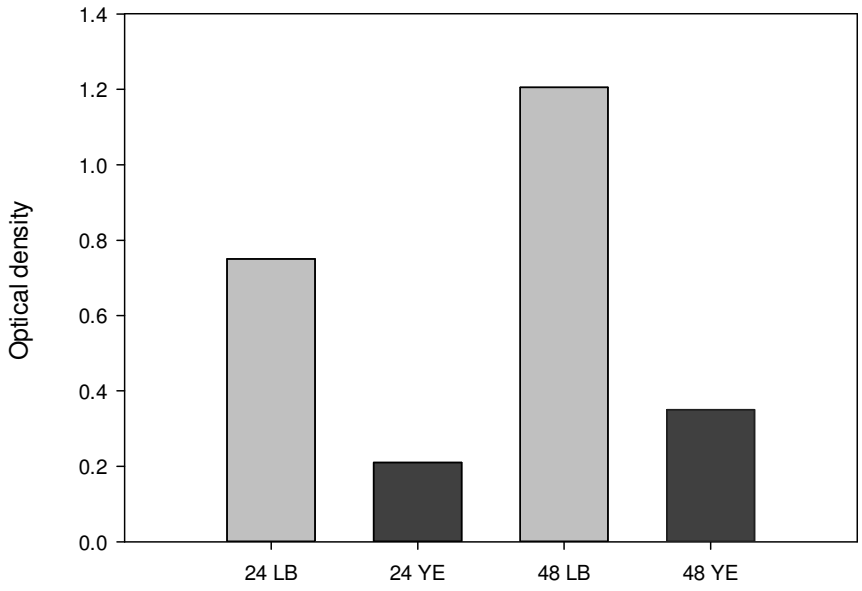


Figure 4-2. The bacterial growth (optical density of culture) in LB and YE medium over time. The reading after 24 and 48 h for Luria Broth and Yeast Extract.

There were highly significant differences between hours and media and the interaction between them (Appendix 2.1). The best bacterial growth was found after 11 h. Therefore this time of bacterial growth was subsequently used for all co-cultivation of explants.

There is highly significant difference between LB and YE media and the interaction between them. The best medium for bacterial growth was Luria Broth (LB). Therefore it was used for bacterial growth in the subsequent transformation experiment.

On the basis of CFU (colony forming units), *Agrobacterium tumefaciens* strain (APX) grew slightly faster, but not significantly, in the LB growth medium than YEB medium but there was no significant differences in the time period but there was a significant difference in the media type.

4.13.2 Determination of *A. tumefaciens* growth of LB medium.

Maximum optical density, and hence the maximum growth of APX strain (1.12) was obtained after 70 hours. A classic sigmoid growth curve was not obtained for APX strain but the log phase of growth (OD_{600} of about 0.6) was detected after 30 hours (Figure 4.3). For transformation experiments it is preferable that the culture are still in log phase and therefore a 30 hours grown culture would appear to be optimal for the *A. tumefaciens* APX strain in this exercise.

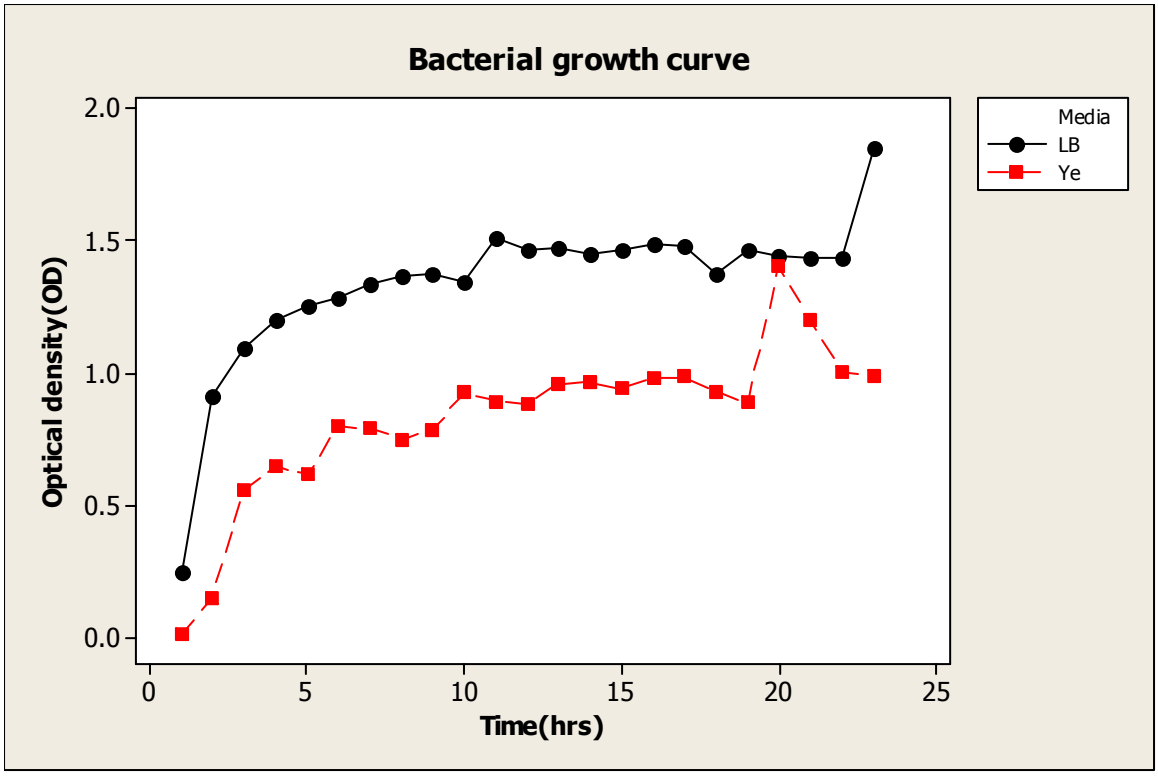


Figure 4-3. Growth curve of bacterial strain (APX) in both media (Lauria Broth& Yeast Extract).P value (<0.005). (reading each 4hrs/unit time)

4.13.3 Selection of transgenic plants

After co-cultivation the infected explants were washed for 30 seconds with 250 mgL^{-1} Cefotaxime to inhibit *Agrobacterium* growth, followed by three washes with sterilized distilled water and transferred to S23 (with 250 mgL^{-1} Cefotaxime in the medium) plus (20 mgL^{-1} Kanamycin + 6.0 mgL^{-1} Gentamycin) or (20 mgL^{-1} Kanamycin + 8.0 mgL^{-1} Tetracycline) or (8.0 mgL^{-1} Gentamycin + 6.0 mgL^{-1} Tetracycline). Within 2 - 4 weeks most untransformed shoots turned either pink or white and no further growth of these shoots was observed, while transformed shoots remained green in this medium.

4.13.4 Optimization of selection condition

For efficient and reliable production of transgenic cauliflower plants, optimization of the most suitable selection conditions is essential. The addition of a selective agent

like Kanamycin in the cultured medium is beneficial for competition of transformed cells with non-transformed ones and to decrease the number of escapes (Plate 4.8). The explants (stem and leaf) of untransformed plants were cultured on MS medium supplemented with 1.0 mg L^{-1} of IBA containing various concentrations of kanamycin (0, 25, 50, 75, 100, mg L^{-1}). Shoot regeneration was inhibited at the concentration of 50 mg L^{-1} kanamycin in stem as well as in leaf segments as shown in chapter four. Concentrations of 100 mg L^{-1} and above resulted in complete bleaching and death of explants. Consequently, concentration of 100 mg L^{-1} of kanamycin was used for selection of the explants. It has been reported that concentration of 50 mg L^{-1} kanamycin completely inhibited shoot formation in non-transformed explants through this work and hence 100 mg L^{-1} kanamycin was used for effective selection of transformed explants (Kaneyoshi et al., 1994).

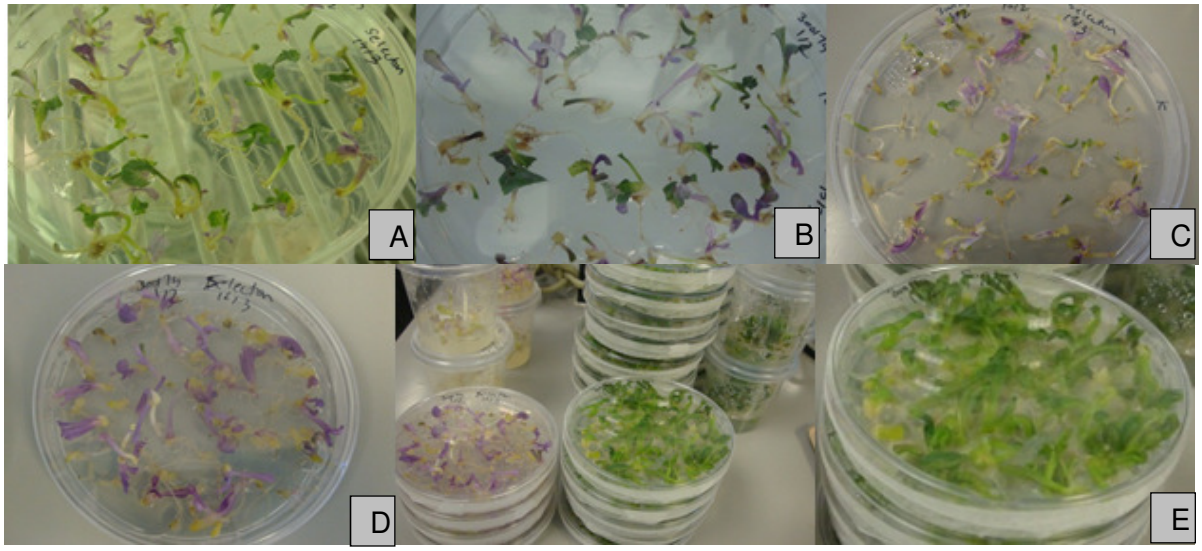


Plate 4.6. Illustration of the regeneration of cauliflower explant in media containing different concentrations of Kan after the plants infected with the APX gene. A) Non-Transgenic plant in media containing Kan (control). B) Non transgenic plants in normal media 0.0 Kan (control). C) Transgenic plants in media containing 25 mgL^{-1} Kan. D) Transgenic plants in media containing 50mgL^{-1} Kan. E) Transgenic plants containing 75 mgL^{-1} Kan.

4.13.5 Histochemical assays for GUS expression analysis

Cauliflower plants transformed with pPRLT2 showed induction of reporter gene expression in roots. In plants transformed with pRLT2, inducible GUS activity, as demonstrated by a blue stain, was evident in young leaves. The results confirm the expression of the target gene.

Histochemical assays for GUS expression analysis explants showed GUS expression after co-cultivation with *Agrobacterium* (Plate 4.9). GUS expression was observed in stably transformed explants (early stages) (Plate 4.9-B), GUS expression was observed in young leaves (Plate 4.9-C) and transformed roots (Plate 4.9-D) of the transgenic plants. The blue staining originating from the GUS was not detected in non-transformed control plant tissues (Plate 4.9-A). Several organs of the transformed plants were stained with X-Gluc. GUS expression was found in all tissues of leaf, root and callus as in Plate 4.10.

Transformation efficiency=Transformed plant/ Total infected plant x 100

TE% = 150/3000 * 100 = 5%

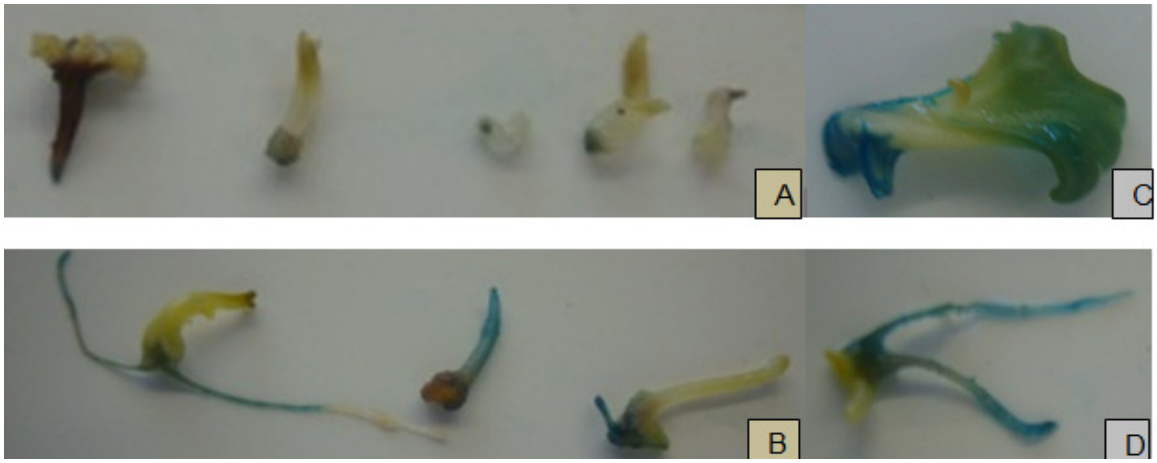


Plate 4.7. Illustration of GUS expression in various vegetative tissues of GUS transgenic and control plants. (A) Non-transformed explant (early stage) (B) Transformed explant, showing GUS expression, (early stage), (C) Transformed young leaf and (D) Transformed root.

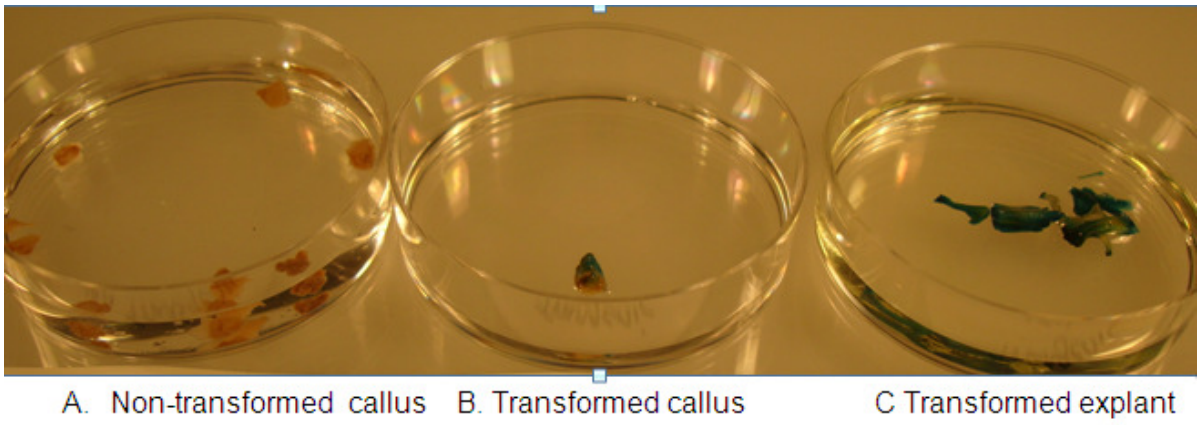


Plate 4.1. Gus at different stages. GUS expression in cauliflower calli (b) in comparison with non-transformed (a). Stable GUS expression obtained from transgenic leaf (c)in comparison with non-transgenic leaf /control.

4.13.6 Molecular analysis of *Agrobacterium* plasmid using PCR

- **Molecular analysis of bacterial strain**

Growth of the bacterial strain with the antibiotics showed a clear band in PCR and confirmed that the use of antibiotics with the bacterial strain was very important since without antibiotics there were no band present (Plate 4.12)

Three constructs were tested in this study. One contained APX inserted into $P^{CGN1578}$ as a vector. Therefore, a PCR using the 35S5` Foreword primer (as described in 4.4.6 PCR reaction) and APX3` Reverse Primer occasionally yields 3 bands. The APX encoding sequences were detected at 478 bp after PCR amplification from the appropriate colonies (Figure 4.13)

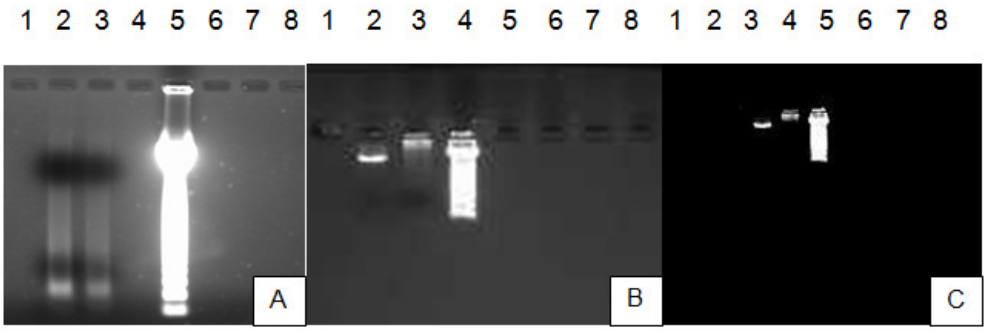


Plate 4.9. PCR detection of insert DNA in recombinant plasmids harboured in *Agrobacterium* but the treatments of bacterial strain without antibiotics where there is no any band(A).while (B&C)With antibiotics where the bands is clear.

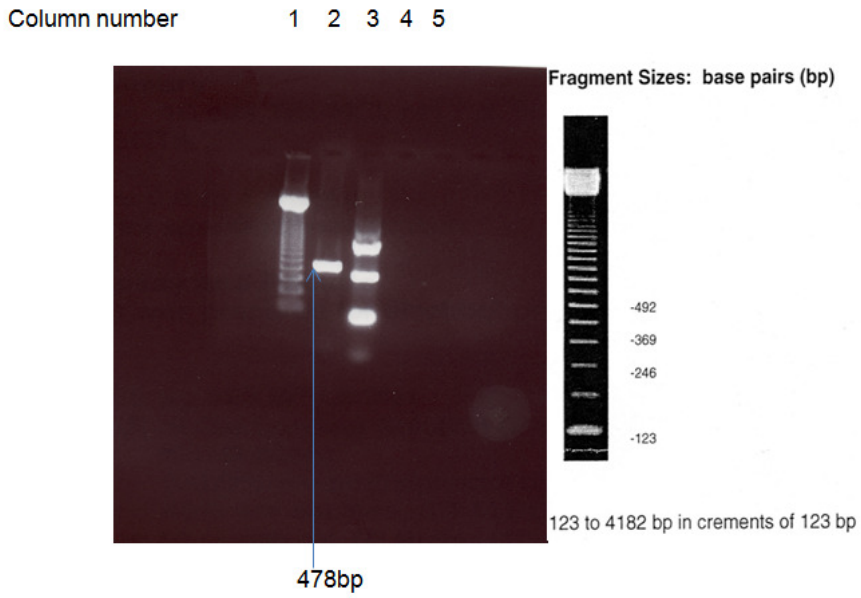


Plate 4.10. PCR detection of insert DNA in recombinant plasmids harboured in *Agrobacterium*. A. PCR product from *Agrobacterium* colonies contained a recombinant plasmid with APX insert. Column 1=Marker, Column 2=APX gene and Column 3=different primer.

