Improving Abiotic Stress Resistance In Cauliflower (*Brassica oleracea var. botrytis L.*) By Mutagenesis And *Agrobacterium* Mediated Transformation

by

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Abstract

Improving abiotic stress resistance in cauliflower (*Brassica oleracea var. botrytis L.*) by mutagenesis and *Agrobacterium* mediated transformation.

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Abiotic environmental stress such as drought, salinity and low temperature are common conditions that adversely affect plant growth and crop production. Breeding for crop resistance to abiotic stress is difficult due to its multigenic nature. An alternative approach is through DNA mutation and DNA transfer. These approaches were employed and tested in this research and comparison between them was carried out.

NEU and NMU induced mutant lines and control plants were sub-cultured many times on maintenance medium and stored at 5 °C for 2 years and then tested for salt and hydroxyproline resistance as *in-vitro* and *in-vivo* plants and proline content was measured. Non-acclimated and acclimated *in-vivo* plants were also assessed for resistance to freezing. Control plants had little or no NaCl or hydroxyproline resistance whilst selected plants showed varying degrees of resistance. *In-vitro* and *in-vivo* responses of selected lines were correlated. Leaf proline content was increased markedly in the mutant lines and the greatest proline contents occurred following NaCl stress with the most respondent line having 100 fold levels compared to the controls. Both non-acclimated and acclimated selected lines showed improved frost resistance over controls. The results clearly demonstrated that NaCl, frost and hydroxyproline resistance were stable traits over repeated *in-vitro* subcultures and prolonged low temperature storage. A complete range of mutants with single, double or triple resistance traits were produced. The level of resistance however was not necessarily correlated with the level of proline and some lines showed resistance without elevated proline. It is concluded that elevated proline is not essential for improved resistance to abiotic stress in cauliflower, but where it does occur it does improve resistance.

Integration of APX and SOD stress genes into cauliflower (*Brassica oleracea var. botrytis L.*) plants was achieved by using *Agrobacterium tumefaciens* – mediated transformation method. The procedure utilized polymerase chain reaction (PCR) amplification of insert DNA directly after isolation of individual colonies without the necessity of separate procedures for DNA isolation and purification. Preliminary selection of transgenic plants was performed on different combinations of kanamycin, gentamycin and tetracycline containing medium. Integration of the introduced stress gene (APX and SOD) in the plants was confirmed by using β-glucuronidase gene (GUS) and leaf disc assays as a gene fusion and diagnostic marker, respectively. The stable integration of the APX and SOD gene at 478 bp was detected by using polymerase chain reaction (PCR) of the putative transgenic plants. Analysis of APX and SOD gene expression under salt treatment showed that putative transgenic cauliflower survived the salinity stress comparing with the control plants.

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Dedication

I dedicate this thesis to my Father, Rabei; my mother, Safa; My wife, Reham my Children, Salma and Adham and to our next baby.

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Declaration

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award.

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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.

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Chapter 1

General Introduction and Literature Review

1. 1 Overview of the Cauliflower (*Brassica oleracea var. botrytis L.*)

Cauliflower originated over 2,000 years ago in gardens of Asia Minor and the Mediterranean. Cauliflower is one variety of the highly polymorphic species *Brassica oleracea,* which also includes cabbage, kale, kohlrabi, Brussels sprouts and broccoli. This vegetable contains high amounts of vitamins C, K, and A (beta-carotene), and folic acid, fiber, and flavonoids, which gives cauliflower its antioxidant and anti-inflammatory properties and is an important source of vegetable plants for man as well as animal feed (Toussaint-Samat, 1994). Having adequate amounts of vitamin C in the diet has 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 artherosclerosis, reduce bruising, and offer a high degree of protection from strokes (Patison, 2006). Eating adequate amounts of cruciferous vegetables such as cauliflower can lower the risk of cancer, particularly bowel, breast and other female cancers, the flavonoids in this vegetable help support the structure of capillaries, and the vitamin A content can help reduce the risk of cataract formation (Anon, 2006). Cauliflower is also a carbohydrate food that is an efficient fuel for energy production, which is useful for athletes involved in prolonged, strenuous exercise (Robert, 2001).

The evolution of the cauliflower probably occurred in the Eastern Mediterranean (Snogerup 1980; Gustaffson 1982) and it has been reported as being under cultivation in Europe since the 15th century and by the 16th century it was eaten throughout Western Europe. It is now grown through out the world with an estimated world production in 2005 of over 16 Mt (Table 1)

Table 1. World production of cauliflower during 2005, (FAO 2006)

Country	Cauliflower production (Mt) (2005)
United Kingdom	160,000
Italy	513,025
France	395,100
India	4,800,000
China	7,339,000
America United State	335,530
Africa	307,130
Egypt	130,000
South America	78,416
World	**16,364,474**

Cauliflower is a cool season vegetable that requirement rich fertile and well-drained soil, sunny location, good moisture ovoid ability and distinct temperature for producing a marketable curd (the edible immature flower buds). In Britain, summer and autumn cauliflower are grown all over the country, but winter cauliflower are grown mainly in mild coastal areas, such as Cornwall, where frost damage is unlikely. The crop is frost sensitive, with exposed curds being liable to frost damage, and unexposed curd also at risk

by prolonged temperature 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 where the relatively mild winters reduce the risk of frost damage. In winter, large quantities of cauliflower are sometimes imported from Spain, Brittany and occasionally Italy.

All *Brassica* species are interrelated by virture of sharing one of 3 genomes (A, B, C) (Figure 1). Using rapid cycling brassicas the resistance of Brassica species to salt has been affected by salt stress. Kumer (1995) and Ashraf *et al.* (2001) suggested that the amphidiploid *Brassica* species, *B. napus* (AC genome), *B. carinata* (BC genome) and *B. juncea* (AB genome) were more tolerant of salinity than their respective diploid progenitors, *B. rapa* (A) and *B. nigra* (B) and *B. oleracea* (C genome). They suggested that salt tolerance has been obtained from A and C genomes. Also, Ashraf and Mcneilly (2004) suggested that higher salt tolerance of the amphidiploid *Brassicas* has been acquired from the A (*B. campestris*) and C (*B. oleracea L.*) genome.

B. rapa ——— **B. napus** ——— **B. oleracea**
(AA) 2n = 20   (AACC) 2n = 38   (CC) 2n = 18

Turnips   Oilseed rape   Cabbages
Turnip-rape   Fodder rape   Kales
Chinese cabbage   Swede   Broccoli
                                             Cauliflowers
                                             Brussels sprouts
                                             Chinese Kale

B. juncea   **B. carinata**
(AABB) 2n = 36   (BBCC) 2n = 34

Brown mustard   Ethiopian mustard

B. nigra
(BB) 2n = 16

Black mustard
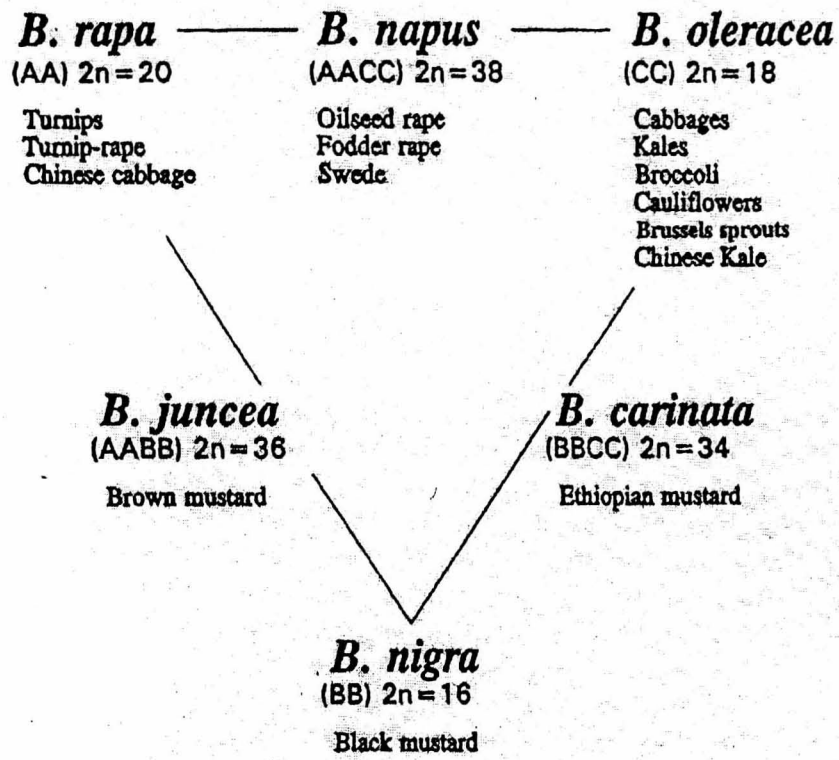
Figure 1: The *Brassica* triangle, showing the origin of the three amphidiploid species from the three pairs of contributing diploid genomes (Simmonds , 1976)

Generally, Brassica Species are moderately sensitive which mean 1) they have moderate energy to get water that would otherwise be used for growth and flowering; 2- they able to adjust internally to osmotic effects of high salt concentration than sensitive plants such as carrot, onion, bean and strawberry; 3- they can be less resistance for specific toxic effect of some element such as sodium, chlorine and borone comparing with resistant plant such as beet and squach; 4- they have a limited ability to adjust and are injured at relatively high salt concentration where expected loss in relative growth and a yield (%) will be 100% if the electrical conductivity is more than 16 dsm^{-1} (Table 2).

Table 2: Soil Salinity level and Yield Potential of *Brassica* species

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

Source: Alan 1994.

In Egypt some 12×10^5 hectares have become saline as result of irrigation from Nile (Heakal *et al.,* 1981). There are about 4.4 billion cubic metes of

reused drainage water with a salinity of 35 mM which is mixed with Nile freshwater and used on the soil (El-Saidi 2000). Moukthar *et al.,* (2000) reported that crop productivity is highly affected by bad soil drainage where the soil are characterised by saline clays with shallow saline water table.

In England the soil are classified to different types, about 70 % from the soil are brown earth, surface water gley and podzolic while the rest of the area are peat, litho morphic, pelsols and ground water. Most of the soils in UK are fine silty, fine loamy and peaty while less of the soil are sandy. Very large concentration occur in the salt affected soils around the wash and the Lincoln Marshes on the east coast. Large concentration are found in the south west Penisula and in west Wales. In south west the Na concentration in agriculture land are above 541 mg kg^{-1} , while in south east and north England are below 187 mg kg^{-1} and it be between 187 to 329 mg kg^{-1} in the west and the rest of the agriculture land area in UK (McGrath and Loveland 1992).

1.2 Abiotic Stresses

Biotic and abiotic stresses are considered as limiting factor for plant growth and productivity (Boyer, 1982; Rathinasabapathi, 2000; Gill *et al.,* 2003 and Seki *et al.,* 2003). Seyle (1936) first developed the concept of stress and Levitt (1980) defined eight stresses: chilling, freezing, heat, drought, flooding, UV, X–rays and salt (Figure 2).

Environmental stress

Biotic
(infection or competition
by other organisms)

.Physicochemical

Temperature   Water   Radiation   Chemical   Wind, pressure, sound, magnetic, electrical, etc.

(4) Deficit
(drought)

(5) Excess
(flooding)

(8) Salts or ions, gases,
herbicides, insecticides, etc.

Low
(cold)

(3) High
(heat)

(1) Chilling   (2) Freezing
(or frost)

IR   Visible   (6) UV   (7) Ionizing
(X-, γ-)
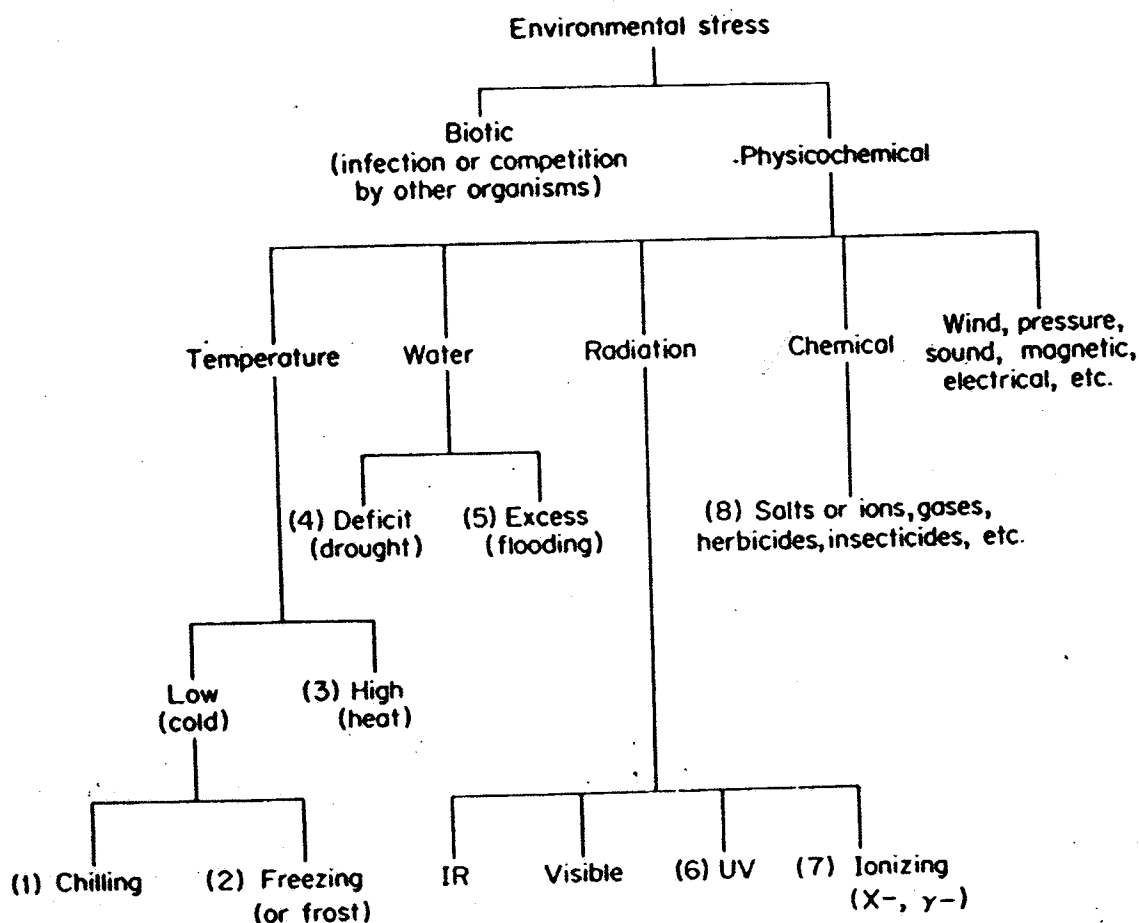
Figure 2: Kinds of environmental stresses to which organisms may be subjected resistance against each of the number stresses is known (Levitt, 1980)

Abiotic environmental stresses are common threats and major limitations adversely affecting 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. Osmond *et al.* (1987) and 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 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 the alterations 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 less hardy to a more hardy status. Acclimation is usually initiated as a mild stress response mechanism that is characterized by transient, physiological, biochemical and molecular perturbations (Levitt, 1980; Sakai and Larcher, 1987 and Kerepesi *et al.,* 2004).

Based on the original concept of stress (Seyle, 1936) and extended ideas given by Larcher (1987) and Lichtenthaler (1996), responses to stress can be differentiated into four phases.

1- Response phase: beginning of stress

- deviation from functional norm

- decline of vitality

- catabolic processes exceed anabolism

2- Restitution phase: (Continuing stress)

- acclimation processes

- repair processes

- 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 severe.

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 mechanisms such as acclimatory metabolic and morphological adjustments. This general alarm syndrome will cause a hardening of the plants by establishing a new physiological standard, which is an optimum stage of physiology under impact of the stressor and corresponds to the plant's resistance maximum.

Under long term stress or with a stress-dose which overloads the plant's 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 progress 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 (Lichtenthaler, 1998).

1.2.1 Salinity

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 effects 7% of the world's land area, which amounts to 930 million ha (Szabda, 1994; based on FAO 1989 data). Munns *et al.* (1999) indicated that 5% (77 million ha) of the 1.5 billion ha of cultivated land is affected by salt. Alteman *et al.* (2000) 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 *et al.* 1980; 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 irrigations. Most irrigated land, which produces about one third of the world's food, is at risk of the saline problem (Munns *et al.* 2000a). Other factors which contribute to excessive salt accumulation in soils are natural processes and poor drainage conditions. In the arid and semi-arid regions there is frequently insufficient rain to leach away soluble salts. Salts occur both naturally in the soil and are added in irrigation water, rain and wind blown dust and by upward movement of ground water. 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 for irrigation, whether from a river or from wells, is never pure but always contain dissolved salt. All soils contain salt but there are differentially taken by plant and levels of sodium can accumulate dispropionately to others and it is high levels of sodium that tends to define the saline problem.

1.2.1.1 Mechanism of salt resistance

Salt resistance means the ability of plants to grow satisfactory in saline soil. Levitt (1980), however, used the term 'salt resistance' in a broader 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 salt gland 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, this reasonable, since tolerance involves an equilibrium state, and avoidance requires development by the plant of a mechanism to avoid equilibrium and to replace it by the steady state. Avoidance is also more efficient adaptation; by avoiding the stress, the plant avoids both elastic and plastic strains. It is, therefore, able not only to survive when exposed to the stress, but also metabolisze, develop, and complete its 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.

Hasegaw *et al.* (2000); Zhu *et al.*, 1997; Shakirov *et al.*, 2003 defined the determinants of salt stress tolerance as effectors molecules (metabolites,

proteins, or other components of biochemical pathways) that control the amount and timing of resistance. Stress adaptation efforts are categorised as those that mediate ion homeostasis, osmolyte biosynthesis, toxic radical scavenging and water transport. Osmoregulation is a common response to salinity stress, which allows maintenance of turgor and thus avoidance of cell desiccation and all associated repercussions of turgor loss (Gracia *et al.*, 1997a). 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 there are numerous mechanisms at cellular tissue, organ or whole plant levels.

Tester and Devenport (2003) showed that mechanisms of tolerance show large taxonomic variation. These mechanisms can occur in all cells within the plant, or can occur in specific cell types, reflecting adaptations at two major level of organization: those that contribute to tolerance of cells and those of the whole plant. High salt disrupts homeostasis in water potential and ion distribution. The disruption of homeostasis occurs both at the cellular and the whole-plant levels, drastic changes in ion and water homeostasis lead to molecular adjustments, growth arrest and even death. The integrity of cellular

membranes, the activity of various membrane, nutrient acquisition and function of the photosynthetic apparatus are all known to be prone to the toxic effects of high salt stress. An important cause of damage is thought to be reactive oxygen species (ROS) generated by salt stress (Zhu *et al.,* 1997 and Poontariga *et al.,* 2000).

Plant 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; Huazhoung *et al.,* 2002).

It is recognized that salt affects virtually all aspect of a plant's physiology at the very first, from ion transport, selectively, excretion, nutrition and compartmentation together with growth, water use and water use efficiency (Koyama *et al.,* 2001). Several studies (Taek-Ryo Kwon, 1997 Muhammed Ashraf 2004; Rosaria *et al.,* 2005 and Harkamal *et al.,* 2005) indicated that

plants under saline conditions establish physiological mechanisms of salinity tolerance such as osmotic adjustment against tissue water loss and ion uptake, and transport control against ion toxicity. The synthesis of compatible organic solutes or accumulation of inorganic solutes achieves osmotic adjustment in the cells, which prevent internal water loss, resulting in the maintenance of water relations.

Garcia *et al.* (1995) 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 photosynthesising 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 a lower rate 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 *et al.* (1985a), on the other hand, found that old leaves accumulate much higher salt levels than young leaves, while Semikhatova *et al.* (1993) suggested that salinity tolerance is a property of whole plants. A major characteristic of solute transport of plants in saline conditions is the degree of selectivity between Na and K (Greenway and Munns, 1980)

Mahmoud (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.

1.2.1.2 Effect of salinity on plant growth

The most common adverse effects of salinity on plants include reduction in height and size, suppression of growth, yield and physiological function and the deterioration in quality of the product. The first and most common

symptom of salt injury is a reduced rate of plant growth (Araus, 2002 and Abebe *et al.*, 2003). A plant growing on saline soil is smaller than normal, may have darker leaves than normal and will wilt from drought sooner than it would in a non-saline soil. As salinity increases plant growth will eventually cease leaf burn, commencing at the tip will occur and ultimately death in highly saline soil (Vyjayantic and Prathapasenan 2000). Plant growth under saline conditions requires additional expenditure of metabolic energy, such as the synthesis of compatible organic solutes, the decrease of salt uptake, and the increase of enzyme activity.

Greenway and Munns (1980) suggest that the deleterious effects of salinity on plant growth were attributed to specific ion toxicity and nutrient ion deficiency. Garcia *et al.* (1997a) indicated that NaCl reduced the growth of plants and caused chlorophyll loss in leaves in 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.

1.2.2 Frost

Frost stress is intimately linked with drought and salt stress where many genes that are regulated by frost are also responsive to drought or salt (Zhu *et al.*, 1997). These three major abiotic stress factors strongly limit plant productivity by reduced the growth of plant, caused chlorophyll loss in leaves due to disrupted chloroplast integrity (Gracia *et al.*, 1997a); decrease the content of dry matter (Heuer and Nadlev 1995); dehydration and disruption of the cell (Thomashow 1999); membrane damage (McKenise and Bowly 1997; protein denaturation (Guy *et al.*, 1998) and growth arrest and even death (Tester and Devenport 2003).

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 (George *et al.*, 1982; Becwar *et al.*, 1981; Steponkus *et al.*, 1998; 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 e.g. in 1987 where £ 3.5 M of winter Cauliflower were lost (Anon, 1987).

1.2.2.1 Freezing injury

The primary problem that plants face when exposed to freezing temperatures is ice formation. Ice crystals can cause severe injuries to the living cells that can 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) relative to the amount available inside living cells (symplastic); 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 intracellularly or extracellularly (Guy 1990). Intracellular freezing is suspected to occur when cooling rates are rapid or after significant supercooling allowed by ice nucleation has taken place. Supercooling is where a liquid is cooled to a temperature below its melting point without ice formation, and the supercooling point is the lowest sub-freezing temperature attained before ice formation (Levitt, 1980). On the other hand, extracellular freezing occurs in the spaces between cells, in water transporting elements or on the external surface of plants. Ice will spread from the initial nucleation point through the extracellular space and, as long as the plasma membrane is intact and cooling rate is slow ($1-2^{\circ}$C/hr), ice will remain confined to the extracellular region and will draw water from the cell until equilibrium of water potential is achieved relative to the temperature and the solute concentration of the cell (Wisniewski and Fuller 1999).

Thomashow (1999) explained that when temperatures drop 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 primarily 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 and Larcher 1987). Uprooted barley 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). Frost cracks in trees are good examples of structural damage as an indirect result of freezing, involving sudden radial splitting of a tree trunk from its center through to the bark (Sakai and Larcher 1987), and cause economic loss in forestry (Sano and Fukazawa 1996).

Multiple forms of membrane damage can occur as a consequence of freeze-induced cellular dehydration including expression induced lysis, lamellar-to-hexagonal-phase transitions, and fracture jump lesions (Steponkus *et al.* 1993). Freeze-induced production of reactive oxygen species contributes to membrane damage (McKerise and Bowly 1997). Also, protein denaturation in plants at low temperature can potentially result in cellular damage (Guy *et al.*, 1998).

Olien and Smith (1997) believed that freezing causes extracellular ice adhesion with 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 damage consistent with tissue death, but acclimated leaves showed reversible symptoms during freezing, including massive extracellular ice formation in specific expansion zones within the midvein.

1.2.2.2 Freezing acclimation and tolerance

Freezing acclimation is the positive effect of exposure to stress of the plant on subsequent resistance to freezing. During exposure of plants to low temperature, many changes in physiological and biochemical parameters have been observed such as: modification of levels and activity of enzyme from various metabolic pathways, accumulation of carbohydrate (e.g. sucrose), (increasing cellular osmotic concentration), proteins developing more resistant low temperature forms, increased proline concentration, altered lipid composition of the cell membrane (Bartels and Nelson, 1994), reduced cell size, increased flexibility of cell walls and changed permeability of membranes (Forbes and Watson 1996).

Blum (1988) defined freezing resistance in terms of the internal interaction among physiological, chemical and physiological reaction during a freeze-thaw cycle of the cell and tissue and believed that the increased osmolality of the cellular solute is an effective mechanism for avoiding intracellular ice formation and cellular dehydration. Resistance of higher plants to freezing has two main components, tolerance and avoidance; as illustrated in Figure 3.

FREEZING RESISTANCE

FREEZING AVOIDANCE   FREEZING TOLERANCE

| AVOIDANCE OF FREEZING TEMPERATURES | AVOIDANCE OF ICE FORMATION AT FREEZING TEMPERATURES | TOLERANCE OF INTRACELLULAR FREEZING | TOLERANCE OF EXTRACELLULAR FREEZING |

| AVOIDANCE OF ANY FREEZING | AVOIDANCE OF INTRACELLULAR FREEZING | AVOIDANCE OF FREEZE DEHYDRATION | TOLERANCE OF FREEZE DEHYDRATION |

| ABSENCE OF FREEZABLE WATER | SUPERCOOLING | LOWERING OF FREEZING POINT |

Fgure 3. The two mechanisms of freezing resistance. Source: (Levitt, 1980)

The ability for plants to survive freezing is dependent on many factors such as; 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 crystallisation, Smallwood and Bowles (2002). Levitt (1980) decided that the ability to survive freezing is dictated by the genetic make up of the plant, stage of development and environmental factors.

Thomashow (1999) demonstrated cold acclimation includes the expression of certain cold-induced genes that function to protect membranes against freeze induced in injury. Guy (1990) suggested that acclimation induced cold tolerance is a quantitative character controlled by number of additive genes.

There are several strategies growers can adopt in the fight against frost damage, these include:

1- Escape   2- Reduction of heat loss   3- Heat input

4- The manipulation ice + bacteria by the addition of competitive bacteria (Lindow, 1995)

5- Selection for frost resistance

6- Genetic engineering.

1.3 Molecular response and transcription factors

Many of the changes in mRNA levels observed during water deficit (salt, cold and drought). Many genes with various function have been described that respond to salt and cold at transcriptional levels (Thomashow 1999; Shinozaki and Yamaguchi-Shinozaki 2000; Zhu 2002). Shinozaki *et al.,* (2003) revealed that many genes are induced by abiotic stress to function in

stress response and tolerance. These products of these genes are involved not only in the protection of cells against stresses, but also in the regulation of gene expression and signal transduction pathway in abiotic stress response. These gene products are classified into groups (Figure 4).

Functional protein

Regulatory protein

Water channel, transport

Detoxification enzymes

Transcription factors

Protein factors of macromolecules (LEA) proteins

Stress

ABA biosynthesis

Key enzymes for osmolyte biosynthesis (Proline, sugar)

Protein kinases, phosphotases, phospholipid metabolism

Chaperones proteases
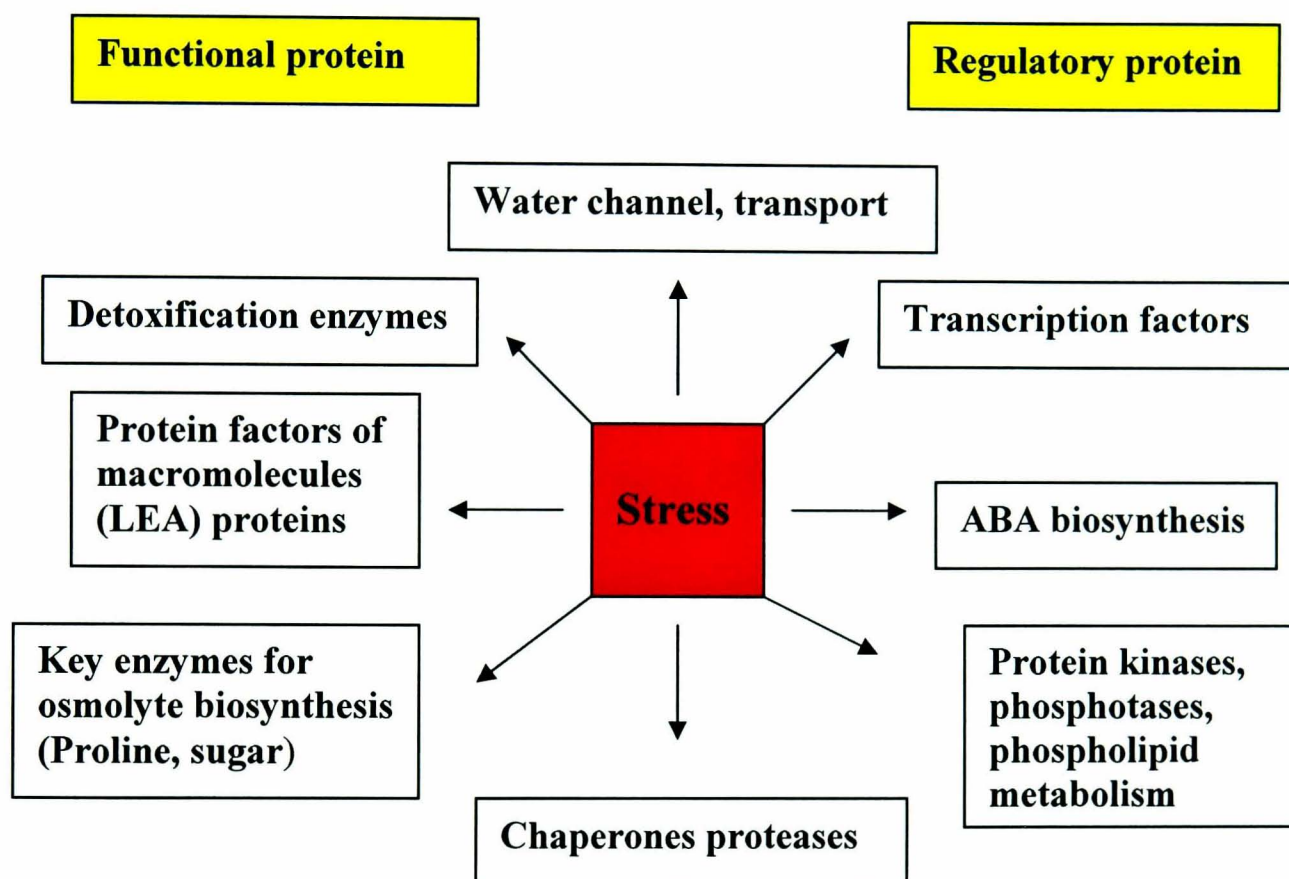
Figure 4. Function of drought and cold stress-inducible genes in stress tolerance and response. Gene products are classified into two groups. The first group includes proteins that probably function in stress tolerance (functional proteins), and the second group contains protein factors involved in further regulation of signal transduction and gene expression that probably function in stress response (regulators protein).