- ***Molecular analysis of transgenic cauliflower plants***

The confirmation of genetic transformation was based on the presence of the reporter gene GUS and selectable marker gene nptII by PCR amplification of expected bands of sizes 478bp, (Plate. 4.14). When the sample of DNA tested was reduced in PCR the result of bands were not clear (Plate 4.15). Only transgenic plants transformed with optimized conditions which survived after selection were selected for the further molecular analysis.

Genomic DNA from independently obtained transgenic plants and a control (non-transgenic) cauliflower plant was subjected to PCR analysis to check the presence of APX gene in the transgenic plants. PCR amplification of the APX gene was carried out on the transgenic lines using primers as described in 4.4.7 APX gene identification PCR to amplify the fragment specific to the APX gene. The PCR result (Plate 4.14) indicated that T-DNA was shown to be stably maintained in transformed cauliflower explants and all of the samples from transgenic plants gave the predicted DNA fragment band of 478 bp for APX gene. No DNA amplification was detected in the sample from the control plant.

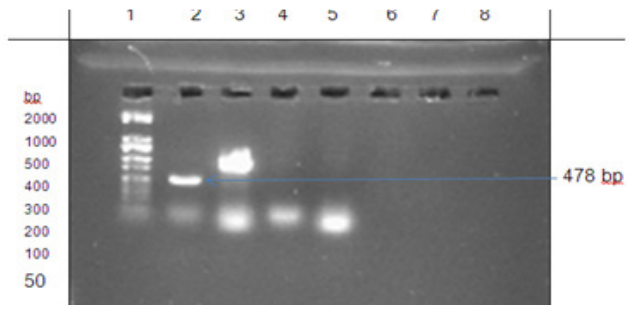


Plate 4.11. PCR analysis to detect the presence of APX gene in transgenic plants. (T2 – T5) developed from independent cauliflower explants. Column1: 1 kb molecular weight ladder (Marker, M), Column 2 & 3 transgenic DNA sample(column 3 uses a different primer), Column 4 & 5: non-Transgenic DNA (Control). 6 & 7 (water sample).

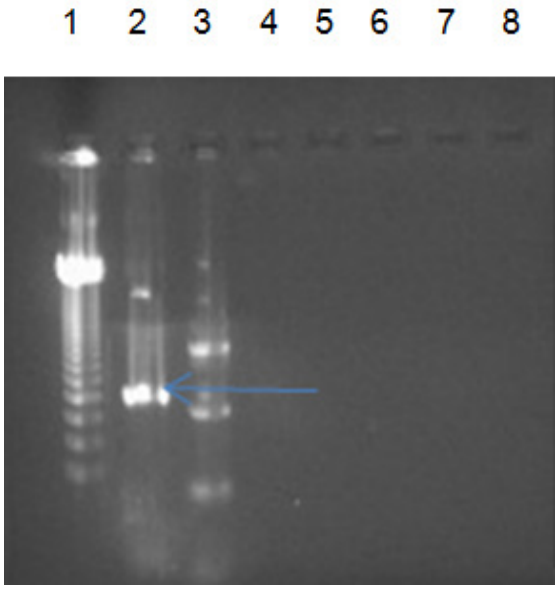


Plate 4.12. PCR analysis to detect the presence of APX gene in transgenic plants. (T1 - T4) developed from independent cauliflower explants. Column 1: 2 kb molecular weight ladder (Marker, M), Column 2 & 3 transgenic DNA sample(column 3 uses a different primer), Column 4 & 5: (Control) non-Transgenic DNA samples.

4.13.7 APX expression - Isolation and cDNA sequence alignment of APX mRNA

The nucleotide sequence of cDNA of the putative APX insert was compared with APX gene sequences reported for other species of *Brassica*. The results (Figure 4.14) showed significant similarities with up to 91% sequence agreement found with sequences from other species (Larkin et al., 2007). Figure 4.3 shows phylogenic relationships of the APX gene with the neighbour-joining phylogenetic tree for Ascorbic peroxidase.

```
2 GTGGTCCTTTCGGAACAATTAAGCACCAAGCTGAGCTTGCTCATGGTGCTAACAACGGTC 300

3 GTGGTCCTTTCGGAACCATCAAGCACCAAGCTGAGCTTGCTCATGGTGCTAACAACGGTC 103
1 ------------------------------------NNNNNNNNNNNNNNNNNNNNNNN
2 ... .  ..... .      ...
2 TTGATATCGCGGTTAGGCTGTTGGAGCCTATTAAGGAGCAATTCCCTATTGTGAGCTATG 360
3 TTGATATCGCTGTTAGGCTTTTGGAGCCTCTTAAGGAGCAATTCCCAATCATTAGCTATG 163
1 NNNNNNTCGCGGTTAGGCTGTTGGAGCCTATTAAGGAGCAATTCCCTATTGTGAGCTATG 84
  ... . **** ******** *********.*****************:** .* *******
2 CTGATTTCTACCAGTTGGCTGGTGTTGTTGCTGTTGAGATTACCGGTGGACCTGAAGTTC 420
3 CTGATTTCTACCAGTTGGCTGGTGTTGTTGCTGTTGAGATTACTGGTGGACCTGAAGTTC 223
1 CTGATTTCTACCAGTTGGCTGGTGTTGTTGCTGTTGAGATTACCGGTGGACCTGAAGTTC 144
  **************************************** ****************
2 CTTTCCACCCTGGTAGGGAGGACAAGCCTGAGCCACCACCTGAGGGTCGCTTGCCTGATG 480
3 CTTTCCACCCCGGTAGGGAGGACAAGCCTGAGCCACCACCAGAGGGTCGCTTGCCTGATG 283
1 CTTTCCACCCTGGTAGGGAGGACAAGCCTGAGCCACCACCTGAGGGTCGCTTGCCTGATG 204
  ********** ****************************:******************

2 CCACTAAGGGTTCTGACCATTTGAGGGATGTGTTTGGAAAGGCTATGGGGCTTAGTGATC 540
3 CCACCAAGGGTTCTGACCATTTGAGGGATGTGTTTGGCAAAGCTATGGGGCTTAGTGACC 343
1 CCACTAAGGGTTCTGACCATTTGAGGGATGTGTTTGGAAAGGCTATGGGGCTTAGTGATC 264
  **** ********************************.**.***************** *

2 AGGACATTGTTGCTCTATCTGGTGGTCACACCATTGGAGCTGCACACAAGGAGCGTTCTG 600
3 AGGACATCGTTGCTCTGTCTGGTGGCCACACCATTGGAGCTGCACACAAGGAGCGTTCTG 403
1 AGGACATTGTTGCTCTATCTGGTGGTCACACCATTGGAGCTGCACACAAGGAGCGTTCTG 324
  ******* ********.******** **********************************

2 GATTTGAGGGACCATGGACTTCTAATCCTCTCATTTTTGACAACTCATATTTCACTGAGT 660
3 GATTTGAGGGCCCATGGACTTCTAATCCTCTCATTTTTGACAACTCATACTTCAAGGAGT 463
1 GATTTGAGGGACCTTGGNCTTCTAATCCTCTCATTTTTGACAACTCATATTTCACTGAGT 384
  **********.**:*** ****************************** ****. ****

2 TGTTGACTGGTGAGAAGGATGGCCTTCTTCAGTTGCCAAGTGATAAGGCACTTTTGACTG 720
3 TGTTGGGTGGTGAGAAGGAAGGCCTTCTTCAGTTGCCAAGTGATAAGGCACTTTTGAGCG 523
1 TGTTGACTGGTGAGAAGGANGGCCTTCTTCAGTTGCCAAGTGATAAGGCACTTTTGACTG 444
  *****. ************ ************************************  *

2 ACTCTGTATTCCGCCCTCTTGTTGAGAAATATGCTGCGGATGAAGATGTTTTCTTTGCTG 780
3 ACCCTGTATTCCGCCCACTTGTTGAGAAATATGCCGCGGGG---------------------- 564
1 ACNNN-NNNCCCCCCCCCNNNNN---------------------------------------- 466
  **  . . . ** *** *.....
```

Figure 4-4. Nucleotide sequences (cDNA) alignment. Alignments were made using ClustalW2 EMBL-EBI(Larkin et al., 2007) Consensus symbols denoted as: "*" means that the nucleotides in that column are identical in all sequences in the alignment. ":" means that conserved substitutions have been observed, "." means that semi-conserved substitutions are observed. The 1APX region is indicated the sequence isolated from *Pisum sativum* (pea) cytosolic ascorbate peroxidase.

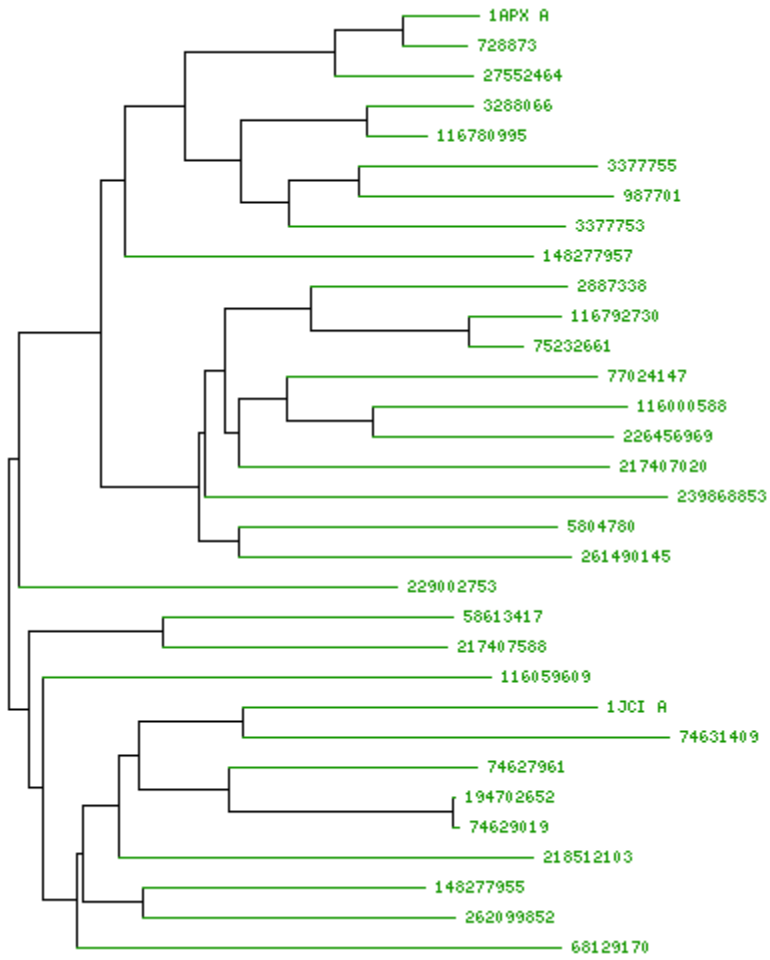
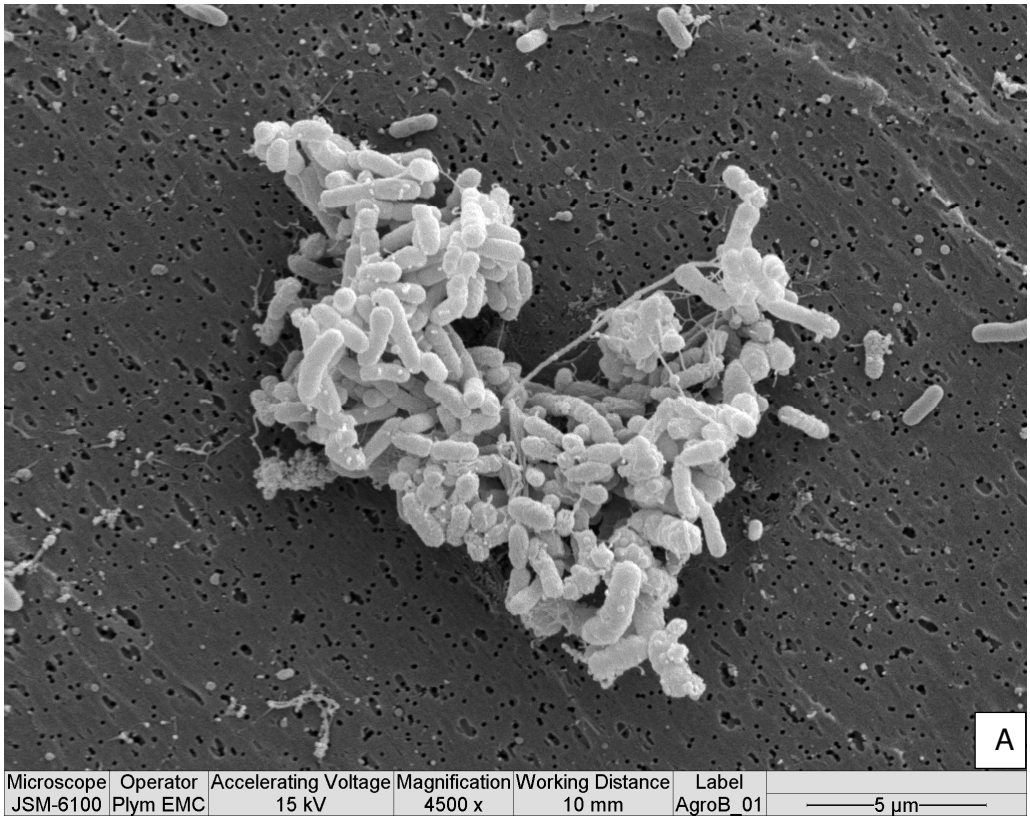


Figure 4-5. Phylogenic relation of the APX gene. The phylogram is based on the alignment of amino acids sequence of *Brassica oleracea v. botrytis* and the following gene from *Pisum sativum* (pea) cytosolic ascorbate peroxidase. The values show tree graph distances.

4.13.8 Attachment of Agrobacterium to leaf explants

SEM observations of explants co-cultivated with *Agrobacterium* for 5 days showed that only a few bacteria clustered on explants co-cultivated on MS medium containing phytohormones with sugar, whereas many clusters of bacteria were present on explants co-cultivated on water and acetosyringone (AS). *Agrobacterium* spp. possess a highly sensitive chemotaxis system that responds to a wide range of amino acids and sugars. From this study, it would appear that bacteria might move to a co-cultivation medium that has a rich food supply, resulting in a rare clustering of bacteria on explants co-cultivated on MS medium containing phytohormone with and without sugar(Broek and Vanderleyden, 1995). *Agrobacterium* motility is induced not only by phenolic compounds (such as acetosyringone) but also by food source in the medium. This suggests that bacterium migration on explants is influenced by the medium composition. The polar attachment of *Agrobacterium* clusters on explants co-cultivated with water or water and AS was thought to be related to their genetic transformation ability. (Kumar et al., 2000) reported that the transport apparatus (Vir), which is important for T-DNA transfer, was gathered at a cell pore. The polar location of the transport complex, therefore, suggested a role for the cell-pole in DNA transfer (Kumar and Das, 2002), agreeing with (Matthysse, 1987), who reported that attachment of bacteria to plant cells was essential for transfer of T-DNA and that those bacteria attached to the plant cell in a polar manner. In this study, the polar orientation of *Agrobacterium* on explants was observed when explants were co-cultivated with AS. When the bacterium attachment characteristic and transformation efficiency of node explants on various cocultivated media were considered, it could be concluded that a cocultivated medium affects the attachment and cluster characteristics of *Agrobacterium* and results in efficient genetic transformation (Plate 4.15).



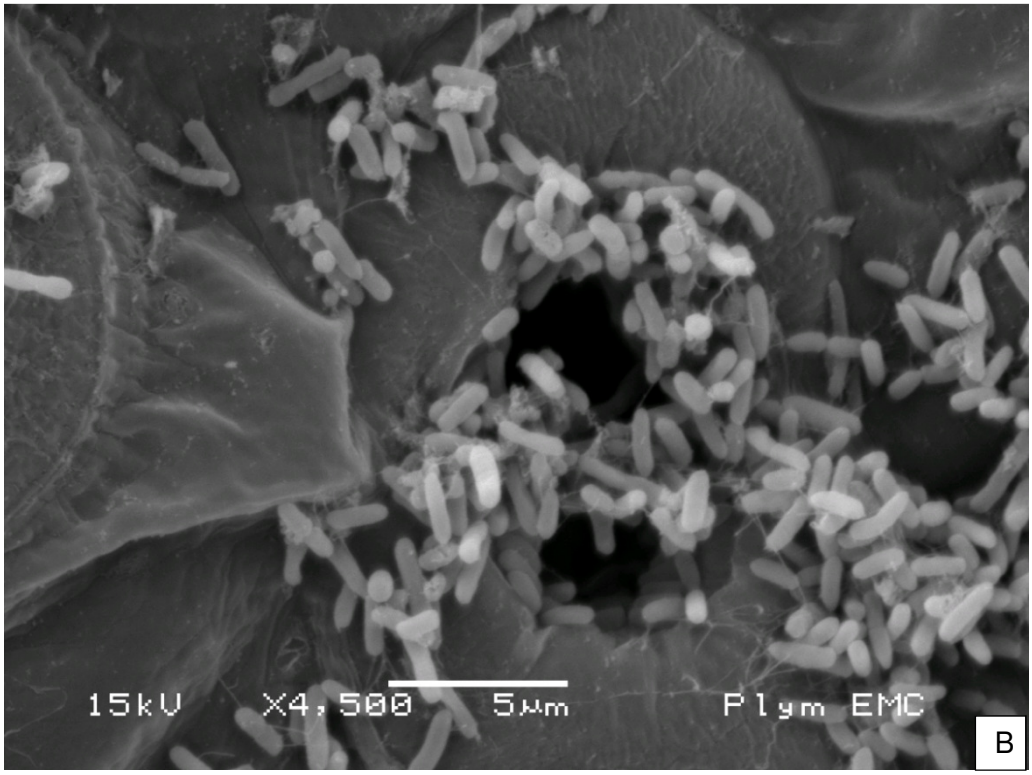


Plate 4.13. SEM observation of colonies of *Agrobacterium tumefaciens*. A) Colony of bacteria, and B) Bacterium cluster on explants; many packed bacterium clusters are observed and they are bound to the explant surface.