Studies on cold-regulator gene expression in Arabidopsis have result in discovery of a family of transcriptional activators, the CBF/DREB1, protein that have a key role in cold acclimation. Expression studies showed that

CBF/DREB1 genes transcriptional induced by low temperature and then their gene products induce the expression of multiple target gene achieving freezing, salt and drought tolerance (Glimour *et al.,* 1998; Shinwari *et al.,* 1998; Medina *et al.,* 1999). Liu *et al.,* (1998) and Nakashima *et al.,* (2000) found that expression of DREB2 was found to be strongly induced in roots and in stems by high salt and dehydration stress, respectively. Also they decided that two independent families of DREB proteins, DREB1 and DREB2, function as trans-acting factors (transcription factor) in two separate signal transduction pathway under low-temperature and dehydration condition. Kasuga *et al.,* (1999) and Glimour *et al.,* (2000) shown that Arabidopsis plant overexpressing CBF3/DREB1a, and consequently the CBF regulon, are not only more freezing tolerant than control plants, but are also more tolerant of dehydration stress caused by either drought or high salinity. Moreover, Glimour *et al.,* (2000) that Arabidopsis plants overexpressing CBF3 not only have elevated levels of COR protein, but also have elevated levels of proline and total soluble sugar. Thus, the CBF/DREB1 regulator proteins appear to be "master switch" that integrate activation of multiple components of the cold acclimation response (Figure 5).

CBF Cold Acclimation Pathway
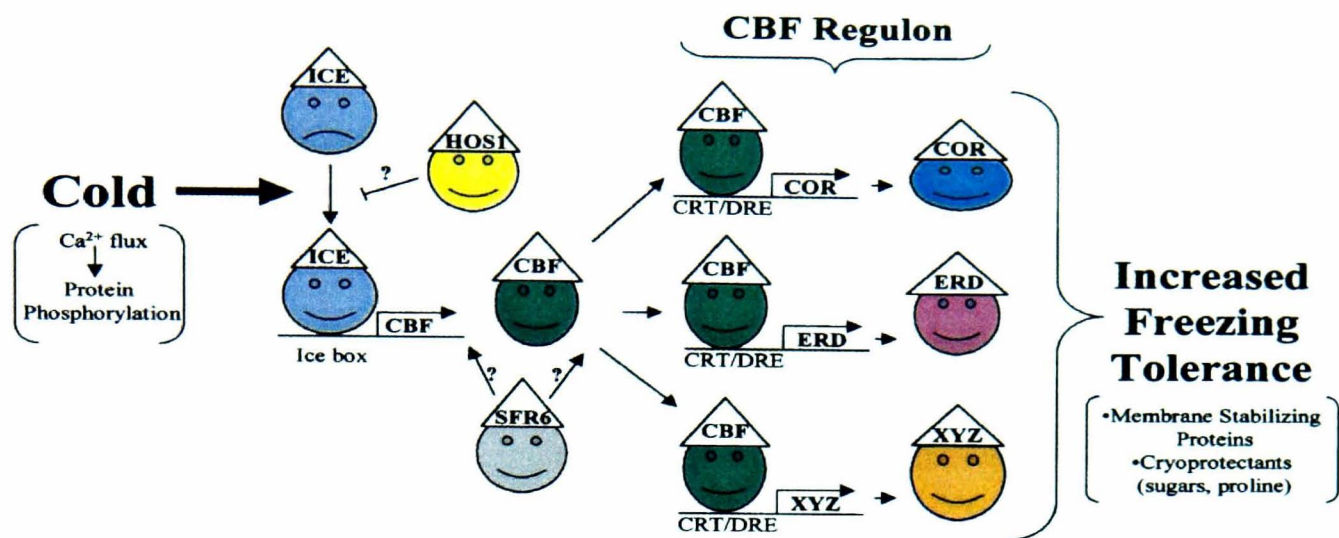
Figure 5. Model of the Arabidopsis CBF cold acclimation pathway (Thomashow 2001).

Low temperature leads to rapid induction of the CBF/DREB1 genes that in turn results in expression of the CBF regulon of CRT/DRE-regulated genes. Action of the CBF regulon, which includes COR, ERD, and presumably yet to be discovered ("XYZ") cold-regulated genes, results in an increase in plant freezing tolerance. Cold-induced expression of the CBF/DREB1genes has been proposed (Glimour *et al.,* 1998) to involve the action of a regulatory protein present at warm temperature designated ICE (inducer of CBF expression).

Low temperature is envisioned to either activate the ICE protein or other protein(s) with which it interacts (Glimour *et al.,* 1998). Such activation may involve alterations in protein phosphorylation caused by a cold-induced influx of calcium .The SFR6 protein appears to act between CBF/DREB1

transcription and induction of the CRT/DRE-regulated genes (Knight *et al.*, 1999) whereas HOS1 appears to act upstream of CBF transcription (Lee *et al.*, 1999 and 2001). Kasuga *et al.*, (1999) showed that overexpression of the cDNA encoding DREB1A in Arabidopsis transgenic plants activated the expression of many genes under normal growing conditions and resulted in improved tolerance to drought, salinity and cold. Gilmour *et al.*, (2000) concluded that both the DREB1A cDNA and the rd29A promoter could be used to improve the dehydration, salt and freezing tolerance of agriculture important plants.

1.4 Oxidative Stress

Several stresses such as high temperature, high light intensity and osmotic stress lead to overproduction of reactive oxygen species (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 causes of plant damage as a result 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 (Yamaguchi-Shinozaki *et al.*, 2002). To minimize the concentration of $1/2$ O_2 and H_2O_2. The enzymes overproduced 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 damage occurs (Van Breusegem *et al.*, 1999). A major hydrogen peroxide-detoxifying system in both plant chloroplasts and the cytosol is called the ascorbate-glutathione cycle, in which APX is the key enzyme (Asada, 1992).

Major reactive oxygen intermediate (ROI) scavenging mechanisms of plants include SOD, APX and catalase (CAT). Biotic stress results in the activation of NADPH oxidase and the suppression of APX and CAT. This leads to the over-accumulation of ROI and the activation of defence mechanisms. Abiotic stress initially enhances ROI production by chloroplasts and mitochondria, however, by inducing ROI-scavenging enzymes such as APX and CAT, ROI levels can then be reduced (Figure 6).

Hernandez *et al.*, (2000) reported NaCl-induced enhanced mRNA expression and activity of Mn-SOD, APX, GR and monodehydro-ascorbate reductase (MDHAR) in tolerant pea cv. Granada, while in salinity-sensitive cv. Chillis, no significant changes in activity and mRNA levels of the above enzymes were observed suggesting considerable genetic variability which may be amenable to genetic selection or manipulation through biotechnology.
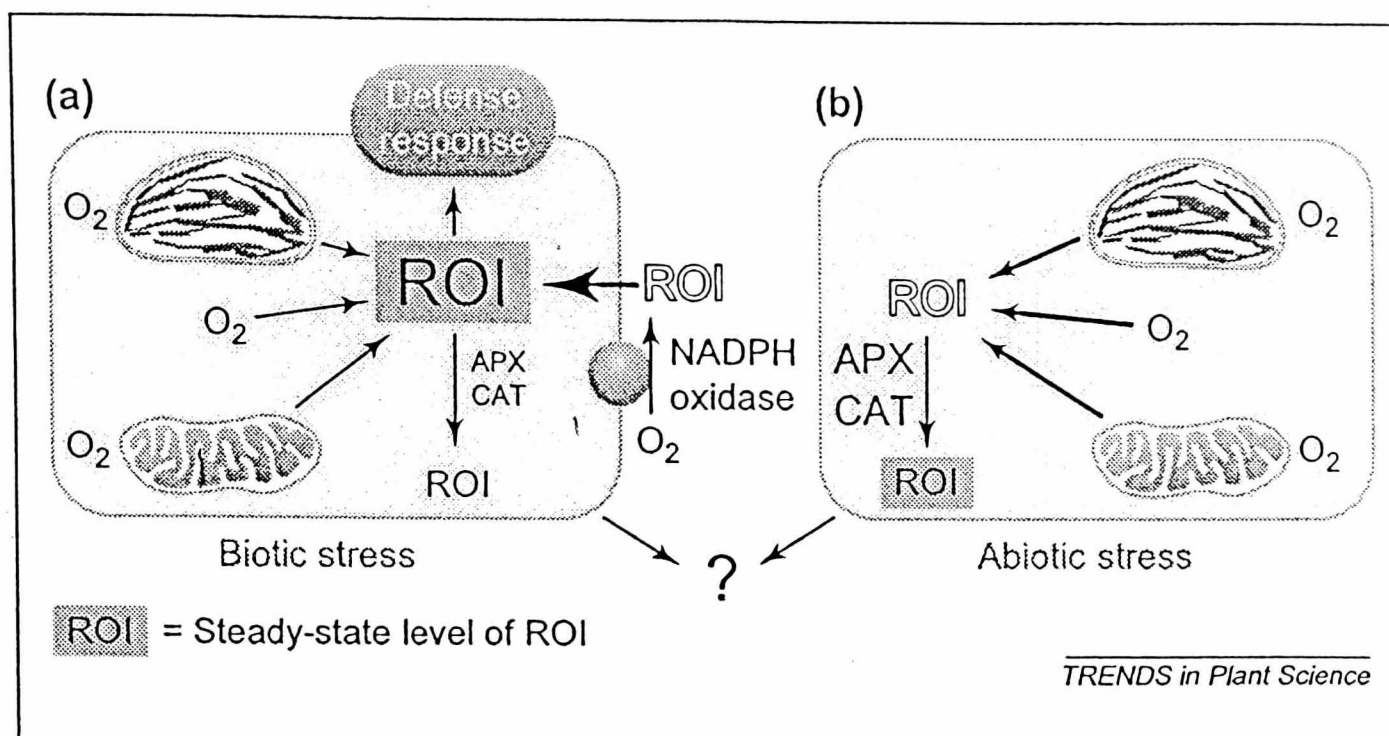
Figure 6. Differences in the steady-state levels of reactive oxygen intermediates (ROI) during (a) biotic stress and (b) abiotic stress. Source: (Mitter, 2002).

1.5 Breeding and selection for salinity and freezing resistance

Breeding strategies for salinity and freezing resistance have generally depended on screening techniques looking for tolerance mechanisms, genetic diversity, genetic mode and heritability (Chaubray and Senadhira, 1994). 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

genetic control of salt and freezing tolerance in any species will be helpful for high 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. Further more 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 =Solanum pennellii*) by Tal and Shannon (1983). Total dry matter production of another F_1 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 salt 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 a plant 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 salt tolerance is a heritable character (Allen *et al.,* 1997; Asraf *et al.,* 1986a). Several experiments have provided evidence that genetic variance decreases with increasing stress levels (Blum, 1988). Bernstein (1977) found a single gene controls sodium and chloride uptake under salinity in grapevine and Scales and Widic (1991) confirmed that a single gene in soybean caused a decrease of salt transport to stem and leaves and it was also shown that the tolerance allele was completely dominant over the sensitive allele. 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: dominance had little influence. Analysis of sodium and potassium accumulation between F_n and F_{n+1} families of 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.,* 1997*a*).

Ramage (1980) 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 tolerance lines. Sacher *et al.,* (1982)

suggested that only one gene might be involved in the inheritance of Na accumulation in hybrid of *Lycopersicon esculentum* and *Solanum pennelli* .

Singh *et al.,* (1989) reported that salinity affects the plant by a series of unstable and stable changes. These changes could be controlled by genetic regulatory mechanisms, which result in both the co-ordinate expression of several genes and in multiple staple genetic changes by perhaps the simultaneous rearrangement of several genes. Mahmoud (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 groups 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.,* (1996b) and Vera-Estrella *et al., (*1999) concluded that it is possible to select salt resistance cell lines, which can integrated in breeding scheme.

Flowers *et al.,* (1995) indicated that although the physiological traits governing the inheritance of salt tolerance is not clear, screening for appropriate diversity in responses 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 (Kazou *et al.* 1999 and Lana *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 and Zhang *et al.* 2000). Jian (2000) indicated that the advances in understanding the effectiveness of stress responses, and distinctions between pathology and adaptive advantage, are increasingly based on transgenic plants and mutant analyses.

1.6 Proline accumulation

Under abiotic stress plants usually accumulate specific types of inorganic and organic molecules. They serve the primary function of maintaining the cytoplasmic osmotic balance and can accumulate to high concentrations without impairment of normal physiological function. There are some physiological parameters which are related with abiotic stress such as; proline, relative water content, water use efficiency, osmotic pressure and

photosynthestic pigments. Ashraf (1994 a) reported that the plants under stress conditions accumulate large quantities of free amino acids, especially proline, and soluble sugars. Martinez *et al.,* (1996) found that salt tolerance in *Salanum juzepczukii* and *Salanum tuberosum* L. was positively related to leaf proline content.

Bhaskaran *et al.,* (1985) highlighted the possible mechanisms of proline accumulation under stress condition: 1- stimulated synthesis from its precursors, 2- low rates of proline oxidation, 3- slow incorporation into protein due to impaired protein synthesis, or 4- accelerated protein breakdown. They considered that a stimulated synthesis might indicate a useful role of proline as an osmoprotectant and a low rate of proline oxidation may indicate a secondary effect of stress.