4.14 Discussion

4.14.1 Factors affecting *Agrobacterium* mediated transformation of cauliflower

Agrobacterium mediated transformation involves collaboration between two biological systems and is affected by many physiological conditions (Prem, 1998). Some factors are critical for successful production of transgenic cauliflower. These include use of young curd explants, *Agrobacterium* density, pre-culture period, co-cultivation period, and acetosyringone.

One of these factors *is Agrobacterium* density which plays a significant role in the transformation process. The current results indicate negative effects between *Agrobacterium* density and transformation rate. The reduction in transformation rate affected by inoculation with high concentration of *Agrobacterium* (OD_{600} = 0.6) related to an obvious hypersensitivity response of explants to the *Agrobacterium.* The same effect of high bacterial concentration has also been reported by (Orlikowska et al., 1995, Cheng et al., 2003b, Ismail et al., 2004). Diluted concentrations (1: 10 and 1:20 dilution) reduced necrosis to a great extent. The maximum transformation rate (5%) was obtained with 1: 10 dilution.

In general the results confirmed that 1: 10 (OD_{600}=0.6) bacteria density helped to improve transformation rate. The reduction of transformation rate caused by inoculation with 1:20 dilution (OD_{600} =0.6) associated with the hypersensitivity response of explants to the *Agrobacterium* or low *Agrobacterium* density, receptively. These results agree with (Srivastava et al., 1988a),whereas (Chakrabarty et al., 2002) achieved maximum transformation efficiency with 1:20 dilution. In the present study explants pre-culture for 10 days old were better for transformation than 1 and 20 day old explants as very few green shoots were recovered when older explants

were used and there was poor survival of the explants during co-cultivation when 1 day old explants were used. In contrast (Polowick et al., 2004, Chakrabarty et al., 2002, Lv Lingling et al., 2005) formed transgenic shoots by using lateral cotyledon buds as explants. Pre-culture duration is an important factor affecting transformation efficiency. Prior to inoculation with *Agrobacterium,* explants were pre-cultured on S23 medium for 1, 10 and 20 days. The results revealed that transformation percentage increased with length of pre-culture period. Improvement in transformation efficiency up on pre-culturing of the explants has also been reported (Molinier et al., 2002, Cardoza and Stewart, 2003b). This means that explants were hypersensitive to the *Agrobacterium* culture without any preculture, while pre-culturing treatment of the explants before co-cultivation allowed explants to overcome the stress resulting from co-cultivation with *Agrobacterium* and consequently improved gene transfer. These results were in agreement with (Molinier et al., 2002). After preculture, the explants were transformed by co-cultivation with *Agrobacterium* for 1, 2, 3 or 4 days. The results indicated that co-cultivating the explants for 2 days significantly increased the transformation efficiency. Co-incubation with *Agrobacterium* for more than 2 days led to most of the explants turning brown and finally dying. The suggested rationale for this phenomenon is that a long period of co-incubation with *Agrobacterium* increased infection and the explants lost the ability to survive the stress. Similar results indicated that a 2 day co-cultivation period significantly increased cauliflower transformation (Chakrabarty et al., 2002, Lv Lingling et al., 2005). Moreover the results are consistent with those of (Fillatti et al., 1987) where about 605 tomato cotyledons co-cultivated with *Agrobacterium* for 48 h produced Kanamycin resistant shoots. In most transformation protocols, 1 day of co-incubation is generally used.

For example, (Horshe et al., 1985) described high frequencies of transformation in tomato and tobacco when they used 1 day of co-incubation.

Two possible reasons could explain the reduction in transformation after 1 day of co-incubation. First, the introduction of too many bacteria may prevent tumour formation, possibly by an excessive synthesis of T -DNA gene product, resulting in a hypersensitive response. Data in support of this possibility have been achieved by studying the crown gall-grape system (Yanofsky et al., 1986). Some grape cultivars evidently have a hypersensitive response when exposed to too much *Agrobacterium.* In this case, cells near the site of inoculation died. The second possibility is that after 1 day of co-incubation, dead bacteria would accumulate. Lysis of cell walls of these bacteria would result in the release of lipopolysaccharide components of cell wall that block tumorogenesis (Whatley et al., 1976). These results are consistent with the findings of (Matthysse et al., 1981), who reported that in their experiment, dead *Agrobacterium tumefaciens* blocked tumorogenesis when co-inoculated with live *Agrobacterium tumefaciens* and tobacco tissue culture. The phenolic compound acetosyringone has been known to induce the vir gene (Shimoda et al., 1990) and the highest transformation rate (13%) was recorded when 80 mM acetosyringone was added to the culture medium. Similar results were achieved by (Sheikholeslam and Weeks, 1987, Bolton et al., 1986, Ismail et al., 2004, Henzi et al., 2000). These results suggest that cauliflower plants do not accumulate sufficient inducing compound for efficient activation of the vir genes. In support of this, (Stachet et al., 1985) suggested that the induction of the vir gene is mediated by acetosyringone which is normally released by the wounded plant cell.

For PCR analysis of the *A. tumefaciens* in this study, the most reliable results were achieved if small quantities of cells and fresh bacterial colonies (one to two weeks

after streaking) were analysed. Bacterial cells were deposited into the microfuge tube by scraping the toothpick along the tube wall to facilitated efficient cell recovery. It may be possible to improve the sensitivity of the PCR by heating the *Agrobacterium,* increasing the annealing temperature or by reducing PCR cycle if non-specific amplification is a problem. The APX gene was detected after PCR amplification from appropriate colonies at 478 bp (Al-Swedi, 2012). This method can be used routinely to evaluate insert sequence constructed into vectors harboured in individual *Agrobacterium* colonies and substantially reduces time and effort required to evaluate the authenticity of inserts in *Agrobacterium* binary vectors (Jones et al., 1987). Although (Prem, 1998) showed that PCR is a fast and sensitive method, it is still relatively expensive and susceptible to cross-contamination. The leaf disc assay on the other hand has an advantage as it is rapid, simple, requires minimal use of chemicals and plant tissues, and causes no permanent damage to the plant.

In order to identify transformed cells or plants that have been growing on a selective medium, it is required to have an easily assayable reporter gene. The most useful reporter genes encode an enzyme activity not found in the organism being studied; a number of genes currently are being used; however one of the most popular is the E. Coli glucuronidase (GUS). In this study, plasmids showed significant GUS activity. The substrate used in this study, X-Gluc, works very well but the quality of the histochemical localization are affected by numerous variables such as tissue preparation and fixation. The results of transformation with the gene construct used here indicated that the GUS assay method is an easy reliable technique of establishing optimal conditions of transformation, (Chakrabarty et al., 2002). All the transformed plants in the GUS histochemical assay showed positive bands in PCR analysis.

4.15 Standard Operating Procedure for Agrobacterium-mediated transformation of cauliflower curd tissues (SOP_{TA}).

1. Grow and select explants derived from curd of cauliflower using SOP_{CM}.
2. Grow and select the *Agrobacterium tumefaciens* strain using SOP_{BA}.
3. Cocultivate the cauliflower explants with an Agrobacterium solution grown for 24h to OD_{600} of 0.6.
4. Wash the explants 3 times with sterile distilled water then leave to dry.
5. For disinfection of *Agrobacterium* from the explants, use 250 mg L^{-1} Carbenicillin/Cefotaxime added to the culture medium.
6. Select transformed explants on medium containing 20 mg L^{-1} Kanamycin. The non-transgenic explants die while putative transformants explants continue to grow under Kanamycin selection pressure (as described above in 4.13.4).
7. Regeneration 1 – place putative transformed explants under continuous selection pressure with Kanamycin to 25 mg L^{-1} in medium to stimulate shoot proliferation.
8. Regeneration 2 – sub-culture shoots produced under Regeneration 1 onto growth medium containing Kanamycin at 50 mg $L^{-1}$to increase selection pressure.
9. Carry out GUS screening on putative transformed shoots as described above in 4.8
10. Carry out PCR confirmation on putative transformed shoots as described above in 4.9
11. Regenerated transformed plants contain APX stress gene is placed into soil for further growth and development as described above in 4.7.

Determination of optimum culture conditions for *Agrobacterium tumefaciens*

Optimum culture conditions for *A. tumefaciens.* The growth of the APX strain was compared in Luria-Broth solid medium (Appendix 5.a) and YE solid medium (Appendix 5.b) supplemented with 80 µM acetosyringone and different combinations of antibiotic (20 mgL^{-1} Kanamycin and 6.0 mgL^{-1} Gentamycin) (chapter 3; table3.1). One mL of culture suspension for APX strain was added to 9 mL of medium in a 10^{-1} dilution.

Using a pipette fresh tip, the dilution was mixed by pipetting up and down three times and then 1 mL was transferred to a second 9 ml of each liquid medium to obtain a 10^{-2} dilution and serial dilutions were made by repeating eight times until the original sample had been diluted to 10^{-8}. Subsequently, 0.1 mL of the final dilution was transferred to a Petri dish either containing 20 mL LB or YE solid medium supplemented with appropriate antibiotic concentration for each strain. The droplet was spread on LB and YE agar plates with a glass spreader and incubated at 28ºC for 48 hours.

The number of colonies grown on each plate after an overnight incubation were counted and compared as colony-forming units (CFU) per mL (as described by Jones *et al*., 1998). The colony count per mL of each dilution was calculated by dividing the mean colony count per plate (C) by the volume (in mL) of liquid transferred to the plate (V) multiplied by the original dilution (M).

Shoots transformed to be selected on kanamycin containing medium. Integration and expression of the introduced transgene were analysed by DNA gel blot and PCR analysis. Factors influencing the transformation efficiency include explants age, the

concentration of bacterium used for infection, duration of infection and co cultivation with *Agrobacterium*.

4.16 Conclusion

The presence of selectable marker genes, especially those which include genes coding for antibiotic resistance and which are essential for the initial selection of transgenic plants. A subject of concern relates to the fact that transgenes integrate at random positions in the genome leading to possible unwanted side effects (mutation) and unpredictable expression patterns.

Generally, selectable marker genes are not required once the transgenic plants are regenerated and the genetic analyses completed. The presence of a particular marker gene in a transgenic plant necessarily prevents the use of the same marker in following transformation and the use of a different marker system is required for each transformation round or event. Thus, any technique that can remove or eliminate a selection marker gene in transgenic crops is highly desirable if for no other reason than that the same procedure can be used in subsequent transformations. For transgene technology to be commercially successful, multiple independent transgenes available need to be added in existing sequence.

At present there is no commercialization of marker-free transgenic crops as it is still in the stage of proof-of-concept (Manimaran et al., 2011). Development of marker-free transgenic would further strengthen the crop improvement programme with prevalent applications in both fundamental research and biotechnology. Overall, the GM crops are expected to contribute globally to the food security.

The stable integration of the GUS gene and the NPTII gene was confirmed by PCR. This study presents an improved and efficient *Agrobacterium*-mediated stable transformation system for cauliflower (*Brassica oleracea* var. botrytis). The transformation procedure involves micropropagation. An overall 5% transformation

frequency was achieved for APX gene. Putative transgenic plants showed the expression of GUS coupled to the APX gene as confirmed by histochemical GUS assay and PCR analysis.

The molecular analysis of the *Agrobacterium* plasmid using PCR can considered as a routine method to evaluate insert a sequence constructed into vectors harboured in individual bacterial colonies and this method substantially reduces the time and effort required to evaluate the reality of inserts in *Agrobacterium* binary vectors.

The elimination of *A. tumefaciens* and shoot regeneration by using Cefotaxime and Carbenicillin confirmed that 250 mgL^{-1} was the best concentration to enhance the regeneration frequency and efficiency and at the same time suppress Agrobacterium growth.

For the best results with recombinant *E. coli* (Escherichia coli has become a "model organism" for studying because: 1) it is easy to maintain and breed in a laboratory setting and has particular experimental advantages; 2) due to its rapid growth rate, simple nutritional supplies, well known genetics and completed genomic sequence) grown in LB (Luria Broth). 1-3 mL of culture should be used for high copy plasmids, or 1-5 mL of culture for low copy plasmids. The harsh vortex mixing will shear genomic DNA, resulting in chromosomal DNA contamination in the final recovered plasmid DNA. The column preparation solution maximizes binding of DNA to the membrane resulting in more consisting yields. Finally if a more concentrated plasmid DNA preparation is required, the elution volume may be reduced to a minimum of 50µl. However, this may result in a reduction in the total plasmid DNA yield.

5 *Chapter Five:* Abiotic stress resistance of transformed lines

5.1 Introduction

Environmental stresses such as drought, salinity, extreme temperatures, toxic chemicals, and excessive ozone or carbon dioxide, are known as abiotic stresses. These stresses cause reduction in productivity and cause an average yield loss of more than 50% for major agricultural crops (Boyer, 1982, Kawaguchi et al., 2003, Shubha and Akhilesh, 2007). A major concern for the scientist is to reduce this loss of yield but is difficult and almost impossible to reduce the stresses.

5.2 Breeding and selection for abiotic stress

Abiotic environmental stresses, particularly salt, freezing and drought are major limitations for plant growth and crop production worldwide. In the case of economically important plants, abiotic stresses can cause significant losses to crop production and breeding for plants tolerant to salt, drought and frost damage has always been an indefinable goal. Breeding for tolerance against cold and salt by classical methods of selection and crossing is a time consuming and, often, inefficient procedure. Improving salt and frost tolerance may however be achieved by direct gene transfer (Zhang et al., 2000) or through DNA mutation (Zhu, 2000). Among mutants lines released as commercial cultivars, 6% were reported by (Micke, 1988) to be tolerant to abiotic stress.

The only achievable principle for selection is the ability of plants to survive various salt levels at different growth stages using biochemical markers as selection tools (Ashraf, 2004). I*n-vitro* selection techniques may provide an alternative way to select new genotypes with improved properties (Fuller et al., 2006b). Norlyn,(1980)

reported that the reaction to salt stress varies with the stage of plant development and a given cultivar may be tolerant at one stage and sensitive at another.

Possible techniques of plant breeding for salinity tolerance include intraspecific or interspecific crosses, chromosome manipulation, somatic hybridization and mutation of plant cell cultures (Ashraf and Wu, 1994, Tal and Shannon, 1983a). During plant breeding, there are three different ways of applying salt treatments for selection: firstly, selection under continuous saline conditions over the entire growth cycle as suggested by (Epstein et al., 1980); secondly, variation of the salinity concentration according to the sensitivity of the growth stage (Noble, 1983); and thirdly, selection at a certain stage of plant growth with an appropriate salt concentration (Noble, 1983).

Ashraf and Harris,(2004) indicated that plant breeders have successfully improved salinity tolerance of some crops in recent decades using plant vigour or seed yield as the main selection criteria. Selection may be more convenient and practicable if the crop possesses distinctive indicators of some salt tolerance at the whole plant, tissue or cellular level.

5.3 Tissue culture as a selection tool

Tal,(1984) suggests a range of advantages of using tissue culture for physiological studies for salt resistance, as follows;

1- Tissue culture can be used for studying mechanisms of salt tolerance at both cellular and whole plant levels,
2- Tissue cultures can be treated uniformly in a controlled way,
3- Experiments can be achieved year-round since the growth of tissue culture is independent of seasonal variations,

4- The contribution of different parts of the plant to the response of the whole plant can be determined by studying their response in culture,

5- In cell culture system millions of cells can be screened and evaluated for their performance in comparatively small area,

6- Relatively homogeneous populations of cells can be settled in tissue culture as associated with heterogeneous whole plants, therefore providing a tool for studying the effect of stress on various components of growth,

7- In culture mutagenic agents can be added to induce variability.

8- Protoplasts are especially useful for studying the involvement of the surface membrane in stress injury.

However there is a disadvantage to using these systems in that surviving callus or tissues might contain of a mixture of resistant and sensitive cell which escaped the selection pressure (chimeras). Moreover, cells of the necessary phenotype may be difficult to discover and may obtain resistance through the production of compounds which may be transferred to adjacent sensitive cells, conferring temporary resistance leading to 'false positives' (Deane, 1994).

Plant cell lines from a number of species have been selected for tolerance to salinity. In most of the examples the improved tolerance was recognized at a cellular level but not at whole plant level. Minimal success has been achieved however in demonstrating the expression of salt tolerance by the plants regenerated from these salt selected cell lines. Flowers and Yeo,(1995) concluded that selection in tissue culture is not a process that can be used simply to generate salt resistant plants in spite of the fact that salt resistant cell lines can readily be selected. There are just two patents for *in-vitro* selection of salt tolerant cell lines and regenerated plants with enhanced salt resistance; one for flax (Patent, 1986) and one for alfalfa (Patent,

1991). The use of cell culture in NaCl resistance studies sometimes leads to putative resistant callus or lines that subsequently lose their tolerance. It would appear that stringent testing and retesting to establish stable and heritable tolerant lines must always follow tissue culture selection.

The correlation between the performance of *in-vitro* and the *in-vivo* growth of plants has been reported to be very poor (Flowers et al., 1985) and it can be concluded that selection for salt tolerance by screening of cultured cells in high NaCI medium is largely unsuccessful. However, it has been demonstrated in several glycophyte species (Lutts et al., 1996b) and halophyte species (Vera-Estrella et al., 1999b), that the responses shown by salt stressed plants is partly determined by cellular properties. Although salinity resistance is a complex trait resulting from interaction between morphological and physiological properties, the possible selection of salt-resistance cell lines to regenerate valuable plant material that could be suitable in crop breeding programs is still regarded as a reality. However, Dracup,(1991) concluded that selection for salt tolerance by selection for salt tolerance by selection of cultured cells which grow at high NaCI has been largely unsuccessful, probably due to unacceptable assumptions that the mechanism of salt tolerance in cultured cells and whole plants are similar.

The change in the DNA sequence of genetic material is known as mutation and this has become an established method of inducing variation within a crop variety. This method contributes significantly to plant breeding (Maluszynski et al., 1995, Nichterlein, 2000) in creating genetic variability, which reduces the time to breed new varieties in comparison with traditional methods (Cornide, 2001). Mutation also offers the possibility of inducing desired attributes that either cannot be expressed in nature or have been lost during evolution (Brunner, 1995). The classical methods of

breeding are time consuming and sometimes inefficient while through DNA mutation or direct gene transfer the cultivar might be improved for abiotic resistance without disrupting the rest of the genotype and the breaking of favourable gene linkages (Zhang et al., 2000).

Even though sizeable DNA deletions and insertions can be detected by PCR and is relatively straightforward, the detection of point mutations, mainly induced by chemical mutagens is challenging because a PCR amplified fragment does not show any change in the size. Single base variation detection has been improved with advances in single-nucleotide polymorphism (SNP) detection technologies (Kwok, 2001). One example of SNP detection technology being applied to reverse genetics is targeting induced local lesions in genomes, in which chemical mutagenesis is followed by screening for point mutations (McCallum et al., 2000). Substances that cause mutations are known as mutagens, and may be either physical or chemical; both are used in conventional plant breeding. The chemical mutagens that induce mutation in plant cell cultures can be divided into two groups - base analogs and alkaline agents.

Alkaline agents include N-nitrose-N-ethylurea (NEU), N-nitrose-N-methylurea (NMU), alkyl sulphate and nitrogen mustards. NEU or NMU are bio-functional agents (Charlotte, 1976) and have been shown to induce gene mutation. They can also cause mispairing of nucleotides with their complementary bases, so introducing base changes after replication (Ashburner, 1990, Haughn and Somerville, 1987). Multiple mutations occur more frequently in NMU-treated plants. There is great scope for increasing both the frequency and spectrum of mutations in treatments with chemical mutagens through suitable modification of the treatment conditions (Savin et al., 1968).

5.4 Dehydration stresses effect on plant

In some cases the plant response to different stresses share similar steps in the mechanism e.g. freezing, drought and salt stresses disturb the osmotic homeostasis of the plant cell and these stresses affect the water relations of plant and cause cellular dehydration and collectively these stresses are known as dehydration stresses (Beck et al., 2007). It was found through a short term treatment experiment that *Arabidopsis* responded to cold, drought and salt stresses in a quite specific way (Kreps et al., 2002) and a cross talk in the signalling pathways appeared in frost hardening by drought or salt treatment and showed cooperative actions for all of these stresses (Beck et al., 2007).

Cell membrane damage occurs during dehydration or rehydration and the capability of a plant to avoid or repair this damage to membrane is essential for plants to survive under dehydration stresses. Some of the mechanisms leading to adaptation to dehydration, have been elucidated by the identification and manipulation of key genes and transcription factors to alter metabolism and increase plant tolerance to dehydration (Chaves and Oliveira, 2004). Dehydration stress tolerance in crops could be improved by engineering and manipulation of osmoprotectent synthesis pathways in the susceptible plant (Rathinasabapathi, 2000). Gene manipulation for the production of osmolytes such as mannitol, fructans and proline. might increase resistance to dehydration (Ramanjulu and Bartels, 2002). Modification of plants for increased dehydration tolerance is mainly based on the manipulation of either signalling or transcription factors or genes that directly protect plant cells against water deficit, but an understanding of the molecular and biochemical mechanisms is still a challenge for scientists (Valliyodan and Nguyen, 2006).

5.5 Frost stress damage in plants

Freezing temperatures damage plant cells and tissues as well as the entire plant, while the cell membrane is the primary site of freezing injury (Levitt, 1980). Ice formation initiates in intercellular spaces and cell walls on exposure to freezing temperature, in some cases the ice nucleating agents such as dust or ice nucleating bacterial proteins facilitate ice formation externally which spreads into plant tissues (Brush et al., 1994).

Freezing temperatures destabilize the cell membrane (Uemura et al., 1995) and the water from the cytoplasm moves outside through the plasma membrane by osmosis to the extracellular ice across a water potential gradient and freezing injury is therefore mainly caused by cellular dehydration (Xin and Browse, 2000). As freezing progresses, certain lipids in the cell membrane form an inverted structure with hexagonal packing symmetry which disrupts the bilayer of the plasma membrane which then becomes permeable to water and solutes and loses osmotic responsiveness (Uemura et al., 1995, Webb et al., 1994, Xin and Browse, 2000). The solute concentration of the cytoplasm plays a vital role and the removal of water from the cell will depend on the solute concentration in cytoplasm acting to balance the water potential gradient with the expanding extracellular ice (Xin and Browse, 2000).

5.5.1 Cold acclimation and frost stress tolerance in plants

Freezing tolerance increases in many plants on exposure to non-freezing low temperature for a certain period, a process known as cold acclimation (Levitt, 1980); (Sakai and Larcher, 1987, Smallwood and Bowles, 2002). Cold acclimation is a

collective whole plant process and plants display a combination of responses in different organs and tissues during cold acclimation (Pearce et al., 1998).

Cold acclimation has provided a way to study how plant cells can tolerate freezing and the biochemical changes that occur during cold acclimation essential for freezing tolerance. However, the biochemical changes occurring during acclimation are not necessarily all adaptive to freezing stress resistance (Gareth, 2001) and there are several plausible reasons for biochemical changes occurring during acclimation (Pearce, 1999). In association with low temperature stress, there may be adaptive responses to other stresses as well, such as attack by snow moulds or ice encasement and developmental responses such as vernalization which also involve novel biochemistry and some biochemical changes may be not adaptive (Gareth, 2001). Some of these changes may make zero or a negative contribution to increased frost tolerance (Gareth, 2001).

Light also plays an important role in the cold acclimation process as moderate to high light is essential for the cold acclimation process. In the absence of light, photo-inhibition leads the plant to form reactive oxygen species causing oxidative stress (Foyer et al., 1994, Wanner and Junttila, 1999).

The cold acclimation capability of plants is a quantitative trait involving a large number of genes and massive reprogramming of gene expression is associated with cold acclimation (Mantas et al., 2010b). It has been estimated that in *Arabidopsis* 5-25% of the plants genes show varied patterns of expression during low temperature (Mantas et al., 2010b, Robinson and Parkin, 2008). The induction of low temperature responsive genes results in a large amount of physiological, metabolic, and biochemical alterations that determine the ultimate level of freezing tolerance

achieved during acclimation (Mantas et al., 2010b). These alterations include the changes in the level of antioxidants, phytohormones, and changes in the level of production of compatible solutes and protective proteins including chaperons proteins and other proteins of unknown function (Aalto et al., 2006, Mantas et al., 2010b).

In this part of the study, transformed clones were sub-cultured every 3-4 months continuously for the maintenance of the material for the project and clones were screened for frost, drought and salt stress resistance. The frost stress resistance was also investigated under cold acclimation conditions.

5.6 Aims and objectives

5.6.1 Aim

The aim of physiological assessments was to confirm the persistence of abiotic stress resistance tolerance in transformed plants over many plant generations. It was important to identify the response of each transformant line prior to further molecular and biochemical characterization of transformant.

5.6.2 Objectives

- To regenerate the transformed plants of cauliflower to maintain materials for project work.
- To screen the cauliflower ***in-vitro*** plants (transformants and control) under frost stress.
- To analyse ***in-vivo*** plants (transformants and control) of cauliflower under frost stress.
- To screen the cauliflower ***in-vitro*** plants (transformants and control) under drought stress.
- To analyse ***in-vivo*** plants (transformants and control) of cauliflower under drought stress.
- To evaluate the cauliflower ***in-vitro*** plants (transformants and control) under salt stress.
- To screen ***in-vivo*** plants (transformants and control) of cauliflower under salt stress.
- To investigate the effect of cold-acclimation on frost stress resistance in cauliflower plants (transformants and control).
- To investigate multi-stress potential of each transformant.

5.7 Materials and methods

5.7.1 Plant screening for abiotic stress resistance

In the present study, highly resistant transformants of cauliflower (*Brassica oleracea var. botrytis*) to abiotic stress were selected from a transformants population. Selection was made on the basis of previous screening results of (Fuller et al., 2006b), and reassessed in the present study for abiotic stress (salt, frost and drought stress) resistance under both *in-vitro* as well as *in-vivo* conditions. The transformant (TA) cauliflower plants were compared with the control (C).

5.7.2 Abiotic stress resistance

After transformation selection of stress resistant transformants was made by the addition of NaCl and Mannitol to the medium. Surviving green shoots were removed from liquid medium and sub-cultured onto S23 solid culture medium (Kieffer et al., 1995a) to develop into shoots. Those shoots with obvious pink or white colour either died or were discarded. Transformants (TA) and control (C) were then subjected to a multiplication phase (S23 + Kinetin 2 mg L^{-1} and IBA 1 mg L^{-1}) to produce clones which were both rooted and regenerated. After continuous sub-culturing for a two year period, the clones were re-assessed for abiotic stresses and further selection was made (Fuller et al., 2006b). Sub-culturing was carried out after each 3 to 4 month period on S23M medium and maintained in *in-vitro* condition.

i. Weaning process

In-vitro clones (TA&C) grown on S23M medium were uprooted and agar from the roots was gently removed by hand. A systemic general fungicide was sprayed on the roots to protect from soil borne pathogenic fungi, and then plants were transferred to pots (6 cm x 6 cm) containing moist compost and kept in a growth chamber at 23^{o}C with 16 hours light (light intensity 160.5 µmol m^{-2} s^{-1}). After 5 days the lids of the

culture pots were holed using a hot needle to reduce humidity inside the pots and left for 5 days, then lids were taken off and for 5 days regular water checking was carried out. The base of each pot was then holed with a hot needle and after 5 days the pots were transferred to the bigger growth cabinet with the same conditions. After 5 days the plantlets along with compost were transferred to bigger pots (12 cm x 13 cm) containing moist compost and allowed to grow in *in-vivo* conditions. This weaning process demonstrated 90% successful transfer of *in-vitro* clones to *in-vivo* conditions.

ii. Evaluation of salt stress resistance

The clones were analysed for salt resistance. Plants of both *in-vivo* as well as *in-vitro* were analysed. Liquid media of seven different concentrations of sodium chloride were prepared in distilled water i.e. 0 (control), 50, 100, 200, 300, 400 and 500 mM NaCl (approximately the concentration of sea water) and labelled as T0, T1 ,T2 T3, T4, T5 and T6 respectively, then 4 gL^{-1} MS salts (Murashige and Skoog, 1962) were added to each of T0, T1, T2, T3, T4, T5 and T6. The pH of all media was adjusted to 5.8, and then autoclaved. Media were poured into sterile Petri dishes under aseptic conditions in a laminar flow hood. Three replicate Petri dishes were used for each clone under each treatment. Two fully expanded upper leaves were detached from each clone. Leaf discs of 1 cm diameter were prepared in a laminar flow cabinet under aseptic conditions and transferred to Petri dishes containing the various liquid media, three discs per Petri dish. Petri dishes were labelled and placed in an incubator at 23 ^{o}C with 16 h photoperiod. Leaf discs from *in-vitro* clones were prepared direct from pots and analysed in a similar way used for *in-vivo* clone analysis. The total number of Petri dishes used for each of *in-vitro* or *in-vivo* clones analysis were 2 samples x 3 replicates x 7 treatments, i.e. 42 plates. The effect of salt concentrations on leaf discs was recorded after 2, 4, 6, 8, 10 and 12 days

treatments. Change in leaf discs colour was scored to differentiate resistance. Colour change of leaf discs was considered as:

4. Dark green (100% greenness)

3. Light green-no white (75% greenness)

2. Half-light green half white (50% greenness)

1. Small amount of light green (25% greenness)

0. White. (0% greenness)

iii. Drought stress resistance investigation

Drought resistance assessment of plant was carried out by leaf disc assays to compare their resistance potential. For this evaluation 4.4 g L^{-1} MS medium was dissolved in distilled water with different test concentrations of Mannitol added (0 (control), 100, 300 and 500 mM) indicated by T0, T1, T2 and T3 respectively. The pH of all of the media was adjusted to 5.8 prior to being autoclaved. The sterilized media were poured into sterile Petri dishes under aseptic conditions in a laminar flow hood. Two fully expanded upper leaves from both *in-vivo* and *in-vitro* clones were tested.

Leaf discs were cut using a 1.0 cm diameter cork borer from the leaf blade areas avoiding the major vascular bundles; leaf discs of each genotype were transferred to a specifically labelled and sterilized petri dish. The discs were allocated to each one of the different media contained in Petri dishes and incubated for twelve days in an incubator at 23^{o}C with 16 h photoperiod. Every two days the score of each disc was scored as outlined above.

iv. Frost stress resistance analysis

Frost resistance analysis of transformants (TA) and control (C) were carried out. The electrical conductivity technique described by Fuller *et al* (1989; 2003) was used. Both *in-vitro* as well as *in-vivo* plants were tested at different temperatures 0, -2, -4, -6,-8,-10 and -12 ^{o}C. Four fully expanded upper leaves from each plant were excised and transferred to the laboratory. Two leaves were used for assessment as non-acclimated and the other two leaves were acclimated by placing them in an incubator (Snijder scientific) at 4 ^{o}C, 8h photoperiod for 14 days. Preliminary experiments demonstrated that excised leaves of cauliflower had the ability to acclimate; acclimation for *in-vitro* plants was carried out by keeping whole plantlet in the same low temperature incubator used for *in-vivo* plant leaves. Three 1 cm diameter leaf discs were cut and placed in boiling tubes. Three replicate tubes were used for each treatment and each genotype. The total tubes used for each of *in-vitro* or *in-vivo* clone analyses was = 2 samples x 3 replicates x 2 treatments (unacclimated/acclimated) x 7 different test temperatures, i.e. 84 tubes. Three replicate tubes without leaf discs (blank tubes) were used for monitoring EC contamination. All the tubes were labelled in test tube racks and put in a freezing chamber (Sanyo).

v. Selection of abiotic stress resistant transformed plants.

The transformed plants were maintained in *in-vitro* condition for about two years by continuous sub-culturing and reassessed for selection of stress resistant plants (Fuller et al., 2006b). From 10 lines of transformed plants, one line was sustained by continuous sub culturing. In the current study, highly resistant lines from a transformed population were selected on the basis of previous screening (Fuller et al., 2006b) and the resistant clones were regenerated, propagated and rescreened

physiologically for the frost, drought, and salt stress resistance. After stress resistance confirmation through physiological screening, the highly resistant plants were subjected to molecular and biochemical investigations, the selected plants were chosen as control (C) and transformants (TA).

5.7.3 Regeneration and propagation of transformants

For regeneration and propagation of transformed plants, S23M media was prepared and 20 mL of medium was poured into sterile plastic pots (5 cm x 4 cm) under aseptic conditions, and then allowed to cool overnight at room temperature.

Curds were used as explant materials for regeneration and plant propagation. Samples were greenhouse raised (Plate 5.1); curds were cut into small pieces and treated with 70% ethanol for 40 seconds and then with 10% bleach for 5 min for surface sterilization and then washed 3 times with sterile dH_2O in order to remove bleach from the surface as in the SOP-$_{CM}$ of curd micropropagation. The explants were then transferred into shoot induction media in plastic pots.

The pots were kept in incubator at 23 ℃ and 8 h photoperiod until shoots were produced. After 5 weeks period the young shoots were excised with a sterile sharp scalpel and inoculated into hormone free S23M media in plastic pots and incubated at 23℃ with 16 h photoperiod in the incubator. When completely rooted, these *in-vitro* clones were sub-cultured every 3 to 4 months on S23M hormone free media to provide the plant materials for all experimental work.

Plate 5.1. Fully developed stock plants in the field.

Acclimatization

Microshoots were removed from the culture vessels (Plate 5.2a), and rinsed with tap water to remove remaining agar from the root system (Plate 5.2b). They were then transplanted into individual commercial plastic pots (Plate 5.2c) filled with a mix of multipurpose compost and John Innes 2 [2:1 (v/v)], covered with a light plastic film and transferred to a growth room. During the first 2 weeks, the plastic was gradually opened to allow air exchange, reduce humidity and enable plant acclimatization. The pots were grown in a culture room under a photoperiod of 16h at 50 μ mol m^{-2} s^{-1} at plant level. Temperature was maintained at 23° C. After 4 weeks survival rate and growth appearance was assessed.

During hardening, the plants were watered and ventilated every day by temporarily removing the plastic bags (Plate 5.3 d). After 10 days, the plastic bags were punctured to allow greater gas exchange, and after 20 days the tops of the plastic bags were cut open.

Plate 5.2. Illustration of the weaning process of *in-vitro* grown cauliflower microshoots: A) transfer of *in-vitro* plants into *in-vivo* conditions, B) Removal of *in-vitro* plants from agar media and fungicide spray on roots, C) *in-vitro* plants transferred to compost under plastic shed, D) plants in growth cabinet, E) plants transferred into bigger pots, F) mature plants.

Frost stress resistance

Both *in-vitro* and *in-vivo* plants were assessed for frost stress resistance at different test temperatures: +1, 0, -2, -4, and -6 ℃ with a hold of two hours at each freezing temperature (Figure 5.1). A leaf disc assay was performed on plants which were cold-acclimated and non-acclimated conditions using the electrical conductivity technique (Plate 5.3) described by (Fuller et al., 1989, Fuller et al., 2003) . For acclimated *in-vivo* plants, fully expanded leaves were excised and transferred to the laboratory in an insulated box containing ice packs. In addition two leaves were used from non-acclimated plants and another other two leaves were acclimated in growing delivery programme incubator (Snijder scientific) at 4℃, 8 h photoperiod for 14 d. In the same incubator the *in-vitro* clones were acclimated for 14 d. The percent relative electrical conductivity was measured as REC% = Post freezing EC/ Post autoclaving EC x 100.