Voetberg and Stewart (1984) found that proline accumulated in barley leaves in response to salinity and its maximum concentration was linearly related to Na concentration. Samaras *et al.,* (1995) reported that proline accumulates in the cytoplasm to balance the osmotic potential of the vacuole under abiotic stress and many plant species accumulate high level of proline, which is thought to function in stress adaptation (Delanney and Verma, 1993). Also, Silveira *et al.,* (2003) showed that a prominent proline accumulation of cashew leaf in response to salt stress.

Singh *et al.,* (1996) proved that proline over accumulation resulting from either loss of the proline oxidase-mediated pathway of proline catabolism or salinity inducible uptake of exogenous proline confers protection against the lethal effects of 150 mM NaCl in the cyanobacterium *Nostoc musorum*. Wyn (1981) noted that proline is only accumulated at concentrations of NaCl which inhibit growth and which decrease the tissue succulence in *Atriplex spongiosa*.

In response to drought and salinity stress, many plant species accumulate high levels of proline, which is thought to function in stress adaptation (Greenway and Munns 1980; Delauney and Verma, 1993). Qasim *et al.,* (2003) observed that Na effect of salt stress was observed on leaf soluble protein but there was a slight increase in total free amino acids of canola cultivars. Serrano and Gaxiola (1994) suggested that proline protects plant tissues against osmotic stress because it is an osmosolute, a source of nitrogen compounds, and protectant for enzymes, cellular structures and a scavenger for hydroxyl radicals. Kishor *et al.,* (1995) reported that constitutive production of proline could confer osmotolerance in transgenic tobacco plants.

Hasegawa *et al.,* (2000) reported that proline is believed to facilitate osmotic adjustment by which the internal osmotic potential is lowered and may then contribute to tolerance. Proline as a compatible solute is typically hydrophilic,

which suggest it could replace water at the surface of proteins, protein complexes, or membrane, thus acting as a non-enzymatic osmoprotectant. On the other hand, Gibon *et al.,* (2000) concluded that proline accumulation was not involved in the maintenance of turgor and it appeared to be more of a consequence of the relative dehydration of stressed tissues in canola and they reported that there was no evidence that proline it self had a protective effect against the consequence of dehydration

1.7 The Relationship between salt treatment and plant sodium, potassium, calcium and magnesium content

The relationship between salinity and mineral nutrition of horticultural crops are extremely complex and there is not a complete understanding of their intricate interaction. Salt tolerances are determined by the ability of the plant to regulate the uptake of salt. The most salt-tolerant species have high internal salt concentration.

The nutritional benefits of Na have been deliberated for many years. Through its positive effects upon growth of some plants have been observed its requirement by plants in general is disputed. Na+ may replace some essential function normally performed by K+. Plants require mineral elements that may be partly or totally satisfied by any element, including Na^+. Na^+ may also

stimulate the uptake of other minerals such that a more favourable balance occurs in the plant. Finally, Na+ may trigger the release of K^+ from sites in plants where it is physiologically inactive. Sodium content has been used in many studies as a selection criterion for crops under salt treatment (Wyn Jones *et al.*, 1984; Lauria *et al.*, 2002; Munns *et al.*, 2003; Davenport *et al.*, 2005 and Tester and Devenport 2003) reported the correlated tolerance of a plant to sodium chloride with the ability to exclude Na from shoots. Despite this, Yeo and Flowers (1986) showed that Na exclusion alone should not be used as an indicator of salt resistance.

Maintenance of adequate levels of K is essential for plant survival in saline habitats. Potassium is the most prominent inorganic plant solute, and as such makes a major contribution to the low osmotic potential in the stele of the roots that is a prerequisite for turgor-pressure-driven solute transport in the xylem and the water balance of plants (Marschner, 1995). Numerous studies with a wide variety of horticultural crops have shown that K concentration in plant tissue decline as the Na-salinity is increased (Francois 1984; Subbarao *et al.*, 1990; Izzo *et al.*, 1991; Graifenberg *et al.*, 1995; PeÀrez-Alfocea *et al.*, 1996; Sophie *et al.*, 2006). He and Cramer (1993b) showed that salt tolerance *B. napus* had a greater concentration of potassium than salt sensitive *B. carinata* following irrigation with seawater. The ion relations of plants and K maintenance or an efficient K retranslocation under salinity is an important

physiological mechanism of salinity tolerance because a salt induced change in potassium uptake by roots is associated with changes in photosynthesis and plant growth (Chow *et al.,* 1990; Yuncai *et al.,* 2005). Flowers *et al.,* (1977) suggested that a preference for K at high salt concentrations is a useful attribute in salinity stress and a good criterion for selection for salt tolerance. Although salinity studies have analysed plant tissues for magnesium, most of the salinity-nutrition studies have directed little attention to magnesium nutrition as affected by salinity. More recent examples are from studies by Ruiz *et al.,* (1997) and Sophie *et al.,* (2006) where they found that NaCl salinity reduced leaf Mg concentration in citrus and *Helianthus paradoxus*. However increases in salinity are not always associated with decreases in leaf Mg. Bernstein *et al.,* (1974) found that increases in salinity (NaCl + $CaCl_2$) only reduced leaf Mg concentration in beet and had little or no effect in leaves from five other vegetable crops that they examined.

Calcium plays an essential role in processes that preserve the structural and functional integrity of plant membranes, stabilise cell wall structures, regulate ion transport and selectivity, and control ion-exchange behaviour as well as cell wall enzyme activities and kinase cascades associated with detection of stress (Rengel, 1992; Marschner, 1995). In those plants whose marketable product consists primarily of large heads enveloped by outer leaves, e.g (cabbage, cauliflower, lettuce) excessive transpiration by the outer leaves

diverts calcium from the rapidly-growing meristematic tissue. Problems of nutrient transport to the shoot meristem arise because this tissue has only rudimentary vascular traces and is supplied with plant resources (mineral nutrients) via diffusion. Furthermore, meristems generally lack a transpirational driving force and must compete with cells through which plant resources are serially transported (Lazof and Lauchli, 1991). Under high level of NaCl salinity, calcium uptake and transport to all organs can be significantly reduced (Sekina *et al.*, 1993 and Sophie *et al.*, 2006)

1.8 Mutation

In the absence of desired characteristics, a common and efficient tool to create new genetic variability in cultivated species is mutagenesis. Mutants have been included in germplasm collections or used in breeding programmes to improve 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 to break desirable linkage 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 tolerant to abiotic stresses (freezing, salinity, drought, alkalinity, high humidity and high soil fertility) (Micke, 1988b).

Mutation can be induced by the use of mutagens which may be either physical or chemical, and both have been used in conventional plant breeding programmea as well as 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 analogues and alkylating agents. Alkylating agents include N-nitrose-N-ethylurea (NEU), N-nitrose-N-methylurea (NMU), alkyl sulphate and nitrogen mustards. NEU or NMU are a biofunctional agent that required metabolic activation to a reactive metabolite (Charlotee, 1976). NEU or NMU alkylate nucleophilic organic macromolecules including DNA. They can induce depurination and depyrimination as well as monoadduct formation. Both NEU or NMU have been shown to induced gene mutation (Deletion), transition mutation, unscheduled DNA synthesis (UDS), sister chromatide exchange (SCE) and they can also induced DNA-DNA and DNA protein crosslink (IAEA, 1977 ; Negrutiu, 1990).

1.8.1 Mutagenesis and selection for stress conditions

Both physical and chemical mutagens have been used to obtain mutant cultivars in crops, the choice usually depending on their variability. Gamma

and X-ray's have been widely used, but fast and thermal neutrons, beta rays and chemicals have also been used. The efficiency of most chemical mutagens is similar (Monti, 1968) (Figure 7). Problems relating to the effective dose assessment of chemical mutagens include non-uniform penetration in multicellular system, breakdown of mutagenic compounds and carcinogenic properties. These factors have tended to determine the preference of many geneticists for physiological agents (Gottschek and Wolff 1983; Micke, 1985). The spectrum of mutations may differ, but nothing is reported specifically on the characteristic related to stress resistance.

The mutagenic agent can be applied to the plant, to individual tissues, to organs (seed, ovaries) or to cells. After exposure of the target material a selection screen is necessary if the desired character is to be isolated from the treated population. A linkage between the nutrient requirement and a desired trait that is expressed at the cellular level provides an effective screen for selection of whole plant characteristics at cellular level (Maliga, 1984). Identification of mutants will provide markers for cellular genetic manipulations and these selected lines will be available for physiological and molecular research and germplasm development (Gottschalk, 1981).
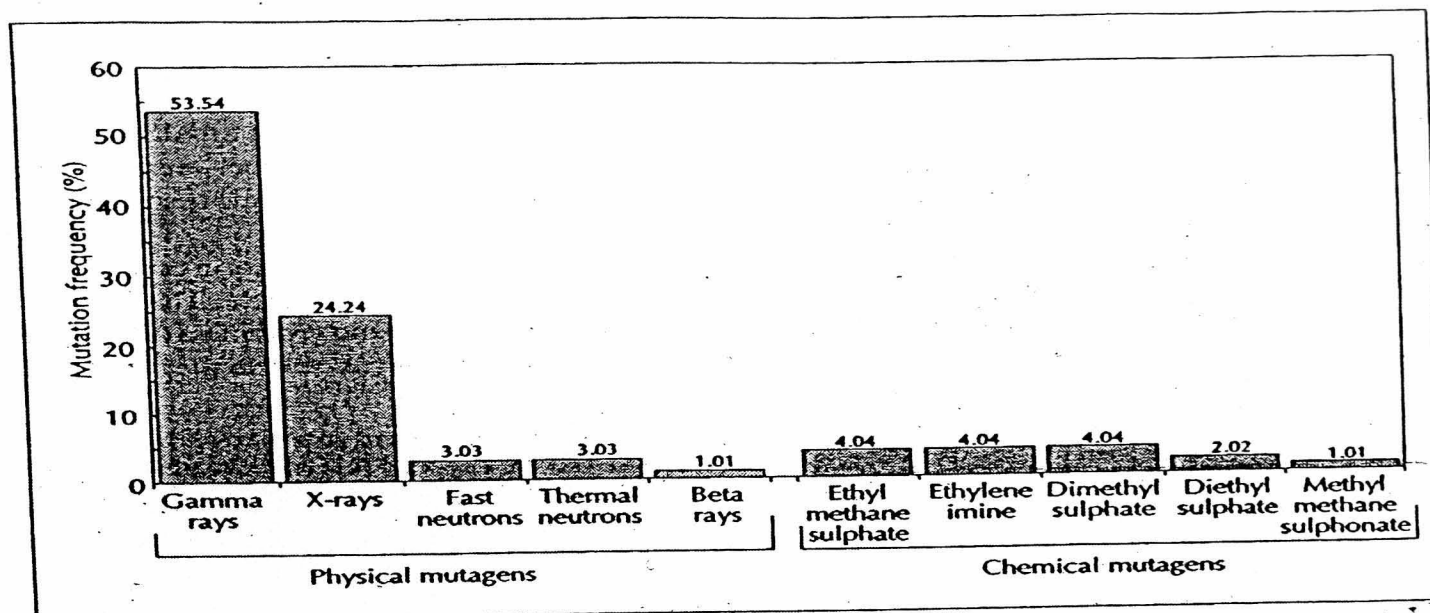
Figure 5. The effect of different mutagens (physical and chemical) on mutation frequency in plants (Saccardo *et al.,* 1993).

László (1981) indicated that the selection of proline as a selective marker for tolerance and proline-over-producing mutants could be more tolerant to salt. Chandler and Thorpe (1989) selected for proline over-production and showed that this technique could be a promising approach to the increase of salt tolerance in crop improvement programmes. Efficiency of selection for proline over-production could be enhanced using single cells to reduce any effects of sectoring and specific metabolic blocks (Dix, 1984) and to remove the necessity for proline determination at each stage of selection. Deane *et al.,* (1995) selected one hydroxyproline resistant shoot from shoots arising from mutagenised cauliflower curd, and found that proline levels were higher than control plants. Gengenbach (1984) considered that mutants could be useful material, not only for the analysis of amino acid metabolism but also for

breeding improved quality, because the corresponding free amino acid accumulates in the mutant. Werner and Finkelstein (1995) separated 6 such mutant lines of *Arabidopsis* which indicated reduction in sensitivity to salt and osmotic stress at germination and later stages of vegetative growth.

Widholm (1976) selected a hydroxyproline resistant carrot cell line from mutagen treated suspension cultures that produced 15-30 times the normal free proline levels and displayed cross-resistance to their proline analogues. Hydroxyproline resistant cells were approximately one hundred times more resistant to proline. A number of auxotrophs have been recovered from mutagenized plant cells requiring various amino acid, nucleic acid and vitamins for growth. The significance of these mutants is the isolation of cell lines with markers that can be used to identify a trait expressed at the cellular level provides an effective screen for selection of whole plant characteristics (Maliga, 1984).

Primarily, mutation breeding is done to enhance the genetic diversity of a crop plant which brings alternation to a single-trait e.g linolenic acid in soybean (Vello *et al.,* 1984). Contrarily, this brings changes to other aspects of the plant, hence it is therefore needful to carry out an extensive agronomic screening and testing of the single mutant that is desired.

1.9 Tissue Culture

Plant tissue culture can be defined as the culture of plant cells, tissue and organs under aseptic condition (Smith, 1992). Tissue culture involves placing small pieces of the explants, (explants is the name given to plant tissue that has been taken from various sites on a plant or the pieces of the plant (propagule) used to initiate the micro propagation or tissue culture process), containing a meristem on an artificial, sterile medium containing minerals, sugar, vitamins, hormones and agar (Forbes and Watson 1996). Cell and tissue culture techniques offer a number of advantages not found in the 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 a well established technique in plant breeding and selection (George and Sherrington 1984). This collection of techniques can be directed either towards the production of identical plants (cloning) or to induce variability (somaclonal 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 cell culture are generally useful in crop improvement programmes through: 1- propagation *in vitro*, 2- meristem culture for virus elimination, 3- secondary product synthesis, 4- production of haploid plants

from cultured anther, and 5- development of new varieties via cellular or molecular genetics (Evans *et al.*, 1984). Tissue culture gives a chance to study some morphological, physiological, 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).

1.9.1 Plant Regeneration

A key factor in the application of *in vitro* techniques to plant improvement is the development of efficient protocols for regeneration of plants from cells, tissue and organs (Seckinger, 1993). Plants can be regenerated from cells or tissues through organogenesis or embryogenesis, depending on plant developmental state and culture medium and conditions. Organogenesis involves the differentiation of organs, e.g., root or shoot primordial, from cells or tissues.

Several factors have been reported to affect regeneration *in vitro* such as culture medium, explants source, growth regulators and the use of antibiotics. Each of these factors and their relationship to regeneration will be discussed in detail. Plant growth regulators is the common term applied to some biochemical products produced in particular tissues or organs of plants and which, despite their small amount, play important role in plant

morphogenesis. Auxin, cytokinins and gibberellins are three major group of plant growth regulator used in different stages of 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 propagule. 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 regulant that is required for different purposes can be generally represented as in Figure 8. Relative proportions of axuins and cytokinins do not always produce the typical results shown in Figure 8. A balance between auxin and cytokinin growth regulators is most often required for the formation of advantage shoot and root meristems (Thomas and Tsvi 2005; Seliskar *et al.,* 2005; 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. For cauliflower the literature on this is summarized in table 3 (after Kieffer , 1999).
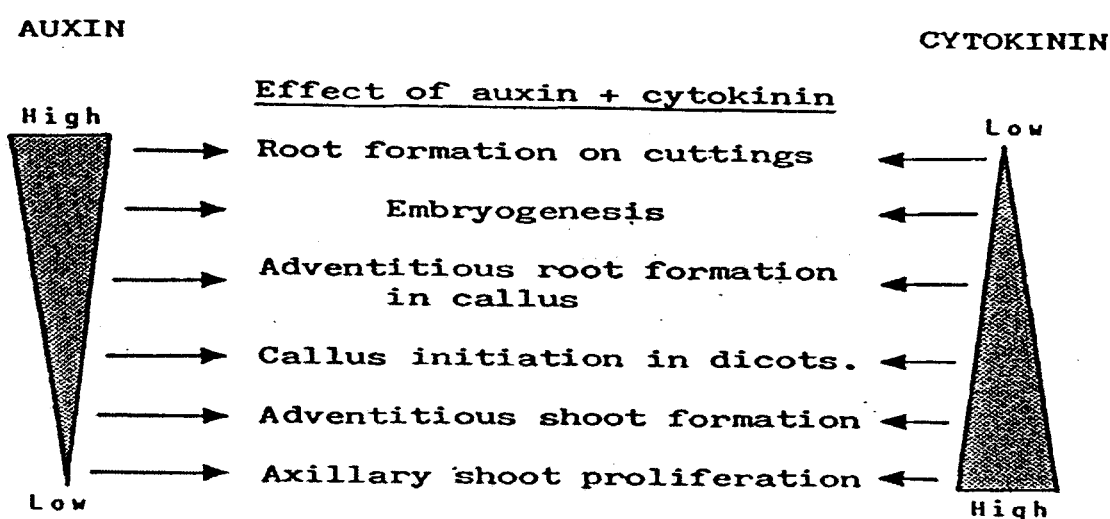
Figure 7: The relative amount of an auxin and a cytokinin that are often required to bring about some kinds of morphogenesis. Source: (George and Sherrington 1984)

Many observations of organ formation in cultured tissues supports the hypothesis that localized meristematic activity precedes the organized development of shoot and roots (Torrey, 1966; Keni and Lewis 2005). In general, buds can be initiated from callus or from cut edges of explants in the presence of high cytokinin: auxin ratio (Gresshoff, 1978; Hiroharu *et al.,* 2001; Helenice *et al.,* 2003; Nathan and Sekhar 2006). In some species, the addition of cytokinins to the medium failed to induce shoots, suggesting that the accumulation of endogenous auxin or other hormones may result in an inhibitory effect on organogenesis which cannot be reversed by exogenously applied hormones (Street, 1977; Khalid *et al.,* 2003). In these cases additional hormones may be required or the cultural conditions, including nutritional and physical factors, may block the onset of the process. The conditions for the induction of roots vary considerably, usually a high concentration of auxin is

favoured to enhance root production, but in some cases exogenous auxin is inhibitory (Thomas and Street 1970; Guichuan *et al.,* 2004).

Ethylene production rates and concentrations may be critical and may have varying effects on different stages of tissue culture, growth and development (Chi *et al.,* 1993). Work with Brussels sprout (*Brassica ver. 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 concentration of silver nitrate led to partial necrosis of turnip rape explants during long culture periods and silver ions have bactericidal activity, even concentrations as high as 15 mg l^{-1} silver nitrate did not have a negative effect on transformation of *Brassica rapa ssp. oleifera.*

Explants 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 most readily, e.g., immature embryos of strawberry have been extremely useful (Wang *et al.,* 1984). However, in some other plants, e.g., *Daucus carota,* regeneration can induced almost from any cell or part of the plant, e.g., excised embryos, hypocotyls, young roots, petioles, peduncles and protoplasts (Ammirato, 1983). In several cases, younger tissues have been found to produce more uniform

regeneration from differentiated cells (Dejong and Custers 1986; Hanisch *et al.,* 1986). A variety of explants including curd, hypocotyls and cotyledons have been tested for their ability to induce shoots of cauliflower (Table 3). Meristem are the basis unit used in plant micropropagation, they are the most genetically stable part of a plant and consequence the most suitable for production of true-to-type propagules. Micropropagation using curd meristems has been used for some time tomaintain cauliflower parent lines (Crisp and Walkely 1974), production of virus-free cauliflower (Walkey *et al.,* 1974), for the early screening of curd quality (Crips and Gray 1979), for chemical mediated mutagensis (Deane *et al.,* 1995), developing and induction mutants (Eed, 2001), and transgenic cauliflower plants (Kiffer, 1996). The technique of curd meristem micropropagation for cauliflower clonal propagation is now well established, but despite a few extensive studies (David and Margara 1980; Torres *et al.,* 1980a; Kumar *et al.,* 1992). Kiffer *et al.,* (1995) developed a new technique for rapid mass production of cauliflower propagules from fractionated and graded curd.

To initiate growth of the explants it is important to provide basic nutrients within the media, this usually consists of a mixture of salts which provide the essential macro and micro elements as well as carbon source (usually sucrose). The most widely used of the formulations available is that Murashige and Skoog media (Jansen *et al,* 1989). Culture medium effects on

Table 3. Summary of work on in vitro cultures of cauliflower (*Brassica oleracea* var. *botrytis*). Data not supplied is indicated by **

Explant	Pattern of culture growth	Basal medium and growth regulators (as quoted)	Topic of study	Reference
Floral meristem	Regeneration to plantlets	Margara + IBA, BA and GA$_3$ at a range of concentrations	Growth regulators and regeneration	Margara (1960)
Floral meristem	Regeneration to plantlets	LS + 0.025 mg/l kin and 8 mg/l IAA	Clonal propagation	Pow (1969)
Floral meristem	Regeneration to plantlets	LS + 2.5 mg/l kin and 8 mg/l IAA	Rapid clonal propagation	Walkey and Woolfitt (1970)
Inflorescence stem	Weight gain-callus	Whites + whole coconut milk and 0.5 mg/l NAA	Metal toxicity	Barker (1972)
**	Callus Differentiated callus	LS + 0.2 mg/l 2,4-D and 3 mg/l kin for callus, 5 mg/l kin for differentiation	Genetics of differentiation	Baroncelli et al. (1973 a)
Leaf vein Excised petals	Callus with differentiation Callus	LS + 0.2 mg/l 2,4-D and 3 mg/l kin As above, but with 5 mg/l kin when differentiation was required	Genetics of growth and differentiation	Baroncelli et al. (1973 b)
Leaf veins Excised petals	Callus Differentiating callus	LS + 0.2 mg/l 2,4-D and 3 mg/l kin LS + 0.1 mg/l 2,4-D and 3 mg/l	Genetics of growth and differentiation	Buiatti et al. (1974 a)
Leaf veins Excised petals	Callus Differentiating callus	3 mg/l kin and a range of 2,4-D concentrations	Genetics of growth and differentiation — growth regulator interactions	Buiatti et al. (1974 b)
Leaf veins Excised petals	Callus Differentiating callus	See Buiatti et al. (1974 a)	Correlation between in vivo and in vitro characteristics	Baroncelli et al. (1974 a)
Leaf vein	Callus	LS + 2 mg/l 2,4-D and 3 mg/l kin	Correlation of in vivo and in vitro characteristics	Baroncelli et al. (1974 b)
Floral meristem	Regeneration to plantlets	LS + 8 mg/l IAA and 2.56 mg/l kin	Clonal propagation	Crisp and Walkey (1974)
Floral meristem	Regeneration to plantlets	See Crisp and Walkey (1974)	Virus elimination	Walkey et al. (1974)
Floral meristem	Regeneration to plantlets	See Crisp and Walkey (1974)	In vitro selection	Crisp (1975)
Floral meristem	Regenerating cultures	See Crisp and Walkey (1974)	Selection against colour defects	Crisp et al. (1975 a)
Floral meristem	Regenerating cultures	See Crisp and Walkey (1974)	Selection against bracting	Crisp et al. (1975 b)
Floral meristem	Regeneration to plantlets	See Crisp and Walkey (1974)	Epicuticular waxes in vitro	Grout (1975)

Floral meristem	Regeneration to plantlets	See Crisp and Walkey (1974)	Transplanting	Grout and Crisp (1977)
Floral meristem	Bud neoformation	See Margara (1969) and IBA, IPA and NAA at a range of concentrations	Effects of auxin on regeneration in vitro	Margara (1977)
Floral meristem	Regeneration to plantlets	See Walkey and Crisp (1974)	Clonal micropropagation	Rahn-Scoher (1977)
Stem internodes	Regeneration to plantlets	See Crisp and Walkey (1974)	Plant regeneration	Trimboli et al. (1977)
Floral meristem	Regeneration to plantlets	See Crisp and Walkey (1974)	Transplanting	Grout and Aston (1977)
Floral meristem	Regeneration to plantlets	See Crisp and Walkey (1974)	Transplanting	Grout and Aston (1978a)
Floral meristem	Regeneration to plantlets	See Crisp and Walkey (1974)	Leaf anatomy	Grout and Aston (1978b)
Leaf lamina	Embryogenic callus	MS + 1 mg/l IAA and 0.5 mg/l kin	Somatic embryogenesis	Pareek and Chandra (1978)
Floral meristem	Regeneration to plantlets	See Crips and Walkey (1974)	In vitro selection for curd quality	Crisp and Gray (1979)
Floral meristem	Regeneration to plantlets	See Crisp and Walkey (1974)	Water loss in plantlets	Wardle et al. (1979)
Floral meristem	Callus — plantlets	MS + 0.1 mg/l BA and 1 mg/l NAA	Plantlet regeneration	Li and Qui (1981)
Floral meristem	Regeneration to plantlets	See Crisp and Walkey (1974)	Transplanting	Wardle et al. (1983b)
Floral meristem	Regeneration to plantlets	MS with various conc of NAA and BAP	Plant regeneration	Maroti and Bognár (1984)
Floral meristem	Regeneration to plantlets	See Crisp and Walkey (1974)	Transplanting	Short et al. (1984)
Seedling leaves	Protoplasts — plantlets	Sequence of media (see Pelletier et al. 1983)	Plantlet regeneration	Jourdan et al. (1985)
Floral meristem	Plantlet regeneration	See Crisp and Walkey (1974)	In vitro photosynthesis	Grout and Donkin (1987)
Cotyledons Hypocotyl Internodes Leaf lamina Stem apices	Shoot bud formation	MS + various levels of IAA, IBA and NAA (3 – 5 mg/l)	Plant regeneration	Pareek (1986)
Floral meristem	Regeneration to plantlets	See Crisp and Walkey (1974)	Transplanting	Short et al. (1987)

Abbreviations: LS, Linsmaier and Skoog medium; *NAA*, Naphthaleneacetic acid; *BA*, Benzyladenine; *IAA*, Indoleacetic acid; *MS*, Murashige and Skoog medium; *IBA*, Indolebutyric acid; *GA₃*, Gibberellic acid; *2,4-D*, 2,4-Dichlorophenoxyacetic acid; *kin*, kinetin.

regeneration have been demonstrated by several studies. Evans *et al.*, (1981) surveyed somatic embryogenesis in crop plants and found that 70% of explants were cultured on MS medium or a modification of MS. 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).

The use of antibiotic in culture media has recently become more widespread with the emergence of antibiotic resistance genes as selectable markers in transformation experiments and in transformation systems. In addition, cocultivation of *Agrobacterium tumefaciens* requires the use of an antibiotic to kill the bacteria. The antibiotic kanamycin, gentamycin and tetracycline have been found to be inhibitory to cell or tissue growth at comparatively low concentrations. Fiola *et al.*, (1990) indicated that the addition of 10 mg l^{-1} or higher 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 (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 control medium and the frequency of regenerating calli were 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 be considered in development of protocols for plant regeneration and more details and sources are overviewed specifically for tissue culture of cauliflower. Care should be given to the choice of explant material.

1.10 Plant Transformation

Until recently, the only available techniques to genetically improve agricultural crops were sexual hybridization and the 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 *et al.,* 2004).

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 (Cardoz and Stewart 2003; Cao and Earle 2003; Cheng *et al.,* 2003). 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 in dryland agriculture, the altered plants showed increased production (Moffat, 2002).

Sato *et al.,* (2001) reported that, when rice seedling kept at 42 °C for 24 h before being exposed to 5 °C for 7 d, did not develop chilling injury, 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. Dariusz (2003), indicated that several lines of transgenic tomato, over-expressing either APX or superoxide dismutase (SOD) have been established and shown to have increased resistance to environmental stress. Randy (1995) showed that Physiological and genetic evidence clearly indicates that the ROI scavenging systems of plants are an important component of the stress protective mechanism. The ability to manipulate the levels of specific enzymes of this pathway using gene transfer technology can be used to improve the stress tolerance of economically important plants.

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 mRNAs 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 defence network against oxidative damage caused by environmental stresses. Transgenic plants expressing *E. coli* catalase to chloroplasts with increased tolerance to oxidative stress indicate that ROI, such as scavenging enzymes, especially chloroplastic 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 abiotic stresses (Shigeru *et al.,* 2002; Ashima *et al.,* 1993).

1.11 Reporter gene (GUS)

All transformation system requires a reporter gene to aid selection of transformation and 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 *Agrobacterium tumefaciens* Ti plasmid (Jefferson, 1987; Jefferson *et al.,* 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. This means that GUS coding sequence is under the direction of the controlling sequence of another gene. Usually the GUS gene is under the control of the cauliflower Mosaic virus 35s promoter. 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-nitrophenyl glucuronide 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.

Further details about using PCR as a technique to detection a desired gene in *Agrobacterium tumefaciens* and evaluate the integration of resistance genes

(APX and SOD) in cauliflower by using GUS gene, Leaf disc assay will be

discussed in chapter 3.

1.2 Thesis aim and objectives

Aim

The ultimate goal of this research is the improvement of cauliflower (*Brassica oleracea var. botrytis L*) to be tolerant of abiotic stress (hydroxyproline, salinity and freezing) via mutation and genetic transformation.

Objectives

1- To evaluate previously created mutant lines under different levels of hydroxyproline, salinity, freezing and study the stability of these mutants.