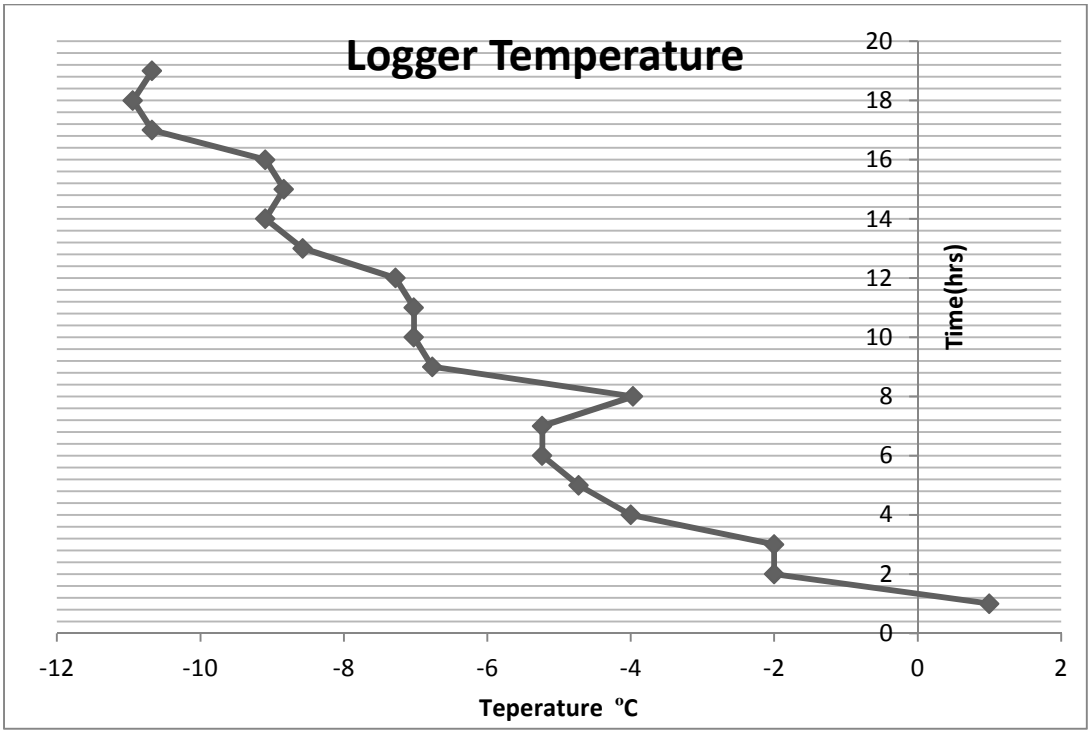


Figure 5-1. Illustration of the temperature of the freezing cabinet. Example of the temperature trace of a frost test at +1, 0, -2, -4, -6,-8,-10 and -12 ℃ to study damage to cell membranes in the growing delivery programme incubator.

Plate 5.3. Illustration of the frost resistance test and general view of the set-up for measuring electrical conductivity.

Drought stress resistance of transgenic plants

Leaf discs assay was carried out for drought stress resistance for both *in-vivo* and *in-vitro* plants. The basal MS medium was used with different concentrations of mannitol added (0, 100, 300, and 500 mM). Leaf discs were incubated in labelled sterilized petri plates at room temperature (Plate 5.4) and incubated for twelve days at 23 ℃ with 16 h photoperiod. Leaf discs were scored for greenness every 2 days.

Plate 5.4. Illustration of drought resistance test

Salt stress resistance

Plants were screened for salt (NaCl) resistance using a leaf disc assay. Leaf discs of one centimetre diameter were prepared from the leaves of both *in-vivo* and *in-vitro* plants. Leaf discs from *in-vivo* plants were surface sterilized in 70% ethanol for a few seconds, followed by shaking in 10% bleach solution (sodium hypochlorite) for approx10 minutes followed by three rinses with sterile distilled water. The *in-vitro* clones were used direct from culture pots.

Leaf discs were transferred to Petri-dishes containing 20 mL sterile liquid medium (M&S salts at 4 g L^{-1}) supplemented with NaCl at concentrations of 0, 50,100, 200,

300, 400 and 500mM. Leaf disc damage was assessed after 2, 4, 6, 8, 10 and 12 days using a five point score based on the percentage greenness of the leaf discs.

5.8 Results

5.8.1 Assessment of transformants for frost resistance after cold-acclimation

Cell damage increased progressively with lowering temperatures below -6 °C with the highest damage observed at -12 °C (Figure 5.2) (the Lower the %EC higher the frost resistance (Figure 5.3). The acclimation process was effective in increasing frost resistance but was more effective in the transformed plants and clear differences existed among acclimated transformed plants with some transformants showing high resistance compared to control plants (Figure 5.3). Other transformants showed moderate resistance compared to controls. The plant C is control and all plants labelled TA are transformants.

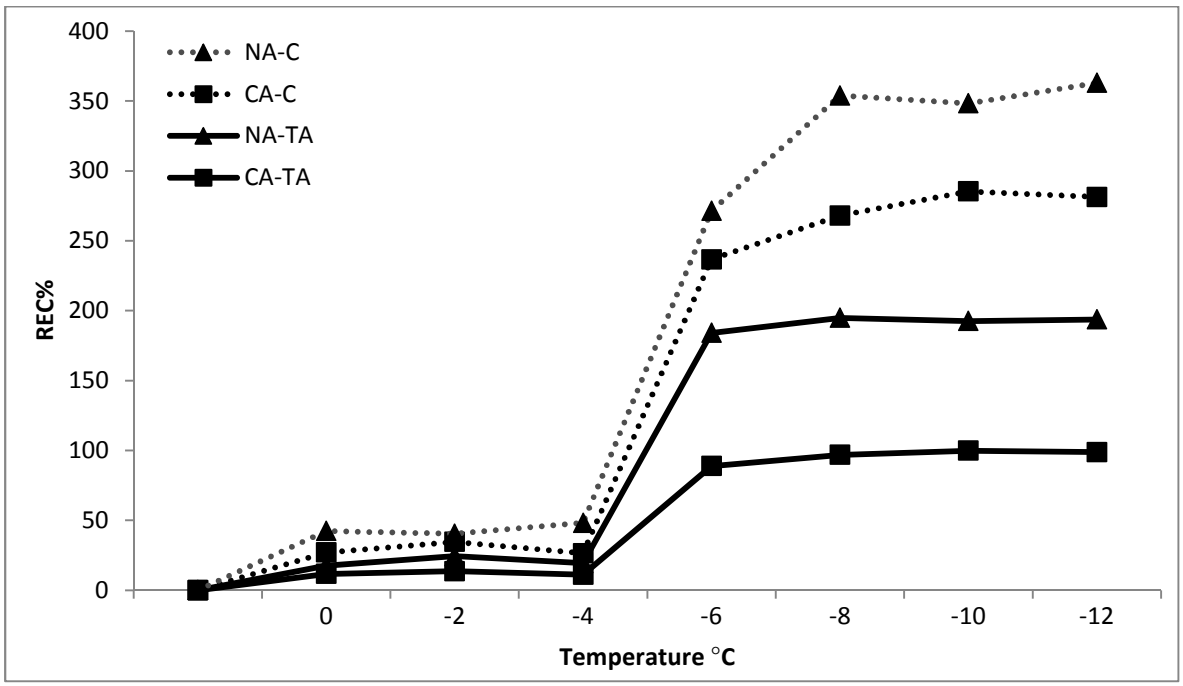


Figure 5-2. Effect of cold acclimation on leakage from cell membrane of *in-vivo* plants at different freezing temperatures. 0 °C, -2 °C, -4 °C and -6 °C,-8 °C,-10 °C,-12 °C. Lower electrical conductivity (EC %) means less damage. C is control and TA transformants. The CA is acclimated and NA is non-acclimated.

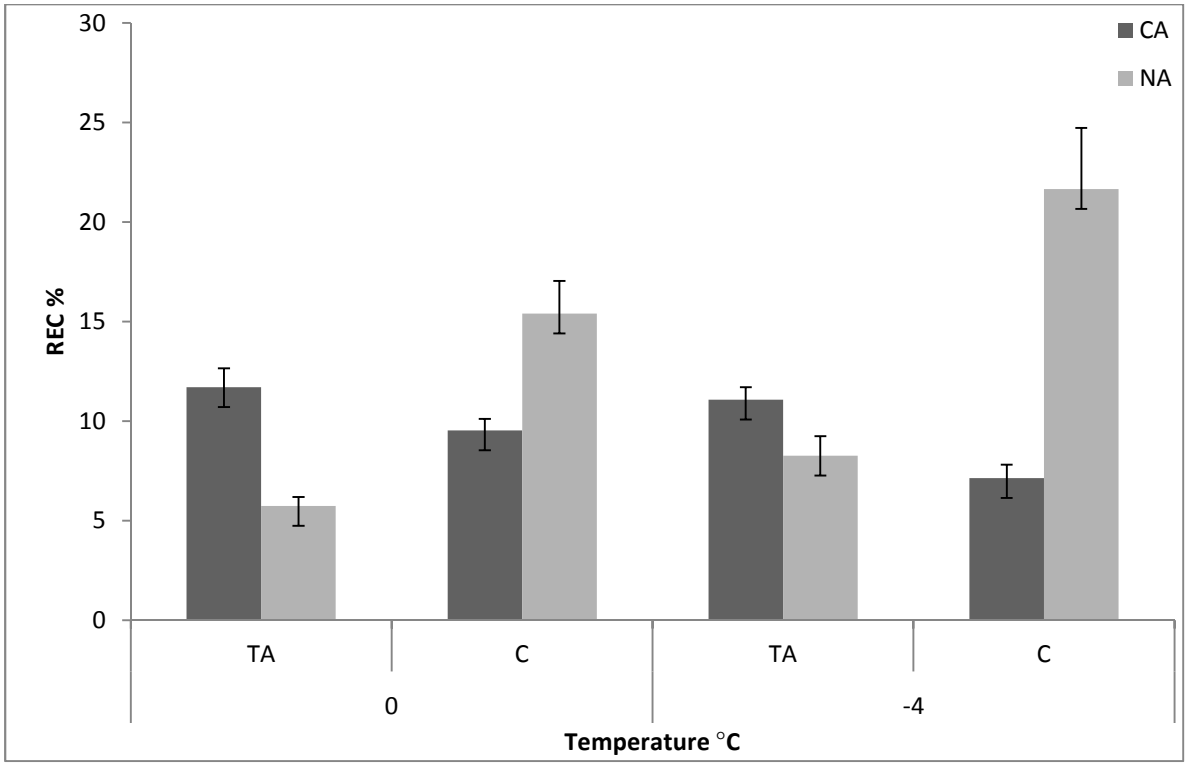


Figure 5-3. Response of cold acclimated (CA) and non-acclimated (NA) *in-vivo* plants at 0 and -4 ℃ respectively.

5.8.2 Evaluation of *in-vivo* and *in-vitro* shoots of transformants for frost resistance

The acclimation process was found to be effective for the increase in frost tolerance (Figure 5.4). All plants in the *in-vivo* state showed higher resistance than in the *in-vitro* state. One reason for this difference between *in-vivo* and *in-vitro* might be the young and soft *in-vitro plants* grown in an incubator under constant optimum temperature and humidity prior to exposure to freezing, while *in-vivo* plants were mature and grown in a varying natural environment where plants are constantly challenged with some minor stresses. It can be concluded from these results that transformants are highly resistant to frost compared to control.

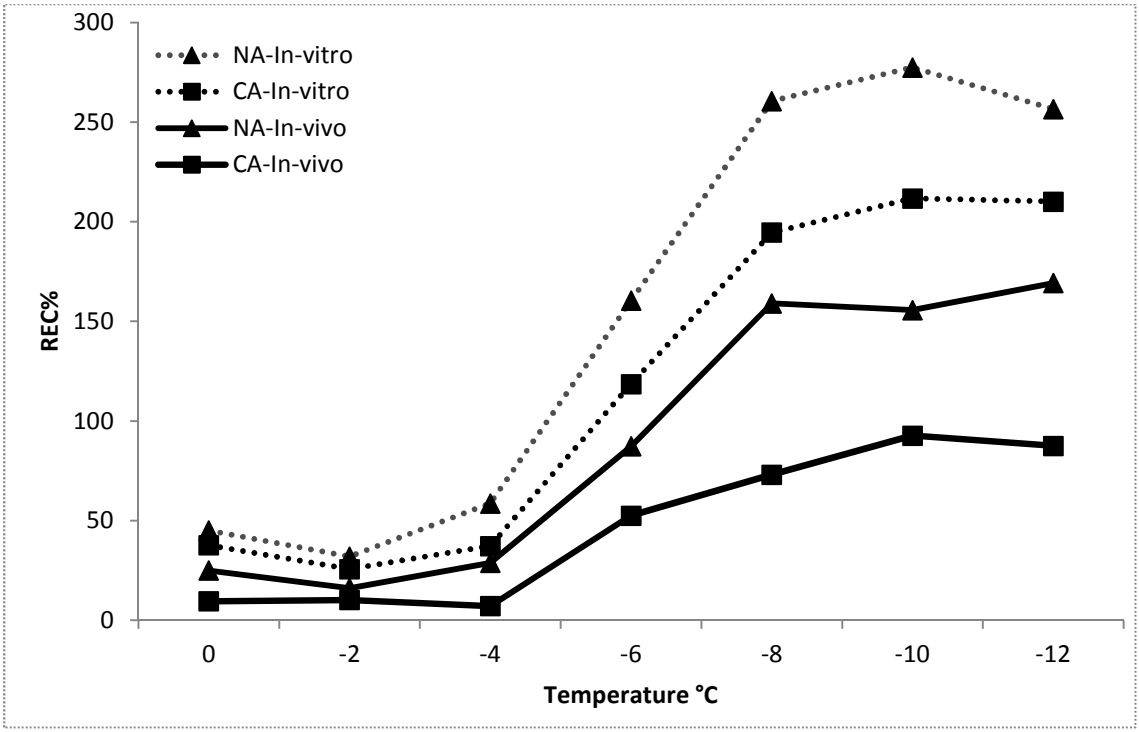


Figure 5-4. The cold acclimation effect on in-vitro clones. Response of clones at different freezing temperature; 0 ℃ -2℃,-4℃ ,-6℃,-8℃,10℃ and -12℃. Lower electrical conductivity (EC %) means less damage. The CA is acclimated and NA is non-acclimated.

5.8.3 Evaluation of *in-vivo* and *in-vitro* shoots of transformants for salt resistance

Both the increase in salt concentration and the time of exposure caused a decrease in greenness in both of *in-vivo* and *in-vitro* grown plants (Figure 5.6 and Plate5.6). After 2 days differences between genotypes were not obvious but by day 4 differences were becoming evident; by day 8 there was very clear differentiation between transformants and control in terms of greenness (Figure 5.5 and Plate 5.5). Leaf discs from some plants had also progressed from green to white and the higher concentration of NaCl 500 mM in liquid media showed clear differences in colour change (Plate 5.6 and 5.6) and control plant discs changed from dark green to white after eight days treatments. All the *in-vivo* grown transformants showed significant differences when compared with their *in-vitro* counterparts (Figure 5.6 and Plate 5.6). Moderately resistant transformants showed a mix of colours of light green and with some discs white. Control leaf discs showed less than 50% greenness after 4days treatment at 400 mM NaCl. *In-vitro* grown clones showed a different general response to *in-vivo* clones with increasing salt concentration and exposure time of treatments (Figures 5.6 and Plate 5.6) and all the transformants showed higher greenness compared to control (Figures 5.5 and Plate 5.5). All the transformants showed more resistance than the control plant line which changed from green to white after 8 days treatment at 400mM salt. These results confirmed that the resistance of transformants had been maintained after a long time in culture and following many sub-cultures.

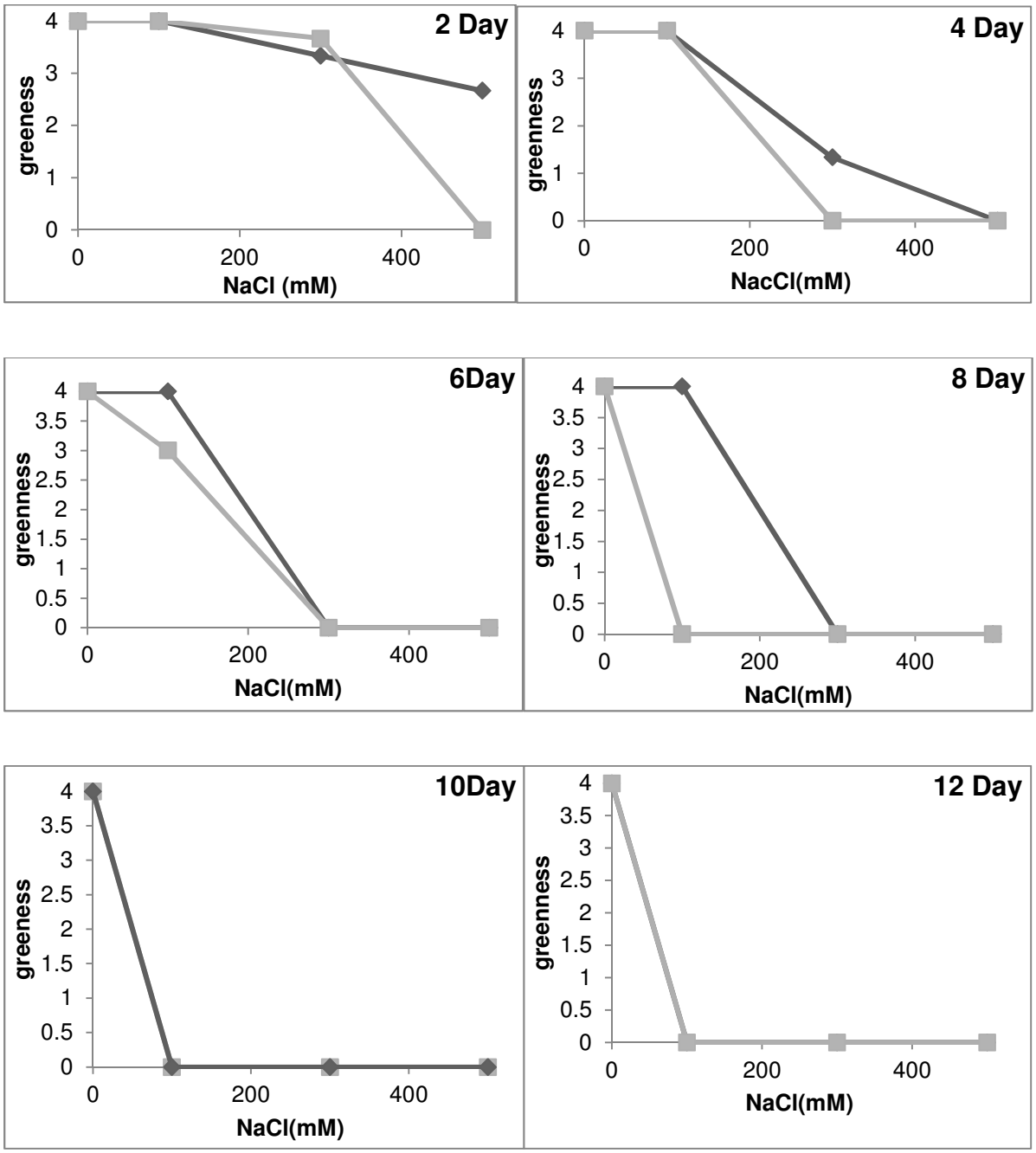


Figure 5-5. Effect of salt treatments on greenness of leaf discs. Salt stress tolerance of plants after 2, 4, 6, 8, 10 and 12 day's treatments with different concentrations of salt. Grey line = control; black line = transformant.

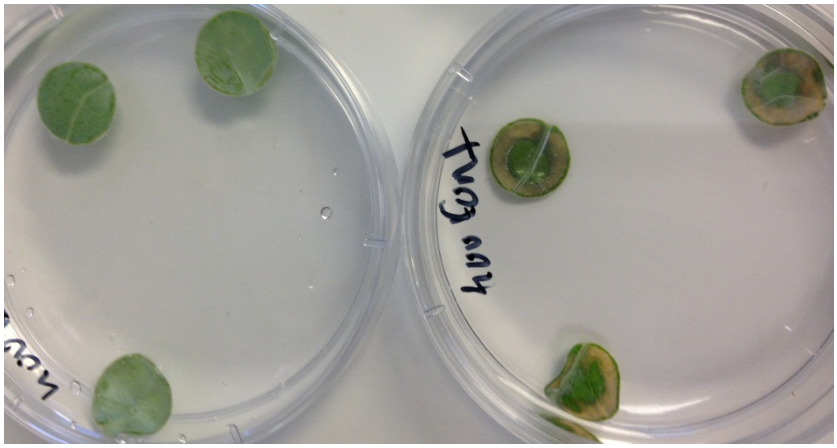


Plate 5.5. Illustration of the response of plants to NaCl stress. Left transformed plant and right control. Salt stress level 400 mM

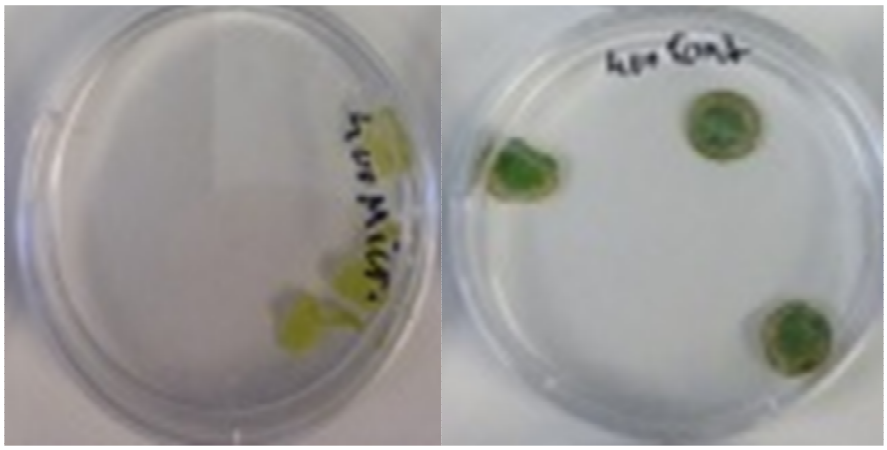


Plate 5.6. Illustration of the effect of salt on leaf discs after 2 days treatment at 400 mM NaCl. Left are *in-vitro* plants, right are *in-vivo* plants. Greenness indicates resistance.

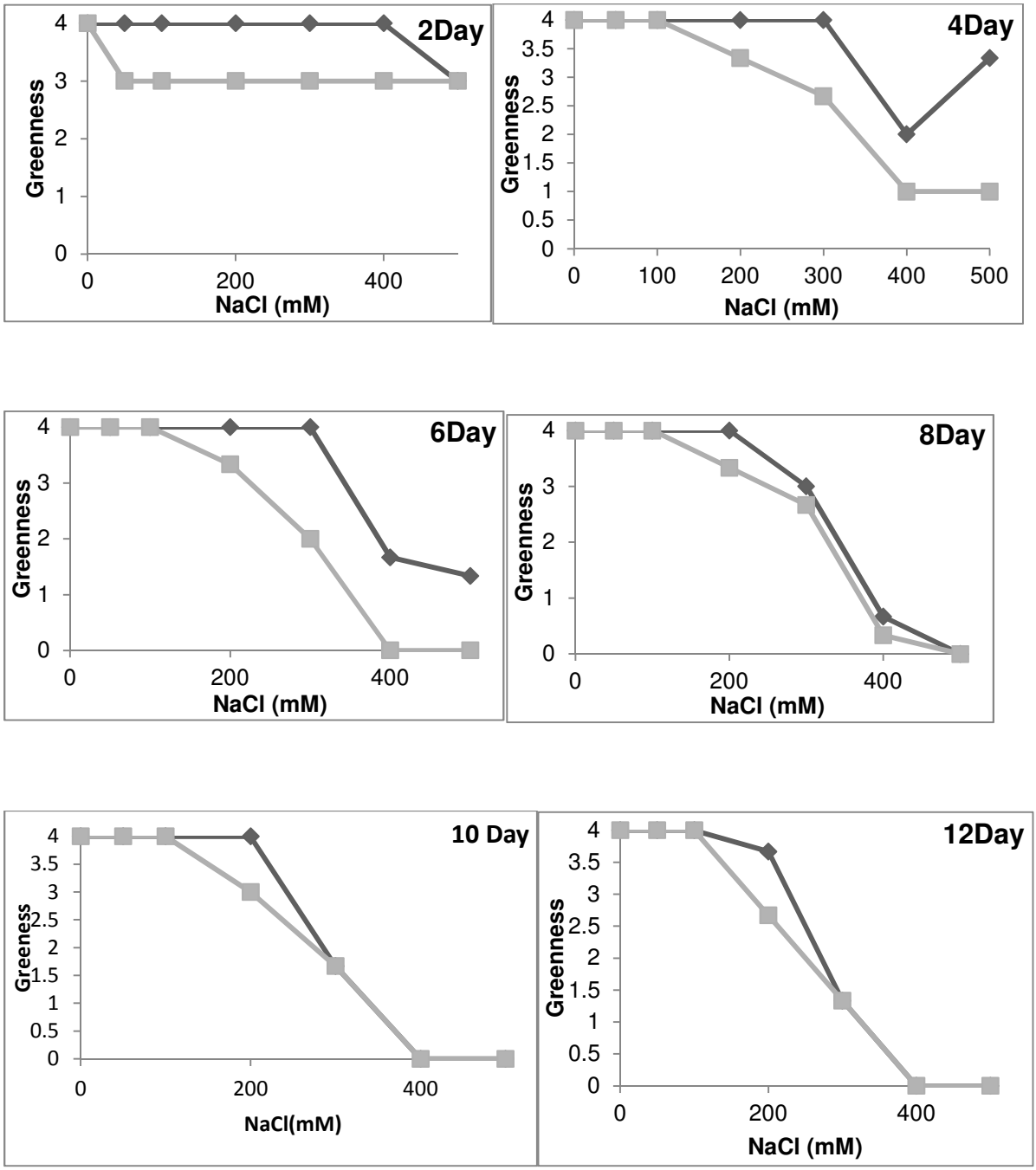


Figure 5-6. Response of *in vitro & in vivo* plants at different salt concentrations. The genotype response of *in-vitro & in-vivo* plants to NaCl after 2, 4, 6, 8, 10 and 12 days of treatments. There are highly significant differences between all treatments (p value=<0.001). *In-vitro* = grey line; *In-vivo* = black line.

5.8.4 Assessment of transformants for drought resistance

Results showed no differences in leaf disc greenness of plants after treatment with different mannitol concentrations (0, 100, 300, and 500 mM). The transformants maintained in all mannitol concentrations were the same as control plants indicating drought resistance. The plants showed similar response to *in-vivo & in-vitro* plants with increasing mannitol concentration. The data analysis showed that even at the highest concentration of mannitol (500 mM) all transformants maintained some greenness but so did the control (C). It can be concluded that selections were equally resistant to dehydration stress provided by mannitol compared to control plant.

5.9 Discussion

The results clearly demonstrated different abiotic stress resistance in transformants of cauliflower compared to control plants. This confirmed the persistence of the resistance over long term storage and repeated sub-culturing of the clones. In addition, the *in-vivo* forms of these transformants related positively with *in-vitro* screening of resistance, indicating a stability of the transformation after regeneration. The transformants used in this investigation had been created by inserting the APX gene and the results showed that this technique is successful in producing lines with improved resistance. This suggests that the new gene could be used in plant breeding programs for *Brassica oleracea var botrytis* (Suprasanna et al., 2009).

The simple leaf disc assay refined in this investigation was found to successfully differentiate the control and transformants clones for frost and salt stress resistance and the selection process used in this investigation clearly show that this type of selection in cauliflower is a very useful way to generate abiotic stress resistant genotypes as seen in other *Brassica* species (Ashraf, 2004, Ashraf et al., 1986, Fuller et al., 2006a).

The Electrical Conductivity Test (EC) was confirmed as a useful test and cold acclimation effectively reduced the EC and indicated an increase in frost resistance, confirming previous findings (Fuller et al., 2006a, Zhu et al., 2007, Thomashow, 1999b).

In all tests there was no significant leakage of electrolytes from leaf discs treated at -2 ℃, but there was a sharp increase in leakage at -4 and -6 ℃. This suggests *B. oleracea* is constitutively resistant to -2 ℃. The transformants showed significant variation in EC values at both -4 ℃ and -6 ℃. Cold acclimation at 4 ℃ with 8 h photoperiod for 14 d appeared sufficient to reduce leakage of electrolytes and activate cold acclimation responses. Low EC% due to no or less leakage of electrolytes is described in term of the stability of cell membranes. An increased leakage of electrolytes reflects the damage to cell membranes (Maheswari et al., 1999, Srinivasan et al., 1996). Guy,(2003) found that the ability of higher plants to acclimate and tolerate freezing stress is a complex quantitative trait.

It was assumed that the electrical conductivity of the leachate was directly proportional to the extent of damage of leaf discs caused by low temperature. There was a wide range of EC values among low temperature treated leaf discs of both unacclimated and acclimated genotypes. This is due to variations in leaf discs thickness and lack of complete homogeneity of the discs, and necessitated the calculation of relative electrical conductivity. Even with the use of relative conductivity, there is variation and therefore replication and randomisation are important in experimental designs.

Generally cauliflower can withstand light frost (-4 ℃) and the results showed that there are possibilities to increase frost resistance in *Brassica oleracea* by exposure to low temperatures (cold acclimation). Genetic diversity also exists in genotype of *B. oleracea* after acclimation (Fuller et al., 1989). The variation in frost resistance and low temperature induced acclimation may be used for selection and breeding programmes for frost prone areas. The results clearly showed differentiation in transformants for salt resistance and this difference was very prominent after 8 days of salt treatment. All of the transformants showed higher resistance compared to the control plants. These results confirm the previous findings of (Fuller et al., 2006a) who reported 80% damage for control populations and a significant degree of resistance with less than 50% damage for selected populations. Kingsbury et al.,(1984) reported that sensitive species were more damaged by salt stress than resistant ones due to reduced photosynthesis and a greater osmotic shock. Munns et al., (2002) also observed that salinity reduces the ability of plants to take up water. Salt stress leads to both an osmotic stress which can be like freezing stress but also to Sodium (Na) poisoning as Potassium (K) channels cannot distinguish between Na and K and excess Na uptake is toxic. A technique used previously by (Fuller et al., 2006a) for salt stress resistance screening of clones was followed in the present study and a similar response was found that confirms the effectiveness of the technique. The screening technique effectively distinguished the clones in term of greenness scoring of leaf discs floated in a saline liquid medium. Leaf discs of control clones lost their greenness resulting on a bleaching effect under salt stress. It might be suggested that leaf discs of control clones lost chlorophyll as a result of salt stress injury or that the plasma lemma is damaged and the cell contents leak out and the cell dies. Gibon et al.,(2000) hypothesised that the loss of chlorophyll was a

result of stress induced senescence and (Huang and Redmann, 1995) proposed the death of leaves due to the build-up of sodium in tissues which would prevent the supply of other nutrients to leaves leading to the death of tissues.

Different selection methods in Brassicas have been used for salt tolerance by using different concentration of NaCl - e.g. (1991) performed *in-vitro* selection for salt tolerance in *Brassica juncea* using cotyledon explants and in callus and cell suspension cultures in Petri dishes containing M&S agar media supplemented with different concentrations of NaCl. The clones found to exhibit salt resistance were suggested by (Parida and Das, 2005) to have some osmo-protective or specific ion toxicity resistance mechanisms. Osmo-protective mechanisms for salt resistance depends upon the genetic make-up of plants (Moghaieb et al., 2004) and specific ion toxicities depend upon adaptation to sodium toxicity (Kingsbury and Epstein, 1986).

Fuller et al.,(2006a) considered that *in-vivo* grown cauliflower plants having the damage of greenness of leaf discs less than 50% to show a significant degree of resistance. Following this criterion at day 8 of NaCl treatment, the *in-vivo* transformed plants with the APX gene showed less than 50% loss of colour and therefore showed salt resistance, while others showed a colour change of 50% or more and were classified as sensitive to NaCl.

Mannitol is an important photosynthetic product in higher plants and some algae, which can enhance tolerance to dehydration stress mainly through osmotic adjustment (Loescher et al., 1992) and many crop genotypes have been screened for drought resistance using mannitol induced drought e.g. *in-vitro* screening of Prunus accessions (Rajashekar et al., 1995), legumes (Grzesiak et al., 1996), and sugar beet (Sadeghian and Yavari, 2004).

The results of the drought resistance tests have however demonstrated no variation in greenness at different concentrations of mannitol, with a similar response evident in each clone at each mannitol concentration. Some transformants showed the same even in the presence of high 500 mM mannitol in the media with no symptoms of necrosis. Chandler and Thorpe,(1987) also reported similarly that up to 400 mM mannitol was not toxic in the screening medium and all unselected explants remained green and healthy. The present findings confirmed that the use of mannitol with *B. oleracea* is not a suitable stressor for induced drought stress resistance screening of cauliflower leaf discs.

Osmotic adjustment in plants under stress has been reported in *Brassica* species (Chandler and Thorpe, 1987, Kumar et al., 1984), in sorghum (Blum et al., 1989) and in wheat (Moinuddin et al., 2005). Cell wall elasticity may also be the cause for variable greenness (Kumar and Elston, 1992) and both osmotic adjustment and cell wall elasticity might have adaptive mechanisms to drought stress.

Osmotic effects are similar in frost, salt (NaCl) and drought (Mannitol) stress but specific ion toxicities are specific to salt stress. Specific ion toxicities are due to sodium and chlorine accumulation in a tissue to damaging levels and damage is visible as a foliar chlorosis and necrosis (Ferguson and Grattan, 2005). Leaf discs greenness scoring for salt resistance was based on the size of foliar chlorosis and necrosis, the damage specific to salt stress resulting poor or no relationship to frost or drought stress, as in frost stress electrolytes leakage (EC%) and in drought stress greenness was measured. In the present work, improved resistance to salinity and sub-zero temperatures was demonstrated but not drought.

5.9.1 Effect of salinity treatment on transgenic cauliflower plants

In general, greenness data of non-transgenic and transgenic plants indicated that shoot greenness was less in salt treatments compared to the control treatment. Shoot greenness was reduced quicker in the control plants than transgenic plants under salinity treatments. The transgenic plants under the highest value of salinity treatment (400 mM and 500 mM) recorded greater resistance than controls.

From these results it is clear that transgenic plants carrying the APX gene which is necessary to protect plants from oxidative damage due to abiotic stresses, are better able to cope with high abiotic stress which agrees with that of (Shigeoka et al., 2002). The constitutively expressed ascorbate peroxidase enzyme in transgenic plants allowed them to develop more tolerant to salinity, treatment than non-transgenic plants.

5.10 Conclusions

The physiological analysis of *in-vivo* and *in-vitro* clones (transformants and control) for frost, drought and salt stress resistance indicated that the transformations were expressed after many clonal generations and the transformants maintained higher resistance compared with control plants.

6 Chapter Six: General Discussion

The improvement of agricultural production and productivity as well as the future adaptability of agricultural production are dependent on the rational application of both established and new technologies. We stand at the combination of an incredible collection of new technologies, such as recombinant DNA technology, information technology and high-throughput genomics, to enhance our understanding of the structure and function of plant genomes with the ability to apply this information for plant improvement. Products arising from modern biotechnology including GM or transgenic crops are providing new opportunities to achieve sustainable productivity gains in agriculture.

6.1 Optimisation of plant tissue culture

The work reported in this thesis was initially challenged to develop an efficient mass micropropagation technique for use with a transformation protocol. This was optimised using a simple and efficient multiplication system manipulating the meristematic potential of curd. Clonal micropropagation of cauliflower is already widely used for maintaining and propagating elite germplasm in plant breeding programmes for example to maintain male sterile or di-haploid lines in F1 breeding, but these only use a very small fraction of the potential micropropagation potential of the cauliflower curd. The objective of this work was therefore to promote direct development of the many pre-existing meristems on the curd, with the final goal of the production of one propagule per explant. Since a cauliflower curd has over 1 million meristematic domes on the curd (Kieffer et al., 1998) the challenge was to release this potential into tissue culture. This required the use of blending and sieving techniques and whilst this crude homogenisation damages a high proportion of meristems it was still capable of releasing many thousands of micro-explants to the tissue culture process. It was then necessary to stimulate their development into

shoots and retain totipotency. Plant morphogenesis is limited by many factors present in the medium but given a balanced nutrition, plant regulators are often found to be key to optimize shoot regeneration from explants. A common feature of the procedures used in many plant species has been the use of high cytokinin and low auxin concentration in the media to achieve adventitious shoot regeneration (de Fossard et al., 1974, Özcan et al., 1993) and for root development of plants a high level of auxin and low cytokinin are used(Dello Ioio et al., 2007). Tissue de-differentiation is also possible in tissue culture and callus cultures are often preferred in many subsequent transformation protocols. Plant tissue culture has generally shown that 2, 4-D was the best hormone to use for callus initiation and maintenance and (Franco et al., 1990) proposed that 2, 4-D was required for inducing cell division and formation *in-vitro* and it has been commonly used for many species (Murata and Orton, 1987, Fuller and M., 1995). There is however a challenge that follows on from this and that is the re-differentiation and formation of shoots from callus. Murata and Orton, (1987) believed that the inability of callus to regenerate shoots might be a result of increase in somatic age or the concentration of genetic abnormalities that accompany callus growth and frequent sub-cultures. Callus cultures also bring a risk to any plant improvement system in that due to mass uncontrolled cell division, somatic mutations can arise which can have morphological or physiological consequences for elite cultivar production (Loeb, 2001). The direct transformation of meristems or meristem cultures is attractive from the point of view of reducing the risk of the somatic mutations associated with callus cultures.