2- To optimize protocol for transform cauliflower with the APX and SOD stress gene using *Agrobacterium* mediated transformation.

Chapter 2

Evaluation of abiotic stress resistance in mutated populations of cauliflower (*Brassica oleracea var. botrytis*).

2.1 Introduction

2.1.1 Tissue culture

Tal (1984) listed some of the advantages in using tissue culture for physiological studies for salt and hydroxyproline (hyp) resistance, as follows: 1- Experiments can be performed year-round since the growth of tissue culture is independent of seasonal fluctuations, 2- Tissue culture can be treated uniformly in a controlled way, 3- Relatively homogeneous populations of cells can be developed in tissue culture as compared with heterogeneous whole plants, thus providing a tool for studying the effect of stress on various components of growth, 4- Tissue culture can be used for studying mechanisms of salt and hyp tolerance on both cellular and whole plant levels, 5-The contribution of different parts of the plant to the response of the whole plant can be determined by studying their response in culture, 6-Protoplasts are especially useful for studying the involvement of the surface membrane in stress injury, 7- In cell culture system millions of cells can be screened and evaluated for their performance in a relatively small area, 8- In culture mutagenic agents can be added to induce variability.

There are however, also some disadvantages to using these systems in that surviving callus or tissues may consist of a mixture of resistant and

sensitive cell which escaped the selection pressure. Furthermore cells of the required phenotype may be surrounded by dead or non-growing cells and may be difficult to detect and acquire resistance through the production of a compounds which may by 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 and hyp. In most of the examples the enhanced tolerance was documented 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 and hyp 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. This opinion was borne out of their search for patents registered between 1992-1993, which revealed only a small number (10) of claims for increased salt resistance. 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 o putative resistant callus or lines that subsequently lose their tolerance.

It would appear that rigorous testing and retesting to establish stable and heritable tolerant lines must always follow selection.

Flowers *et al.,* (1985) indicated a very poor correlation between the performance of cells *in vitro* and the *in vivo* growth of plants and it can be concluded that selection for salt tolerance by screening of cultured cells in high NaCl medium is largely unsuccessful. However, it is has been demonstrated in several glycophyte species (Luttes *et al.,* 1996a; Luttes *et al.,* 1996b) and halophyte species (Vera-Estrella *et al.,* 1999), that the responses shown by salt stressed plants is partly determined by cellular properties. The implication of this is that although salinity resistance is a complex trait resulting from interaction between morphological and physiological properties, it is possible to select salt-resistance cell lines to regenerate valuable plant material that could be beneficial in crop breeding programmes. However, Dracup (1991 & 1993) concluded that selection for salt tolerance by selection of cultured cells which grow at high NaCl have been largely unsuccessful, probably due to erroneous assumptions that the mechanism of salt tolerance in cultured cells and whole plants are similar.

!.1.2 Breeding and selection for abiotic stress

\biotic environmental stresses particularly salt, freezing and drought are

najor limitations for plant growth and crop production worldwide. In the

:ase of economically important plants, abiotic stresses can cause

;ignificant losses to crop production and breeding for plants tolerant to

;alt, drought and frost damage has always been an elusive goal. Breeding

'or tolerance against cold and salt by classical methods of selection and

:rossing is a time consuming and, often, inefficient procedure. Improving

;alt and frost tolerance may however be achieved by direct gene transfer

Zhang *et al.,* 2000) or through DNA mutation (Zhu, 2000). As a

:omplementary approach to breeding activity, these methods provide an

)pportunity to improve a cultivar for a particular trait without disrupting

he genotype or breaking desirable linkages between existing genes.

\mong mutants lines released as commercial cultivars, 6% were reported

o tolerant to abiotic stress by Micke (1988b).

'he only feasible criterion for selection is the ability of plants to survive

'arious salt levels at different growth stages using biochemical markers

s selection tools (Muhammad, 2004) and *in-vitro* selection techniques

1ay provide an alternative way to select new genotypes with improved

roperties (Eed, 2001). Al-Shamma (1979), Madder (1976) and Norlyn

1980) reported that the reaction to salt stress varies with the stages of lant development and a given cultivar may be tolerant at one stage and ensitive at another.

Possible techniques of plant breeding for salinity tolerance are ntraspecific or interspecific crosses, chromosome manipulation, somatic lybridization, mutation or plant cell cultures (Nobel, 1983; Tal, 1983). During plant breeding, there are three different ways of applying salt reatments for selection that relate to differences in tolerance during intogeny, firstly, selection under continuous saline conditions over the intire growth cycle which was suggested by Epstein *et al.*, (1980); econdly, the variation of the concentration of salinity according to the ensitivity of the growth stage (Noble, 1983); and thirdly, selection at a ertain stage of plant growth with an appropriate salt concentration Noble, 1983).

Mahmoud (1991) disapproved of the use of growth measurements as election criteria for many reasons including: 1- this was a destructive lethod which was unacceptable for selection in early generations; 2- ometimes varieties show different responses at different salinity levels or variety may show a changed response in different environmental onditions at the same salinity level; 3- a genotype recognized as tolerant

nay already be vigorous or high yielding under normal conditions so that inder salinity conditions its yield, although reduced drastically, will emain better than other lower yielding genotypes.

Selection and breeding approaches to increase resistance might be more successful, with respect to achieving maximum attainable resistance, if selection is based directly on the relevant physiological mechanism. However, since the relevant physiological mechanisms have yet to be agreed upon, this makes this approach difficult.

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. Dix (1993) reported that improved stress resistance was achieved by selection for hydroxyproline resistance in cell culture. Hydroxyproline is a toxic analogue of the amino acid proline. Proline tends to accumulate in response to acclimation (Hasegaw, 2000). Van Swaaij *et al.,* (1986 & 1987) were the first to succeed, by means of hydroxyproline, with the *in-itro* selection of potato plants with increased frost tolerance.

2.1.3 Proline accumulation under abiotic stress

Conventional breeding programmes have used proline accumulation as a biochemical marker for increased frost and salinity tolerance in crops (Winkel, 1989; Samaras *et al.,* 1995; Delanney and Vernu, 1993; Silverira *et al.,* 2003). Many plants accumulate compatible solutes, such as proline, in response to non-damaging levels of abiotic stress and these are thought to play a role in acclimation, reducing the effects of stress as it becomes more serious (Aspinall and Paleg 1981; Hasegaw *et al.,* 2000). Proline levels can be manipulated in some species by *in-vitro* selection in the presence of hydroxyproline (Van Swaaij *et al.,* 1986 & 1987; Dix 1993). Selection in the presence of hydroxyproline can lead to proline over-accumulation in cell lines and eventually to plants with heritable stress resistance (Riccardi *et al.,* 1983; Dorffling *et al.,* 1997). Selection for high proline has also been used as a biochemical marker for increased frost and salinity tolerance in conventional crop breeding programmes (Winkel, 1989; Samaras *et al.,* 1995; Delauney and Verma 1993; Silverira *et al.,* 2003). Whilst proline it is not universally accepted as a successful selectant for all species some commercial proline over-accumulating cultivars of barley with improved frost resistance following hydroxyproline selection have been released (Tantau *et al.,* 2004).

!.1.4 Acclimation under frost stress

Freezing is a major environmental stress, inflicting economic damage on

crops and limiting the distribution of both wild and crop species, freezing

tolerance in plants is not a constitutive trait but rather is induced by

exposure to low but non-freezing temperature (acclimation).

Plant species have different potentials for cold acclimation. For example,

Chen and Li (1980) reported that various *Solanum* species differed in

their hardiness when grown under non-acclimating conditions. They also

differed in cold acclimation potential. Within a single plant species,

cultivars differ in cold hardiness, for example in wheat (Limin and

Fowler 1983).

Multiple mechanisms appear to be involved in membrane stabilization.

Protection against freeze dehydration induced damage is a major process

n freezing tolerance. This is achieved both by changes in membrane lipid

composition (Steponkus, 1984; Steponkus *et al.*, 1988, & 1993; Uemura

t al., 1997) and by accumulation of substances in the surrounding

cytosol. Solutes accumulating during cold acclimation include sugar

(Gilmour *et al.*, 2000), proline (Sahra *et al.,* 2000 and Zhizhong *et al.,*

2002) and betaines (Xin and Browse 2000).

!.1.5 Measurement of freezing tolerance

[here are several technique used to evaluate freezing injury in plants such

 is re-growth (Levitt, 1980), visual estimates of discoloration/browing

Kraut *et al.,* 1986), vital staining (Stadelmann and Kinzel 1972),

)rotoplasmic streaming and the ability of the cell to plasmolyse (Arora

ind Palta 1988), triphenyl tetrazolium chloride reduction (TTC) (Levitt

.980), leakage of amino acids (Pomeroy, Simimovitch and Wightman,

.970), different thermal analysis (Quamme *et al.,* 1972) and electrolyte

eakage (Levitt, 1980).

n cauliflower plants, it is possible to use just part of the vegetative

naterial such as a leaf or leaf segment and assess the frost-hardiness of

his using the electrical conductivity method (Dexter *et al.,* 1973; Fuller

t al., 1989; Deane 1994; Deane *et al.,* 1995;). Electrolyte leakage is the

)asis of the electrical conductivity test developed by Dexter, Tottingham

.nd Garber (1937) and is commonly used in conjunction with

)bservations of the degree of browning/water-soaking of tissues. The test

s based on the assumption that where there is cell membrane injury there

vill be greater efflux of solutes from cells (ions, sugar and proteins).

:lectrical conductivity is recorded after freeze injured tissue is incubated

.nd shaken in distilled water. Conductivity is recorded again after the

amples have been heat killed in the same solution which provides a

easure of total ions present in the tissue. Freezing injury is calculated as

he release due to freezing as a percentage of total ions. The advantage of

his method is that data can collected immediately or a few hours after

reezing. There are, however, a few problems with the conductivity

nethod; 1) a substantial amount of solute leak out can occur with

infrozen tissue when incubated in water, 2) sometimes not all the solutes

each out from heat-killed tissue, even when tissue is irreversibly injured

lue to freeze-thaw stress (Wisniewski and Arora, 1993). Zhang and

Villison (1987) and Fuller *et al.,* (1989) modified the conductivity

nethod to overcome these problems, by using vacuum infiltration to

xclude air which facilitates the filling of extracellular spaces with

istilled deionised water and improves ion leakage.

auliflower curd is known to be very responsive in tissue culture and a

0 cm diameter curd may carry up to 10 million meristems (Kieffer *et al.,*

998). Previous work has also demonstrated that cauliflower is

esponsive to mutagenesis and hydroxyproline selection resulting in

roline over-producing *in-vitro* lines (Deane *et al.,* 1995). This earlier

ork was restricted by the culture methods available and more recently a

rotocol for the production of thousands of responsive meristematic lines

f cauliflower in liquid culture has greatly enhanced the opportunity for

eveloping selection procedures (Kieffer *et al.,* 1995 & 2001). The work

ı this chapter reports the assessment of abiotic stress resistance of

auliflower lines following mutagenesis and hydroxyproline selection

sing this new protocol. Preliminary results have already been reported

Fuller and Eed 2003) and the current chapter reports the results of a

ırger population of lines and their stability following up to 15 routine

ub-cultures and 2 years storage at low temperature (5 °C). With the

ırger volume of data generated it was possible to compare the responses

f the lines both *in-vitro* and *in-vivo* and investigate correlations between

old, salt and hydroxyproline resistance and proline accumulation.

2.2 Aim and objectives

Aim

To screen one hundred cauliflower lines (mutant and control) under abiotic stress to assess putative resistance patterns previously reported (Fuller and Eed 2003; Eed 2001)

Objectives

1- To screen cauliflower lines (mutant and control) under salt stress *in-vitro*.

2- To screen cauliflower lines (mutant and control) under hydroxyproline stress *in-vitro*.

3- To screen cauliflower lines (mutant and control) under frost stress *in-vivo*.

4- To examine the effect of different abiotic stress (salt and hydroxyproline) on plant growth of cauliflower lines.

5- To determine the proline response in abiotic stress.

6- To estimate the stability of resistant cauliflower mutant lines against abiotic stresses after several sub-cultures.

7- To investigate the correlation between resistance to salt, hydroxyproline and cold treatment.

2.3 Materials and Methods

2.3.1 Plant materials

The January heading Roscoff F1 hybrid cauliflower Medaillon (courtesy of Elsoms Seeds Ltd) was grown in the field on the Seale-Hayne Estate, University of Plymouth, Devon, UK, according to good commercial practice (Anon, 1982) and curds harvested and taken to the laboratory. *In-vitro* microshoots were produced in liquid culture according to the technique described by Kieffer *et al.,* (1995 & 2001). This technique produced a high volume of single or double curd meristem explants in the size range 300 – 600 µm.

2.3.2 Mutagenesis and selection. (This was carried out previously by Eed 2001 but is included here for completeness)

Mutagenesis using N-nitroso-N-ethylurea (NEU) and N-Nitroso-N-methyl-urea (NMU) at 1 and 2.5 mM for 90 minutes was carried out in liquid culture 24 hours after preparation of the microshoots. Mutagens were removed by decanting and washing the microshoots three times in fresh liquid culture medium (Kieffer *et al.,* 1995). All techniques were carried out according to the safe working practices established by Mcabe *et al.,* (1990).

Selection was carried out by the addition of 3 mM hydroxyproline to the final liquid culture medium and incubating for 3 weeks. The experiment was

repeated 7 times and approximately 1.5 million microshoots were exposed to the mutagens. Green shoots surviving selection were removed from liquid medium and sub-cultured onto S23 solid culture medium (Kieffer *et al.*, 1995) without hydroxyproline to develop into shoots.   Shoots with obvious morphological abnormalities either died or were discarded. Selections were then subjected to a multiplication phase on S23M (S23 + Kinetin 2 mg l^{-1} and IBA 1 mg l^{-1}) to produce clones of each selection which were either rooted (Kieffer *et al.,* 1995) and regenerated or put into cold storage (5 $^{\circ}$C). A population of non-mutated/selected control clones was also prepared from the same curd material. These clones were periodically subcultured every 4-6 month. A number of clones were tested for their resistance to various stresses (Fuller and Eed 2003) and then maintained *in-vitro* over a period of 2 years and reassessed for their resistance to various stresses.

Prior to any physiological assessments the *in-vitro* cultures were sub-cultured to reinvigorate them. Existing plantlets were removed from culture pots and the apical meristem + 1 emerging leaf, and stem sections with a single node, were cut from the plantlets and sub-cultured onto S23 medium. Where clones of lines existed then at least one clone of each line was sub-cultured to the multiplication medium (S23M) in order to build up clone numbers for experimentation. Cultures were maintained in a growth room at 23 $^{\circ}$C, 16 hour photoperiod, 50 μmol^{-1} m^{-2} s^{-1} light intensity and plantlets with 3 to 4

leaves were ready for testing for stress resistance 4 to 5 weeks after sub-culturing.

In-vivo clones of most of the lines were obtained by weaning of plantlets (Plate 1) 3 - 4 weeks after subculture in S23 medium. Plantlets were immersed in fungicide solution (Dithane 2 g l^{-1}) for 5 minutes prior to potting in trays filled with peat-based compost. The trays were placed in an incubator and shaded with a greenhouse shading material (Plate 2) to encourage establishment and reduced plate-bleaching maintained in a glasshouse under natural daylight supplemented with a 16 h photoperiod and a minimum temperature of 15 °C. After 10 days the vents of the incubator were opened to allow a gradual reduction of relative humidity. After 2-3 weeks *in-vivo*, roots had developed and the plants were removed from the incubator (Plate 3) and re-potted into 14 cm diameter pots and grown-on in the glasshouse (Plate 4). Plants typically had 3 to 4 leaves at the point of testing for stress resistance and were 8 to 9 weeks old post-weaning.

Plate 1. Process of transfer *in-vitro* plant growing on agar medium to *in-vivo* condition

Plate 2. Cauliflower plantlets under weaning processes

Plate 3. Cauliflower Plantlets after weaning (3 weeks-old)

Plate 4. Cauliflower plantlets after 9 weeks-old in glasshouse

2.3.3 Analysis of salt resistance

Plants were screened for NaCl resistance using a leaf disc assay. One centimetre diameter leaf discs were cut from the leaves of plants of both *in-vitro* and *in-vivo* lines. Leaf discs from *in-vivo* plants were first surface sterilized in 70 % ethanol for 10 seconds, followed by continuous shaking for 2 minutes in 10 % commercial bleach solution (sodium hypochlorite) followed by three rinses in sterile distilled water. *In-vitro* grown plants were used direct from culture vessels. Fifteen leaf discs were cut from each plant and placed in 3 replicate petri-dishes containing 20 ml sterile liquid medium (M&S salts at 4.4 g l^{-1}) supplemented with NaCl at concentrations of 350 mM and 550 mM for *in-vitro* and *in-vivo* plantlets respectively. Salt damage was assessed, as greenness, after 7 days using a four point score as follow: 0=white, 1= slightly green. 2= moderately green, 3= green and the data was presented as a score.

2.3.4 Analysis of hydroxyproline resistance

Leaf strips approximately 5 mm wide, were cut from the first and second leaves of both *in-vitro* and *in-vivo* plants. *In-vivo* leaves were first surface sterilised (as for NaCl testing). Five leaf strips were placed in petri dishes containing solid S23 medium, supplemented with two concentrations of hydroxyproline: 3 mM and 10 mM for *in-vitro* and *in-vivo* shoots, respectively. These two concentrations were applied in this experiment as in

other published work on cauliflower where Eed 2000 and Deane *et al.,* 1996 found that 3 mM and 10 mM are a critical level for selection *in-vitro* and *in-vivo,* respectively. After 4 weeks leaf strips were scored for resistance categorized by whether they displayed total, partial or no resistance evidenced by bleaching (disappearance of chlorophyll) ,as described in 2.3.3, where the data was presented as a score: 0=white, 1= slightly green. 2= moderately green, 3= green.

2.3.5 The effect of salinity and hydroxyproline on growth of cauliflower lines

*In-*vitro shoot tips (apex plus 1 emergent leaf) of the cauliflower lines were subcultured onto S23 medium supplemented with either 3 mM hydroxyproline or 350 mM NaCl and grown for 4 weeks in the culture room. After for 4 weeks the damage was assessed using five points vigour score where:

0=dead (no growth); 1= weak growth (plant just growth not more than 1 cm over culture medium with yellow leaves); 2 = moderate growth (length of shoot not more than 3 cm with few leaves); 3= good growth (plant growing well with few leaves, length of shoot between 3-4 cm); 4= strong growth (plant cover all the pots area with lot of leaves and long shoot).

2.3.6 Estimation of free proline content

As described in 2.3.5, the first fully expanded leaf was then removed for determination of leaf proline content. Leaves were immediately frozen in a -80 °C freezer and then dried in a freeze drier to constant weight. Dried leaf (0.04g), with 2 ml ultra clean water, was finely ground in a pestle, taken into an eppendorf tube and centrifuged at 15000 rpm for 15 minutes. Three aliquots of the resultant supernatant (100 μl) were diluted to 1 ml in 3 crimp top sample vials. HPLC was used to determine proline levels using a Dionex AAA-Direct Amino Acid Analyser System. A series of proline standards analysed by the acid ninhydrin method (Bates *et al.*, 1973) and the Dionex system, found a good linear fit between the two methods (Fuller and Paisey personal communication) and concluded that the Dionex system is reliable, versatile, convenient and a suitable replacement for the more regularly used acid ninhydrin method. At first, 2 vials containing water were run on the Dionex to clean the column and stabilize the detector's reading. Then, 5 standard samples were run to obtain a calibration curve for all analyses. Afterwards, vials containing prepared samples were placed in the auto sampler and run overnight. *Peaknet*[TM] software gave output curves from which the concentration of proline was integrated.

2.3.7 Analysis of frost tolerance

Frost tolerance was assessed using 6 to 7 week old *in*-vivo plants. Both non-acclimated (direct from glasshouse) and acclimated (after 14 d at 2 °C, 8h photoperiod 150 μmol^{-1} m^{-2} s^{-1} irradiance) plants were assessed. Leaf discs, 1 cm in diameter, were cut from the newest fully expanded leaf avoiding the main veins and leaf margins. Twenty discs from each plant were randomly divided into sets of five discs and placed into glass test tubes. The tubes were chilled to 2 °C and ice added to each tube to ensure ice nucleation. The tubes were placed in a Sanyo incubator programmed to run to 2, -3, -5 and -7 °C (freezing rate 5 °C h^{-1}) with a two hour hold at each temperature and duplicate samples removed at the end of each 2 hour hold. Tubes were allowed to thaw overnight at 4 °C and then 12.5 ml of distilled water was added to each tube. After 5-6 hours incubation at 20 °C, the electrical conductivity of the water was determined using a Walden precision conductivity meter fitted with a platinum electrode (Fuller *et al.,* 1989). The tubes were autoclaved at 15 psi for 5 minutes and cooled to 20 °C and the conductivity re-measured. The degree of damage caused by freezing was expressed as a relative conductivity reading after correcting for the background conductivity of distilled water,

Relative Conductivity percentage (RC %) =

Test reading / Final reading x 100.

2.3.8 Statistical Analysis

Scheirer-Ray-Hare (SRH) test is anon parametric equivalent of a two way ANOVA with replication which it recommended and used to analysis data presented as a score (Calvin, 2003). By using Minitab programme this test was running to analysis this type of data. Analysis of variance (ANOVA) for other data (parametric data) was employed and performed using Minitab and means compared using the least significant difference (LSD) test. Relations between data sets were investigated using simple sequential polynomial curve fitting to maximum R^2 values. The data was examined to ensure that it is normally distributed by employed normality test (Anderson Dorling) using Minitab.

2.4 Results

2.4.1 Assessment of salt resistance in leaf discs

Overall, leaf discs from both *in-vitro* and *in-vivo* plants exposed to NaCl treatment for 7 days showed over 80% damage for the control population whilst the selected population showed a significant degree of resistance with less than 50% damage (Figure 9; Plate 5 & 6). The data was normally distributed and among the selected population there was a significant linear correlation between the *in-vitro* and *in-vivo* scores (Figure 10). There was considerable variation in resistance demonstrated by individual lines and selections S2 and S17 showed a very high degree of resistance (Figure 10).
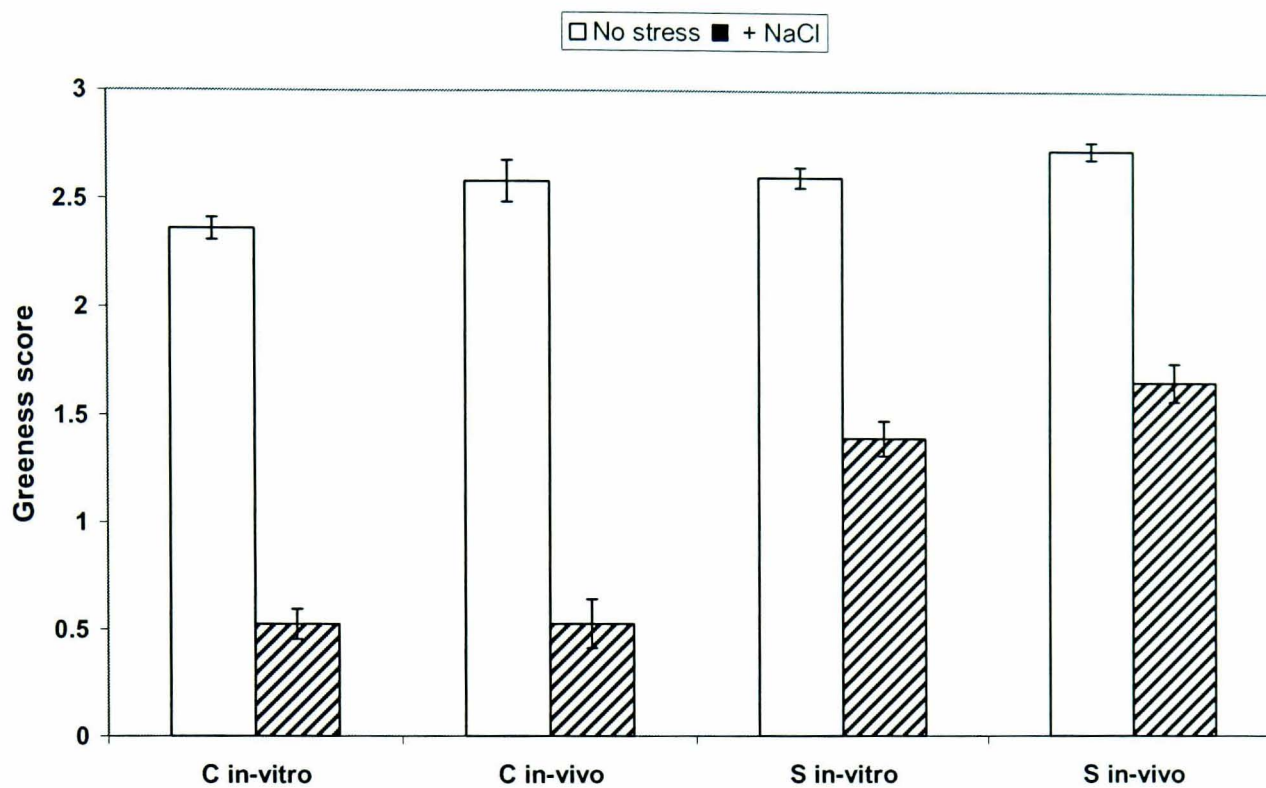
Figure 9. The effect of NaCl on the greenness of leaf discs of control(C) and selected populations (S)of cauliflower lines for *in-vitro* and *in-vivo* plants. I bar = SE and n=15