The procedure here produced thousands of cauliflower propagules from one curd at a low unit cost especially in comparison with the cost of micropropagated plant and seed derived plantlets (Kieffer, 2001). Therefore the first objective was achieved and

it was necessary next to assess the regeneration system for its capacity to assist in an improved gene transfer system for cauliflower, so that a sufficient number of shoots could be produced to allow the necessary level of replication in and transformation and regeneration experiments.

Curd micro-explants would appear to be a useful material to test Agrobacterium delivery of transgenic plants firstly because of the efficiency of the procedure releasing a large number of micro-explants which can be treated in Agrobacterium co-cultivation and secondly because their capacity for shoot regeneration is high. Consequently the chance to regenerate plants arising from a rare event such as stable transgene integration is considerably increased.

The study of the factors limiting *in-vitro* shoot regeneration from curd tissues highlighted that meristems are not determined to produce flowers and this supports molecular evidence (Smyth, 1995). Their organogenic properties appeared to be under several levels of control: curd genetic background (summer heading varieties were less responsive than winter varieties) and physiological status (young curds were more responsive than older ones), explant physiological and physical parameters (size), and the *in-vitro* culture environment. The key limiting factors when using material in the optimal physiological state were nutrient supply, plant growth regulators and explant size. Optimisation of these parameters reduced the influence of the others and dramatically simplified the culture system. The refined protocol for mass production of cauliflower propagules was hereafter developed as a standard operating procedure (SOP). The SOP is based on semi-mechanical explant production using a homogenisation treatment for production of pieces of curd of optimum size 0.2 - 0.3 mm in diameter. For a single curd over 17,000 explants can be produced. Their culture is then carried out in a liquid culture medium at an

adjusted culture density (74 µL of micro-explants in 30 mL liquid culture) to optimise nutrient supply. For winter heading cauliflower at the optimal explant size-class at least 10,000 propagules can be produced per curd. However, for these genotypes increasing the explant size to 0.3 to 0.9 mm improved shoots regeneration capacity because each micro-explant carried more than one meristem. Propagule production took 40-50 days from culture initiation to strong weaned plantlets at a low unit cost. However plant strength, homogeneity and genetic stability have yet to be fully characterised and homogeneity of strength is the most difficult factor to control as it seems to be essentially controlled by the variability in the size of the initial meristems on the curd.

The problem of clonal multiplication of the genotype being the only option would be solved by the use of the protocol for mass production of micro-propagules presented. The protocols described here are at different stages of their development and aim to become new tools for cauliflower breeders, they highlight only some of the multiple potentialities of curd meristematic tissues for *in-vitro* manipulation.

6.2 Transformation protocol for cauliflower

6.2.1 Can cauliflower be transformed with the curd protocol?

Development of an efficient gene transfer system largely depends on a rapid and reliable *in-vitro* regeneration system for the desired plant species. The preceding discussion established that a simple methodology was refined allowing the production of tens thousands of micro-explants capable of regenerating shoots, from curd meristematic tissue which would allow the necessary level of replication for transformation. This was subsequently used in optimizing the transformation protocol.

Agrobacterium-mediated transformation comprises the interaction between two biological systems and is affected by numerous physical and physiological

conditions (Bhalla and Smith, 1998). Initially this transformation technique did not work because of bacterial overgrowth and for the curd meristem culture high levels of contamination meant that no meristem was able to survive the infection with *Agrobacterium* during the initial study. Later however, when the contamination problem was overcome by optimization and the conditions of transformation were established some transformant lines were produced. Previously, most successful reports of transformation in Brassicas were published using hypocotyl and cotyledonary explants (Chakrabarty et al., 2002, Prem, 1998) and this applied also to cauliflower (Leroy et al., 2000). Generally, the results reported here confirmed earlier reports that lowering bacterial density (Srivastava et al., 1988a, Henzi et al., 2000) and preculture of explant on callus inducing medium (Sangwan et al., 1992) support improved transformation frequency. The control of *Agrobacterium* overgrowth or *Agrobacterium* elimination using Carb or Cef was found to be the main factor controlling this aspect. Both Carb and Cef prevented *Agrobacterium* overgrowth without being phytotoxic to the cauliflower explants. Mathias and Boyd, (1986) suggested Cef binds with the penicillin binding proteins of the inner membrane of the bacterial envelope that are essential for cell division. However Cef is also metabolized to an unknown compound with growth regulator activity which can influence plant culture development (Borrelli et al., 1992). Carb breakdown products may also act as growth regulators, therefore regulating definitive tissue culture response (Lin et al., 1995). There was no evidence however of any growth regulator-like response with cauliflower micro-explants. Another possible mechanism for these antibiotics is that they cause DNA hyper methylation more readily in prokaryotic cells (the *Agrobacterium*) than in eukaryotic cells (the plant cells). Whatever the

mechanism, it meant that these antibiotics can be used to eliminate *Agrobacterium* without serious phytotoxicity in cauliflower as in other plant species.

Optimization and identification of the factors affecting T-DNA delivery in the recalcitrant species of cauliflower were needed. A reduced rate of transformation was affected by inoculation with a high volume of explants which is in contrast to many other transformation protocols (Hansen and Wright, 1999). It was very important to reduce the concentration of bacteria with an explant age of 10 days which is commonly recommended for transformation experiments (Punja Z.K, 2001). Furthermore a short pre-incubation period increased the transformation rate and appeared to support the ability of the explants to overcome the stress resulting from the co-cultivation with *Agrobacterium* (Punja Z.K, 2001). A co-incubation time of 2 days was the most effective in cauliflower transformation and 4 days co-cultivation reduced transformation rate. The introduction of too many bacteria has been reported to induce a hypersensitive response (Heath, 2000) and that was also the case with cauliflower micro-explants. Long periods of incubation tend to lead to death of bacteria and release of lipopolysaccharide components of the cell wall that block transgenosis (Whatley et al., 1976). There was data in support of this with cultivars of cauliflower clearly demonstrating a hypersensitive response when exposed in excess of 10 days co-incubation. These results were also confirmed in tobacco tissue culture which showed that the effect only occurred with live *A. tumefaciens* , dead *A. tumefaciens* did not show the hypersensitive response (Matthysse et al., 1981).

Gene transfer from *Agrobacterium* to the host plant cells appears to be dependent on the bacterial virulence mediated by the virulence (vir) gene coded on the Ti plasmid and organized in six operons (Hooykaas and Beijersbergen, 1994). It has

been found that the vir genes are inducible by the phenolic compound acetosyringone and leads to improved transformation frequency (Shimoda et al., 1990). Although addition of acetosyringone to the bacterial culture medium has been reported to be not beneficial, its presence during the co-culture stage was found to improve transformation efficiency (Henzi et al., 2000). With cauliflower microshoots it was found that the use of 80 µM acetosyringone improved transformation rate when added at the co-culture stage.

The confirmation that DNA has been transferred and integrated into the cauliflower genome became the next challenge in the transformation protocol. Histochemical, fluorometric and leaf disc assays of Gus as well as molecular analysis were all investigated. Enzymatic assays based on GUS expression were expensive procedures mostly due to the cost of X-Gluc and also proved to be time consuming. PCR is a fast and sensitive DNA method, but it is also expensive and susceptible to cross-contamination and requires optimization, but this method can be used routinely to evaluate insert sequences constructed into vectors in individual bacteria colonies and substantially reduces time and effort required to evaluate the authenticity of *Agrobacterium* binary vectors. The sensitivity of PCR was improved by increasing the annealing temperature and by reducing PCR cycles when non-specific amplification was a problem. On the other hand the advantages of both of GUS assay and leaf disc assay used in this research is they are rapid, require minimal use of chemicals and plant tissues, and histochemical analysis has been used to determine the localization of gene activity in cells and tissues of transformed plants.

The presence of selectable marker genes, especially those which include genes coding for antibiotic resistance such as kanamycin or Hygromycin are the simplest method for the initial selection of transgenic plants and these were used successfully

here. Whilst these antibiotic selectable genes are very useful and convenient to use in the transgenosis process the genes are not required once the transgenic plants are regenerated and the genetic analyses completed. The presence of a particular marker gene in a transgenic plant necessarily prevents the use of the same marker in following transformation and the use of a different marker system is required for each transformation round or event. Thus, any technique that can remove or eliminate a selection marker gene in transgenic crops is highly desirable if for no other reason than that the same procedure can be used in subsequent transformations. For transgene technology to be commercially successful, multiple independent transgenes available need to be added into existing sequences.

The presence of antibiotic genes in transgenic crop varieties has been criticised as a real threat to the adoption of GM crops with a fear that the increase in the frequency of antibiotic genes in the environment threatens the long term use of certain antibiotics (Houndt and Ochman, 2000). However this has been addressed in a wide ranging investigation and the conclusion was that this was not a serious threat of the widespread adoption of the process (Chapotin and Wolt, 2007).

At present there is no commercialization of marker-free transgenic crop varieties as this is still in the stage of proof-of-concept (Manimaran et al., 2011). However the development of marker-free transgenic would further strengthen crop improvement programmes with prevalent applications in fundamental research and could help lead to the wider acceptance of GM crops and a positive contribution to food security.

Another subject of concern relates to the fact that transgenes integrate at random positions in the genome leading to possible unwanted side effects (mutation) if the gene is integrated into an existing gene sequence leading to unpredictable

expression patterns. However, much of the genome of a crop plant is made up of non-coding sequences and the likelihood of this occurring is rather small. In practical plant breeding terms, such aberrant lines would simply be culled in the subsequent breeding programme.

6.3 Abiotic stress improvements in cauliflower by transformation

There is an extremely large body of literature concerning plant abiotic stress resistance and to improve resistance various methods have been put forward, but to date only a limited number of techniques have shown any enduring success. Some successes have been recorded using traditional plant breeding methods in cereals and other crop species (Gengenbach, 1984 , Maliga, 1984) but there are only a very limited number of successes through genetic modification (Cardoza and Stewart, 2003b, Cao and Earle, 2003b, Cheng et al., 2003b). Moreover in genetic modification there are only a limited number of researchers working with crop species especially in Europe where the anti-GM campaigns have led to a number of scientists moving away from GM research. Most research work using transformation is being carried out in the model species *Arabidopsis* mostly in order to understand gene expression (Caspar, 1991, Rosso et al., 2003).

In the current study transformation success was demonstrated using commercial cauliflower varieties and it was confirmed that this is one method of producing new lines with improved stress resistance. The technique succeeded in achieving its goal to produce GM cauliflower lines tolerant to abiotic stress through targeted gene supplementation by *Agrobacterium* mediated transformation delivering constitutive APX expression. *Agrobacterium* mediated transformation proved to be rapid and simple in comparison with other breeding procedures. It required minimal use of

chemicals and plant tissue culture, is low cost, causes no damage or contamination to the explants and maintained abiotic stress resistance over a 2-3 year period.

Some limitations can be overcome by techniques use in tissue culture in the application of transformation and DNA transfer techniques. The combination of *in-vitro* culture with transformation can speed up breeding programmes from the generation of variability, through selection to the multiplication of the desired genotypes. The presented study clearly confirmed that a short selection procedure with relatively high selection pressure can produce beneficial stable transformants very quickly. This kind of selection confirms that GM transformation is useful in creating genotypes resistant to abiotic stress (Ashraf, 2004).

6.4 Transformant line screening under abiotic stress (salt and frost)

The screening results of cauliflower lines under high NaCl concentrations using an in-vitro leaf disc resistance assay provided advantages over the study of whole plants. The assay took place over a short time period, under well-controlled experimental conditions and provided excellent repeatability. Ait Barka and Audran, (1997) and Vijayan et al.,(2003) reported similar techniques as powerful tools for studying plant responses to different abiotic and biotic stress.

Some transformants lines were resistance to salt and/or frost (Zhu, 2000) showed that many genes that are essential for salt tolerance may not actually be induced by salt stress. The molecular evidence has to be supported by physiological evidence for many genes that are involved in the resistance to abiotic stress (Zhu et al., 1997, Pearce, 1999, Guy, 2003, Vinocur and Altman, 2005).

These gene products are involved not only in the protection of cells against stress, but moreover in the regulation of other gene expression in the abiotic stress

response. Thus most essential metabolic enzymes synthesis variant isoforms which can operate under stress conditions (Wong, 1995).

Stress resistance metabolism in plants and the precise role of increased compatible solutes for the protection of cells during dehydrative stresses still remains a gap in our knowledge (Vinocur and Altman, 2005) and research efforts are being matched to study this in model species (Meinke et al., 1998). For crop species the results of such efforts will then need to be corroborated and the presence of the range of transformants described in the research work reported here will greatly support future corroborative studies. In the meantime, agronomic evaluation of resistant transformant lines described here needs to be carried out to determine the stability of the transformants traits *in-vivo*. However, most of the transformants lines confirmed good resistance to stress resistance to both salt and frost demonstrating cross-talk between these stresses. Many previous studies have also shown increased resistance to abiotic stress such as salt and freezing (Riccardi et al., 1983, Ashraf, 2004, Ashraf and Wu, 1994, Kueh and Bright, 1981, Dix et al., 1984, Tantau et al., 2004) but very few of these have led to the introduction of resistance varieties in commercial production.

A reliable transformation system in the present study provides for integration of APX gene into the cauliflower genome and the introduced gene provides an opportunity to improve agronomic traits such as abiotic resistance (salt, frost and drought).

In this study putative transgenic cauliflower plants and non-transformed plants were tested under NaCl stress. Measurement of fresh shoot indicated that transformed plants were more tolerant to salt stress compared with non-transformed plants under salt stress treatment. The stress resistance in this result indicated strongly that in transgenic plants was caused by APX gene expression. Raised level of APX enzyme

has been correlated with increased levels of oxidative stress resistance in several cases (Jahnke et al., 1991). In other plant species it has been previously shown that over expression of antioxidant genes provides enhanced resistance to oxidative stress (Allen et al., 1997, Gueta-Dahan et al., 1997, Payton et al., 2001, Roxas et al., 2000). The results support these previous reports and show that expression of the APX gene in cauliflower confers increased resistance to oxidative damage caused by exposure to salt. The suggestion of data cited in literature that antioxidant enzyme, such as APX possibly will serve as good physiological and molecular markers in marker assisted breeding programs aimed at increasing resistance to environmental stress.

Finally, this study confirmed that the transformation was efficient, gene stable, low cost, and gave no contamination or damage to explants and provides a low risk method for the improvement of abiotic stress resistance. This result suggests that transformation methodology should be used to improve abiotic stress resistance in cauliflower.

6.5 Conclusion

This study has enabled the development of improved and new plant tissue culture protocols for cauliflower using curd meristematic tissues due to a better understanding of both *in-vivo* and *in-vitro* biology. The research showed that a `marketable' curd can carry millions of meristems highlighting the potential of this tissue for micropropagation. This better understanding of curd structure gave new insight into phenomena occurring *in-vitro* and enabled the design of new approaches to optimise the micropropagation system. Furthermore this contribution to the understanding of curd biology should help to clarify the genetic determinism of curd production (Anthony et al., 1995, Kempin et al., 1995, Smyth, 1995) by enabling precise comparison of transformants with the normal cauliflower phenotype studied here.

6.6 Limiting factors of micropropagation

6.6.1 Curd explants transformation via *Agrobacterium tumefaciens,*

The main question modeled was; is it possible or not to transform cauliflower? It is clearly demonstrated here that it is possible to transform curd explants with *Agrobacterium tumefaciens* strains harbouring the co-integrated Ti-based vectors $P^{CIN+ARS}$ carrying the APX gene.

The shoot regeneration and elimination of *Agrobacterium tumefaciens* by using Cefotaxime and Carbenicillin confirmed that the best concentration was 250 mg L^{-1} to improve the regeneration frequency and efficiency and at the same time suppress *Agrobacterium* overgrowth growth during selection procedures.

Optimal conditions for transformation of curd explants were: density of bacteria culture 1:10 dilution (OD_{600} = 0.6); explant age (10 days); 2 days of per-culture 2 days co-cultivation; 80 µM acetosyringone. The frequency of transformation was still low (5%) but the protocol was successful to mobilize APX stress gene into cauliflower.

Several methods could be used to improve this further e.g.:

i. By manipulating curd physiology using phytohormone treatment (cytokinin) in the growth phase (Fujime, 1983) before explant isolation.
ii. By creating a phase of meristem multiplication *in-vitro* to allow selection of improved strength of meristems before multiplying them using the protocol (Ziv, 1991).
iii. Modification of the phytohormone balance in favour of auxin to reduce the occurrence of neoformed shoots, and thereby limit phenotypic variability.

In the present state, the protocol is very competitive with published cauliflower micropropagation protocols (Crisp and Walkey, 1974, Kumar A, 1993) and its low

cost should lead to the improvement of micropropagation techniques for multiplication of hybrid F1 parents and for transformation protocols.

Specifically this regeneration system seems to open new perspectives in terms of transformation as originally postulated by (Deane et al., 1995) and genetic transformation techniques such as *Agrobacterium* mediated transformation *in planta* reported by (Bechtold, 1993).

6.7 Improved abiotic stresses after transformation

Transformation provides a promising approach to improved abiotic stress resistance in cauliflower compared with traditional plant breeding approaches. In this research the transformation approach was successful in producing plants with improved stress resistance.

The *Agrobacterium* mediated transformation approach has an advantage compared with the general breeding approach as it is rapid, simple, requires minimal use of chemicals and plant tissues, is low cost, requires less time, and produces stable products (retained abiotic stress resistance over 2-3 year period was demonstrated) and caused no contamination or damage to the explants.

To evaluate insert sequence constructed into vectors harbored in individual bacterial colonies through conduct of molecular analysis of *Agrobacterium* plasmids using PCR can be considered as a routine method.

The results of APX gene constructs with transformation showed that the transient GUS assay method is also an easy reliable method of establishing optimal conditions of transformation.

The screening of cauliflower, using leaf discs, is an easy and efficient method to identify salt adapted genotypes within a limited space and time period. It is

concluded that raised NaCI is not essential for improved resistance to abiotic stress in cauliflower, but where it does occur it does improve resistance.

Transformant lines also demonstrated improved frost stress under non-acclimated conditions and this difference increased when plants were acclimated. Some of the transformant lines proved to be resistance to both salt and frost and this cross-resistance suggest a common resistance mechanism, which requires further investigation.

In the current study it was presented that the resistance to salt is stable in transformants lines during many *in-vitro* subcultures. Efekodo,(2004) has proved similar work and these differences were heritable and expressed in the F1 population of a mass pollinated cross of selected lines.

In this study a leaf disc assay as a method to differentiate between transgenic and non-transgenic plants and showed the advantage as being simple, rapid and requiring minimal use of chemicals and plant tissues that cause no permanent damage to the plant.

It has been shown here that it is possible to transform meristematic cells on cauliflower, as has been shown for oilseed *Brassica campestris* (Mukhopadhyay et al., 1992), sugarcane (Elliott et al., 1998) and Arabidopsis (Sessions et al., 1999). However, due to the very low efficiency of stable transformation, it is not expected that all the cells of a meristematic dome could be transformed and this is particularly relevant for curd meristematic domes which are of an unusually large size (Medford et al., 1991). Combining the powerful capacity of plant regeneration of curd micro-explants with an improved gene transfer via *Agrobacterium*, followed by careful selection, is a very promising protocol which will be able to overcome the problem of low rates of stable transformation.

The expression of high levels of ascorbate peroxidase isozymes in transgenic plants is hypothesized to protect cells from oxidative damage due to abiotic stress through increased activity of the antioxidants system. These plants however still require assessment by measurement of ascorbate peroxidase activity either using a gel assay method (Chen and and Pan, 1996) or spectrophotometer method (Nakano and Asada, 1981).

6.8 The research Overview

The outputs of this 3 year research project were successful in confirming transformation as a possible technology for the genetic manipulation of cauliflower but it cannot be claimed to be the most efficient or effective. It could be that other constructs prove to be more effective and/or reliable in future studies. This thesis illustrates more that these technique can be effectively applied to this recalcitrant species. Thus the thesis does not claim to be exhaustive study but in more a proof of concept.

6.9 Limitations of the research and future work

The finding from the present study is not the answer for the whole mechanism of stress resistance in cauliflower. This investigation provides a base for further research to explore the molecular mechanism and the gene expression in cauliflower. Due to limitation of time and finance, drought hardiness was not exhaustively explored in this project but the techniques can open the door for further research to use new techniques for drought resistance improvement in cauliflower.

Dehydration stress resistance is a complex mechanism and gene is response to dehydration stresses (such as frost, drought and salt stress) and more research are required to explore and understand the resistance mechanism to enhance resistance

in cauliflower. In the present study a number of transformants were screened physiologically for frost, drought and salt stress resistance but not for molecular gene expression because of limited time. These transformants along with physiological resistance showed variable resistance to frost and salt stress, further research to explore the molecular mechanisms of these stress resistances is recommended with these transformed plants.

The mechanisms of salt and frost resistance are very complex and are clearly quantitative traits controlled by several factors, and more investigation is needed to understand and to confirm the mechanism(s) of resistance and which factor is the main factor in resistance. Also more investigations are necessary to optimize further the transformation protocol for curd explants and improve the transformation rate.

Due to the limitation of time, funds and facilities (i.e. no GM greenhouse) the transgenic plants were not cultured under *in-vivo* conditions and it is necessary to transfer the transgenic plants to *in-vivo* conditions to evaluate their agronomic characters under abiotic stress and to assess their gene stability and to ensure no gene escape possibilities.

The higher ambition for this project was to try to address the mechanism of cold acclimation and development of freezing tolerance, salt tolerance and drought tolerance of novel crop varieties to allow the use of marginal lands for agricultural production. Whilst this higher ambition cannot be realised within a single PhD study, it has provided techniques which brings science one step closer to achieving this goal. Physiology, molecular biology and biochemistry through further intensive efforts are required to understand the complex quantitative traits of abiotic stress resistance (Mantas et al., 2010a). The major challenge to agriculture in the coming years will be a shortage of food production for the increasing population of the world (Jones, 2011)

and the development of crops with increased environmental stress tolerance will greatly help in this regard. Further achievements and investigations in this area will not only be exciting and a deep scientific achievement, but will greatly aid efforts in agriculture to continue providing food to feed the world (Thomashow, 2001).

7. Chapter Seven: Appendices

Appendix; 1(GM1)

UNIVERSITY OF PLYMOUTH
UOP-GM1

Genetic Modification
Registration of Authorized Workers

Surname	**Forenames**	**Title**
Al-Swedi	Fadil	Mr.

Qualifications (e.g. B.Sc., Ph.D.	**Present post or grade**
BSc	Research student

Faculty/School/Department

Biomedical and Biological Sciences

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RM 404 B Portland Square
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Fadil.al-swedi@plymouth.ac.uk

Visitor? Yes/No **Normal Place of work (Visitors only)**

No

Period of visit (Dates)

Date of commencement of GM work	**GM Reference Numbers(s)**
	GM 06/2010

**Give the local reference numbers of the projects on which you will be working.*

Containment level for which authorisation is sought (circle): (1) 2 3 4

I agree to abide local genetic modification safety rules. I undertake to notify the Genetic Modification Safety Officer (GMSO) of any changes to the information presented above.

Signed _____ Date _____

Reference Number (to be entered by GMSO)
(Proposal N^{o} / Calendar year)

UNIVERSITY OF PLYMOUTH

UOP- GM2 *(revised February 2008)*

INITIAL GENETIC MODIFICATION RISK ASSESSMENT

Proposal to carry out activities involving genetically modified organisms

- Please return completed to the University's Genetic Modification Safety Officer.
- Please complete the form in typescript.
- *"Guidance"* refers to the ACGM Compendium, which can be viewed on the WWW at http://www.hse.gov.uk/hthdir/noframes/acgmcomp/acgmcomp.htm;

1. Title of proposed project

Genetic Modification of Cauliflower For Improved A biotic Stress Resistance

2. Genetic Construct(s)

Please specify each host/vector/insert combination

• *Host*	• *Vector*	• *Insert*	*Guidance*
Cauliflower	Agrobacterium tumefaciens	1- APX -Ascorbate peroxidase. 2- SOD- Super oxidase dismutase.	2A, 2B

3. Summary of intended work

Give a brief summary of the aims of the project, and the procedures and materials to be used. **The biological functions of the insert(s) *must* be explained.**

The inserts aim to improve the anti-oxidant handling capacity of the host and enable it to resist and recover from a biotic stress more readily. Proof of concept has already been shows in other plant hosts.

4. Describe the validated waste treatment procedures, for both liquid and solid waste and ***procedures in case of spillage.***

Specify named disinfectants and state working concentrations.

Solid waste: *Autoclave at 121˚C*	Guidance: 3A (See also the Local Rules). Disinfection MUST be by validated means.
Liquid waste*: Disinfectant*	
Disinfectant to be used, exposure time and working concentration: *1% Virkon*	

Source of validation data: (e.g. manufacturer's data or own studies)	

5. Does the work involve a recipient organism that is accepted as inherently safe? Examples: *E. coli* K12, Yeasts, Mammalian cells. YES / NO

6. Can the gene product(s) of the gene(s) being cloned be considered non-harmful? √ YES / NO

If you have answered YES to both the above questions, do you believe you have sufficient information at this stage to classify the project as Activity Class 1, as defined in the Contained Use Regulations 2000. If YES, sign Declaration A, complete the rest of this form and omit Form UOP-GM3. If NO, please complete Form UOP-GM3 as well and sign Declaration B.	YES / NO

- To make this classification you need to be confident that **under no circumstances will the GMM be likely to cause disease to humans, or cause harm to the environment.** √
- If you are sure that the proposal meets these criteria you can omit the detailed risk assessment (form **UOP-GM3**), sign **Declaration A** and return it to the Genetic Modification Safety Officer.
- **If using or creating a transgenic plant or animal you must complete UOP-GM3 Part 2 for environmental risk and state containment measures to be used.** √
- If you are uncertain whether the proposal meets the above criteria, you should proceed to complete, a detailed risk assessment (form **UOP-GM3**), and sign **Declaration B**.

DECLARATION SIGN AT "A" or "B"

• **A**	**This project meets the above criteria for classification as Activity Class 1 and is being submitted to the Genetic Modification Safety Committee on that basis, without further risk assessment.**
Signature of Proposer:	*Date*:

• ***B***	• ***Final Activity Class from form UOP-GM3:***
Signature of Proposer:	*Date:*

Name of proposer:	**Telephone Number**	**E-Mail**

Faculty / School / Department Address

Laboratories in which the work will take place:

Names and job titles of all workers on this project

Anticipated start date of project

This proposal has been considered by the GMSC of which I am the authorised representative and whose views are accurately set out below		
Name:	**Signed:**	**Date:**

GMSC Comments:

UNIVERSITY OF PLYMOUTH UOP-GM3

Part 1 - Genetically Modified Organisms - Risk Assessment for Human Health

<table>
<tr><td>1. Consider:

• hazards associated with the host / recipient / viral vector (“access & expression”)
• hazards from the inserted gene (“expression & damage”)
• hazards from the alteration of existing pathogenic traits
• could an otherwise non-harmful inserted sequence cause harm as a result of gene transfer to another organism?
Could the predicted GMO be hazardous to human health?
Guidance 2A (bacteria & cell cultures), 11-22 & Annex I, II, III, V 2B (human & animal viruses), 9-26 & Annex III, IV</td><td>If NO: √

Select containment level 1 at (2) below.

(Questions (3) and (4) do not apply)</td><td>If YES:

Explain the possible hazards to human health then go to (2) below.
•</td></tr>
<tr><td>2. Assignment of a provisional Class / containment level based on:
• the hazard classification of the host
• any identified hazards
• the severity of any harmful consequences should they occur
• estimated containment level to control the hazards
-Guidance 2A, 28-31 2B, 37-41</td><td colspan="2"><u>Comments:</u></td></tr>
<tr><td>3. How likely is it that the GMO could actually harm human health in the event of exposure? Consider:
• likelihood of rare events occurring e.g. recombination events
• ability of GMM to establish infection in vivo; assessment of the GMM’s “fitness”
-Guidance 2A, 23-27 2B, 27-36</td><td colspan="2"><u>Comments:</u></td></tr>
<tr><td>4. Considering the nature of the work, are any additional controls necessary to protect human health?
- Guidance 2A, 32-36, 2B, 42-47</td><td colspan="2">• YES / NO Additional controls (if applicable):</td></tr>
</table>

Part2 - Genetically Modified Organisms: Risk Assessment for Environmental Harm

1. Is the GMO able to survive, establish, disseminate and/or displace other organisms? *Guidance: 2A, 37-47*	**If NO: go to (2)** √	**If YES: go to (3)**
2. Can the gene product persist in the environment and cause harm, or is it possible for passive transfer of the gene to other organisms in which it might be expressed? *Guidance:2A, 46*	If NO: no further containment is needed to protect the environment. Go to (9). Gene product would not produce harm.	If YES: Escape of Vector(Agrobacterium) Could cause transmission of the gene to be transferred to other plants- Risk, but risk is negligible
3. Assess pathogenicity to animals and plants *Guidance: 2A, Annex IV*	Comments Non-pathogenic Agrobacterium strain	Likelihood: negligible Consequence: negligible Risk: efficiency zero
4. Assess potential for transfer of genetic material between MM and other organisms (e.g. presence of conjugative plasmids; consider locality) Guidance: 2A, Annex IV	Comments Agrobacterium selective medium used, will not support conjugative related bacteria.	Likelihood: negligible Consequence: negligible Risk: efficiency zero
5. Assess products of gene expression that could be toxic to other organisms (e.g. bio pesticide) Guidance:2A, Annex IV	Comments Product of gene construct is not toxic.	Likelihood: negligible Consequence: negligible Risk: efficiency zero
6. Assess any other identifiable negative effects on organisms Guidance: 2A, Annex IV	Comments Could influence stress tolerance of transformed plants increasing completiveness.	Likelihood: negligible Consequence: medium Risk: efficiency zero
7. Assess phenotypic and genetic stability (e.g. could genetic instability be hazardous?) Guidance: 2A, Annex IV	Comments Unlikely to affect genetic stability &not hazardous	Likelihood: negligible Consequence: negligible Risk: efficiency zero

8. Management of risk: is the containment level designed to protect human health adequate to protect the environment? Guidance: 2A, 55-56	Proposed containment procedures 1. Transformed plants exist only in tissue culture not in –vivo. 2. No transformed plants allowed to flower or shed pollen. 3. Transformed plants&vector contained in designated incubators&growth chambers under Lock&key. Class; with elevated security measures aligned to a class 2.
9. ASSIGNMENT OF FINAL ACTIVITY CLASS Guidance: 2A, 57-63	

Estimation of risk:

	Likelihood of hazard			
Consequence of hazard	***high***	***Medium***	***low***	***negligible***
severe	high	High	medium	effectively zero
medium	high	Medium	medium/low	effectively zero
low	medium/low	Low	low	effectively zero
negligible	effectively zero	effectively zero	effectively zero	effectively zero

Proposed Project
Genetic modification for improved stress resistance in cauliflower.

Proposer : Prof. Micheal Fuller
Mr Fadil Al-Swedi

22/02/2010

Contact Address RM B 404 Portland Square
School of Biomedical& Biological Sciences
University Of Plymouth

Reference Number (to be entered by GMSO) 06/2010
(Proposal N^{o} / Calendar year)

This proposal has been considered by the GMSC and signed by an authorised representative

Name:	**Signed:**	**Date:**
M.L. Gilpin	*M.L. Gilpin*	2/03/2010

GMSC Comments:

Appendix 2(M&S)

Ingredients of tissue culture medium developed in 1962 by Murashige and Skoog, which is still one of the most widely, used culture media today.

contents	mg L^{-1}
Ammonium Nitrate	1650
Boric Acid	6.2
Calcium chloride Anhydrous	332.2
Cobalt Chloride Hexahydrate	0.025
Super Sulphate Pent hydrate	0.025
Disodium EDTA Dihydrate	37.26
Ferrous Sulphate Hyptahydrate	27.8
Glycine(Free Base)	2.0
Magnesium Sulphate Hyptahydrate	180.7
Manganese Sulphate Monohydrate	16.9
Myo-Inositol	100.0
Nicotinic Acid(Free Acid)	0.5
Potassium Iodide	0.83
Potassium Nitrate	1900
Potassium Phosphate Monobasic	170
Pyridoxin Hydrochloride	0.5
Sodium Molybdate dihydrate	0,25
Thiamine Hydrochloride	0.1
Zinc Sulphate Hyptahydrate	8.6

This powder is extremely hygroscopic and must be produced from atmospheric moisture. 4.4 g powder required for preparing 1 L of medium.

Appendix of Statistical analysis;

0-1. Statistical analysis of the effect of time and media on bacterial growth.

Tests of Between-Subjects Effects

Dependent Variable: RESPONSE

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
HOURS	3.504	22	.159	11.018	.000
MEDIA	2.950	1	2.950	204.093	.000
Error	.318	22	1.445E-02		
Corrected Total	6.772	45			

Appendix 2.2. Statistical analysis of the effect of dilution (10^-7)

Tests of Between-Subjects Effects

Dependent Variable: Res. 10-7

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
HOURS	1592543.870	22	72388.358	1.149	.374
MEDIA	259951.391	1	259951.391	4.126	.054
Error	1386163.609	22	63007.437		
Corrected Total	3238658.870	45			

Appendix 2.3. Statistical analysis of the effect of dilution (10^-8)

Tests of Between-Subjects Effects

Dependent Variable: Res.10-8

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
HOURS	587241.217	22	26692.783	1.463	.189
MEDIA	106368.348	1	106368.348	5.832	.024
Error	401283.652	22	18240.166		
Corrected Total	1094893.217	45			

Appendix 2.5. P and LSD values of the source of variation

Source of variance	**Shoot**		**Root**		**Weight**	
	P value	**L.S.D (0.05)**	**P value**	**L.S.D (0.05)**	**P value**	**L.S.D (0.05)**
Treatment	0.000	4.394***	0.000	7.732***	0.000	0.034***
Days	0.000	2.537***	0.000	4.464***	0.000	0.020***
Treatment*Days	0.000	8.788***	0.000	15.464***	0.203	0.069$^{n.\ s}$

***- Highly significant, n. s - non-significant.

Appendix 3(PPM) Plant Preservative Mixture (PPM)

Content	mg L^{-1}
MethylChloro-isothiazolinone	1.25
Methyl- isothiazolinone	0.35
Magnesium chloride	10
Magnesium Nitrate	10
Potassium sorbate	10
Sodium Benzoate	10

Appendix 4(S23) Liquid medium

Content	g L^{-1}
M&S	4.4
Sucrose	30
Kinetin	0.002
Indol-3-Butyric Acid(IBA)	0.001
Plant preservative Mixture(PPM)	0.001

PH=5.8

Solid medium was added 7 g L^{-1} Agar to the liquid medium.

Appendix (5) Antibiotic stock solution

Kanamycin: 50 mg L^{-1}

0.5 g Kanamycin was dissolved in 10 ml distilled water

Gentamycin: 10 mg L^{-1}

0.1 g Gentamycin was dissolved in 10 ml distilled water

Tetracycline: 12 g L^{-1}

0.120 g Tetracycline was dissolved by adding some drops from 50% ethanol and the volume was made up to 10 by distilled water.

Carbenicillin or Cefotaxime 500 m g L^{-1}

5 g Carbenicillin or Cefotaxime was dissolved in 10 ml distilled water

Appendix (6) Luria-Broth medium (L.B)

Content	g L^{-1}
Bacto-tryptone	10
Bacto-yeast extract	5
NaCl	10
Agar(solid only)	12
Distilled water	Complete volume to 1000 ml
PH	7.2 adjust with 1 m NaOH or HCl

Yeast Extract Broth medium (YE)

Content	g L^{-1}
Beef Extract	5
Yeast Extract	1
Peptone	5
Sucrose	1
MgSO4	2 m/l of a 1 M solution
Agar(solid only)	12
Distilled water	Complete volume to 1000 ml
PH	7.2 adjust with 1 m NaOH or HCl

Appendix 7(NaOH)

1. a. NaOH 1M;
   4 g NaOH + 100 ml water

1. b-HCl 1M;
   8.59 ml HCl (36%) then volume was made up to 100 ml by adding distilled water

1. c-IMS 70%;
   70ml Ethanol absolute + 30 ml distilled water

Appendix 8; CTAB buffer:

2 x CTAB buffer:	To prepare 1000 ml buffer
2% CTAB	20 g CTAB
100 Mm TRIS PH 8.0	12.11.g Tris
20 Nm EDTA	7.44 g EDTA
1.4 M NaCl	81.82 g NaCl
2% PVP 40	20 g PVP-40

Preparation of 50 x TAE Buffer (Tris-Acetic Acid EDTA buffer, pH 8.0)

40 mM Tris
10 mM Na-Acetate
1 mM EDTA
This solution is 50 x TAE
For agarose gel 1 x TAE is needed

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