$y = 0.6205x + 0.733$
$R^2 = 0.5102$
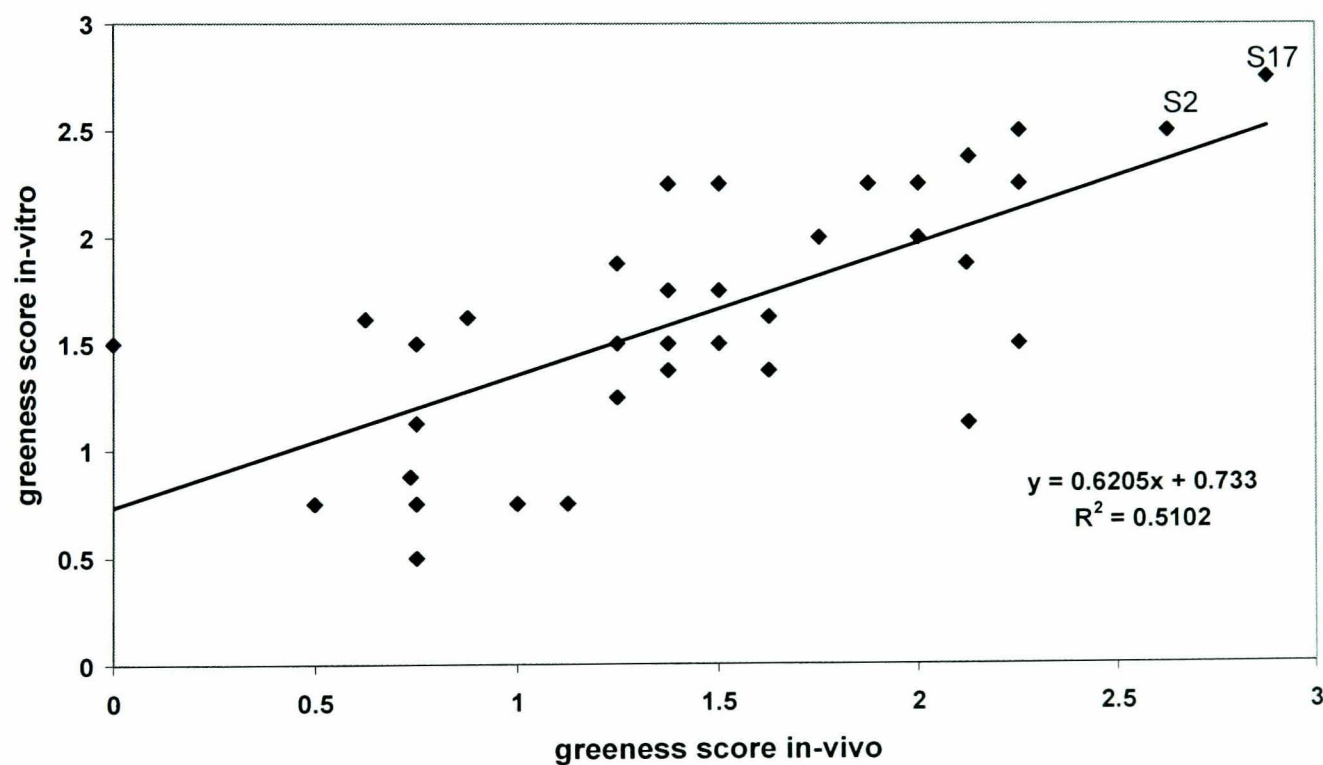
Figure 10. The relationship between *in-vitro* and *in-vivo* responses to the salinity treatment in the selected population (S)
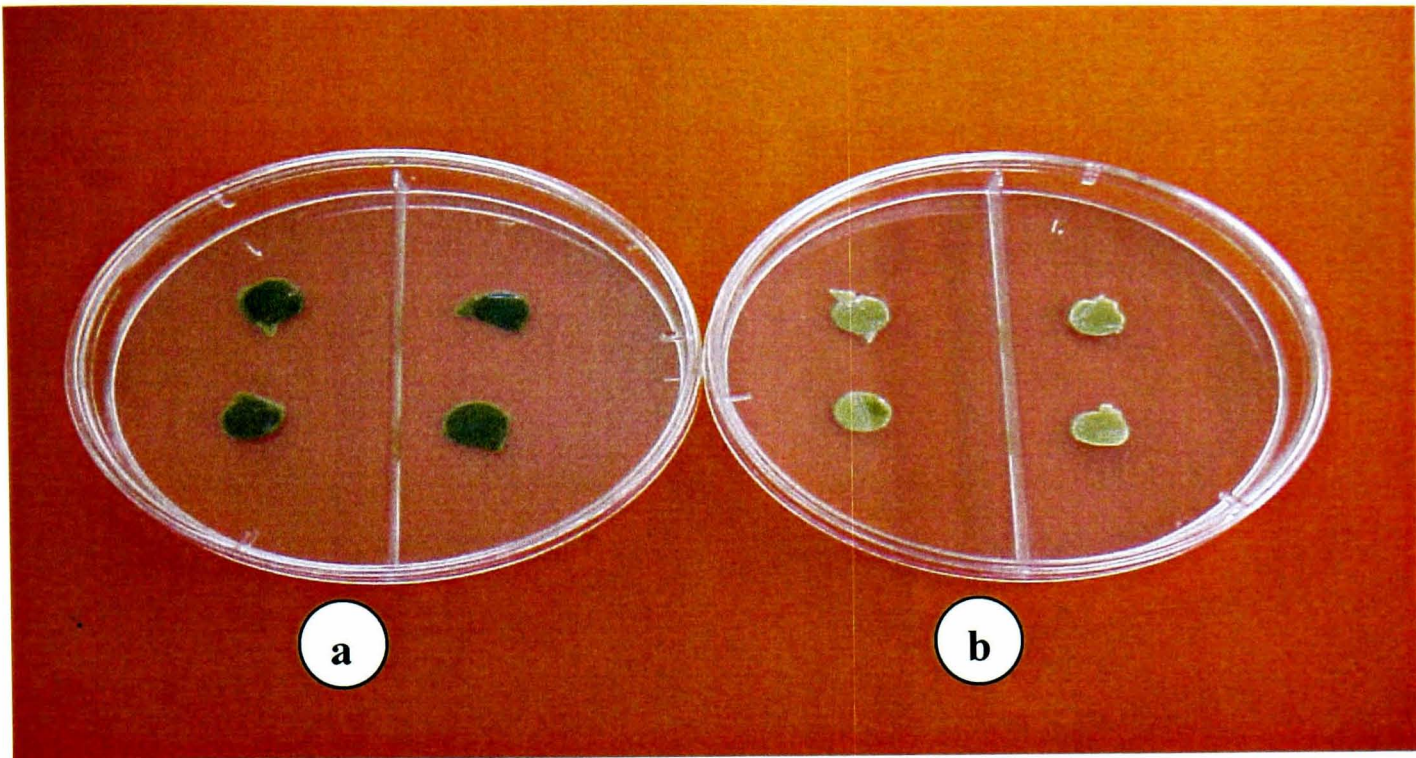
1)
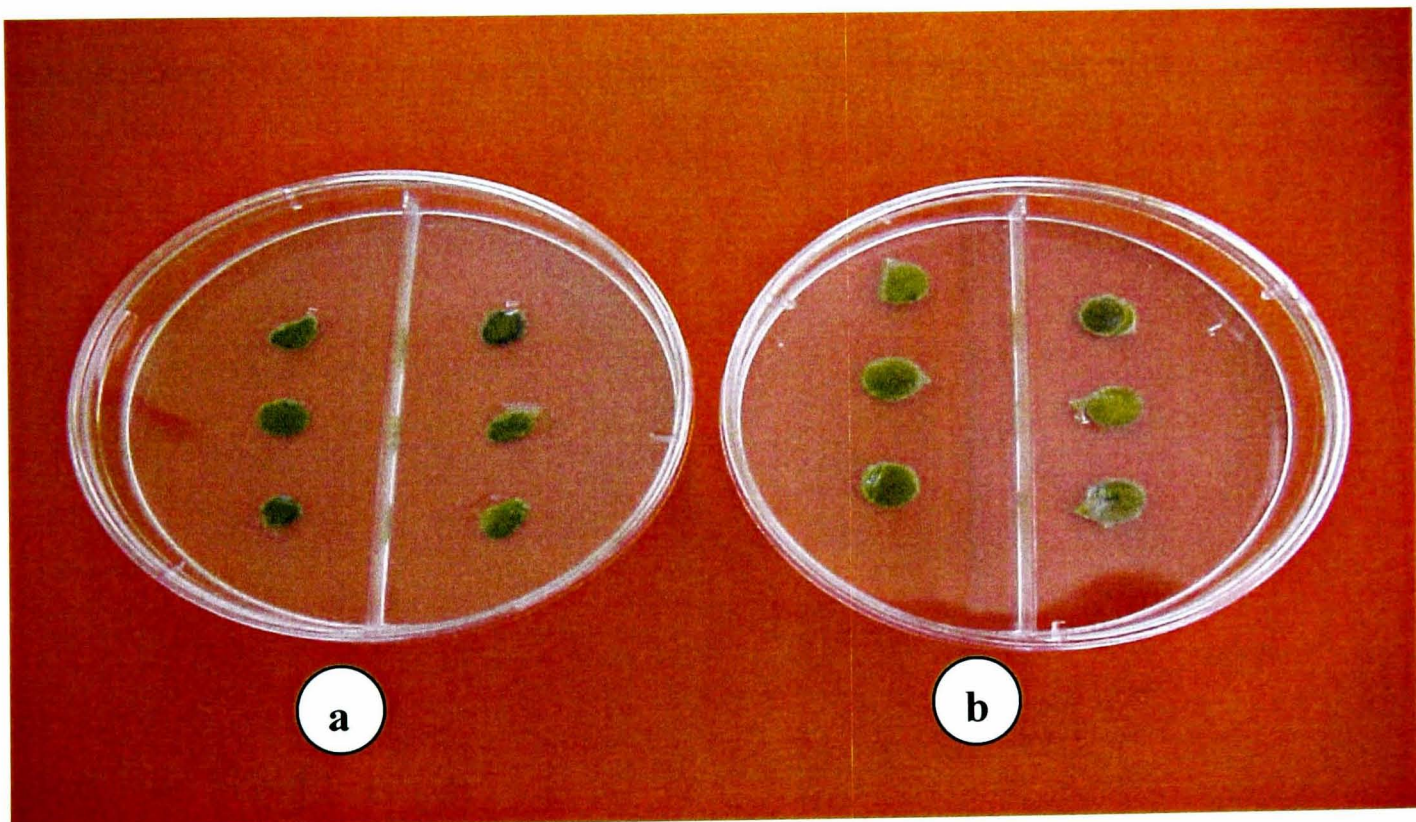
2)

Plate 5. The effect of salinity treatment 350mM NaCl on the leaf discs of cauliflower 1) control (C) and 2)mutant lines (S) for *in-vitro* after 7 days for the treatment. a) no-NaCl b) 350 mM NaCl
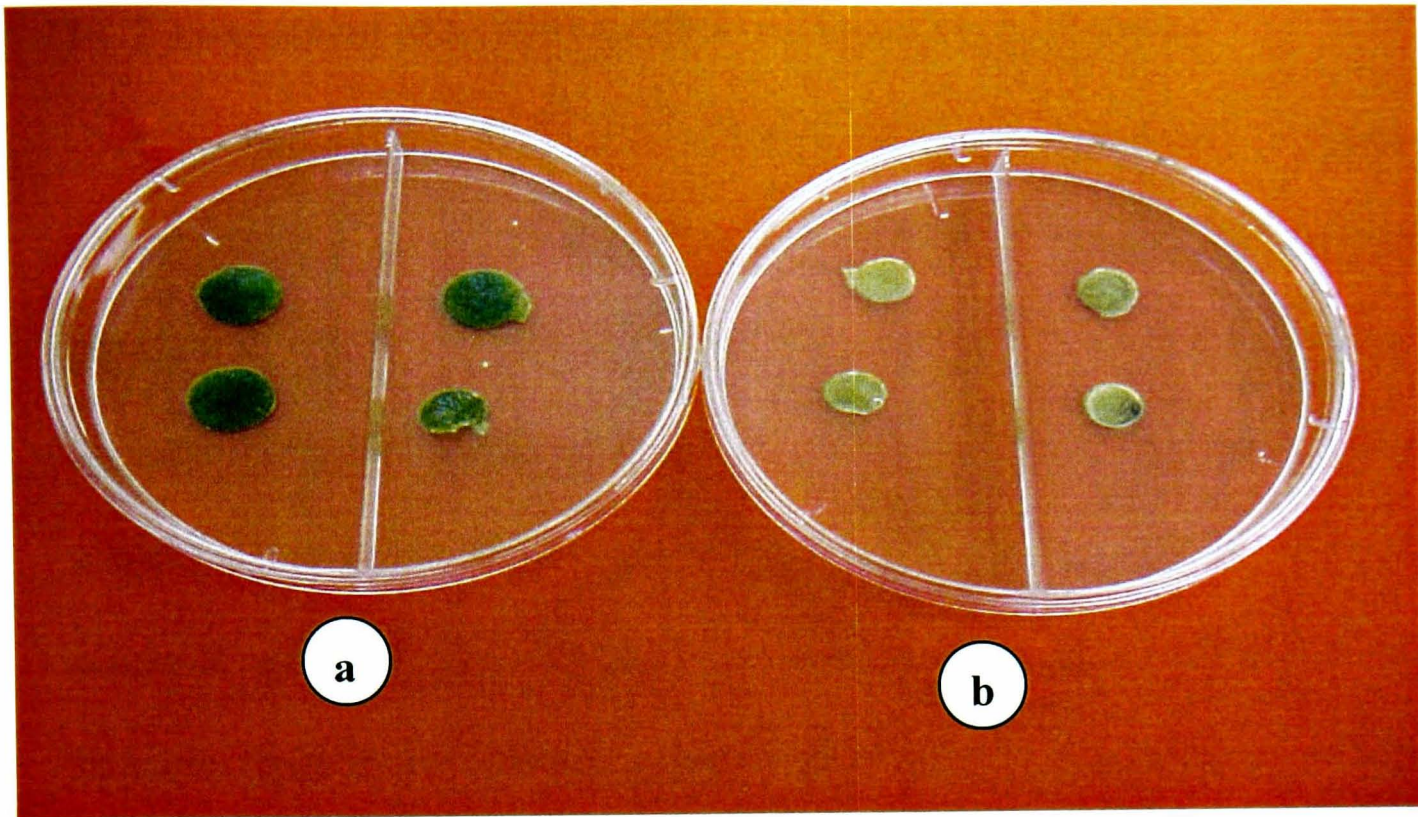
1)
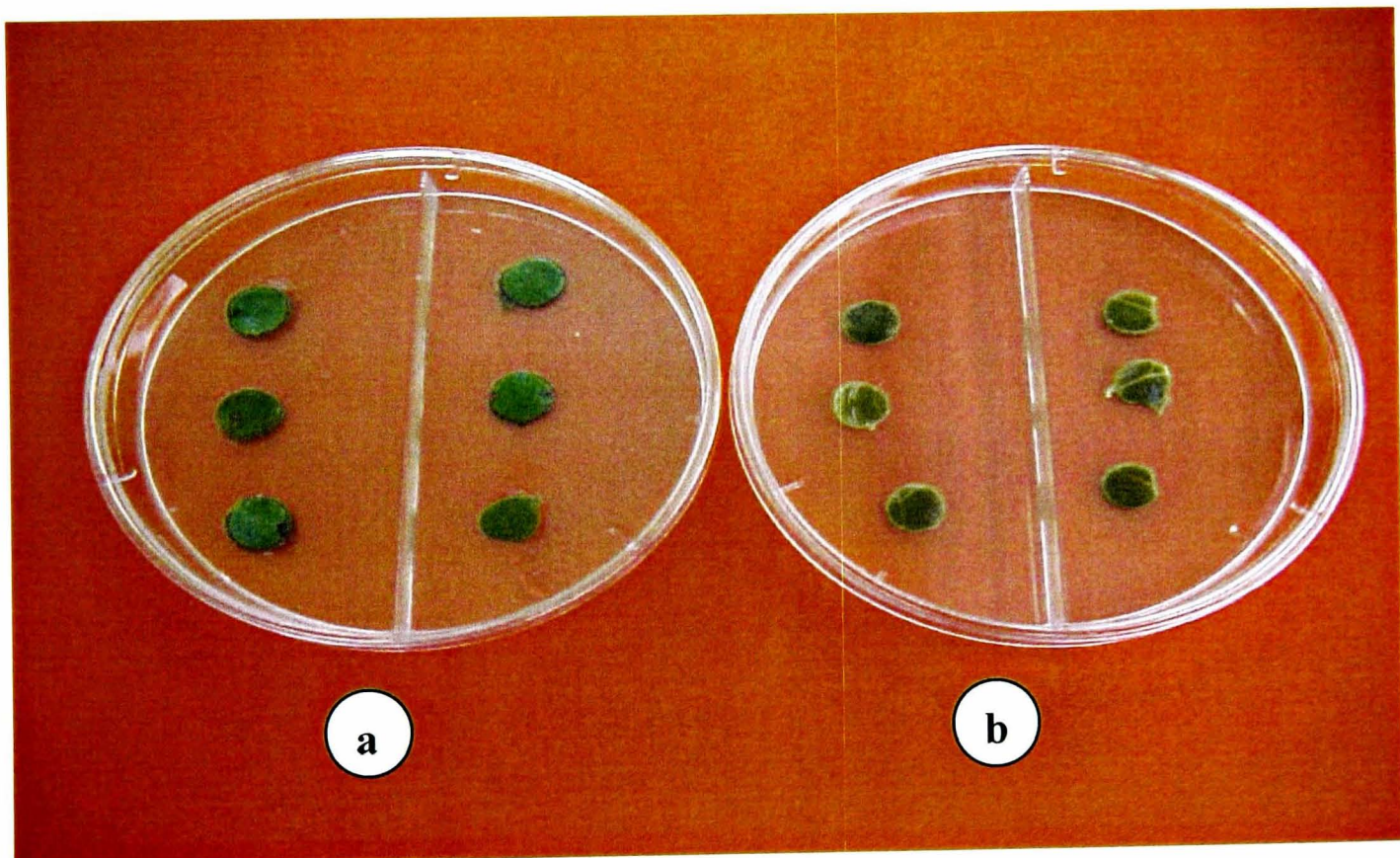
2)

Plate 6. The effect of salinity treatment 550mM NaCl on the leaf discs of cauliflower 1) control (C) and 2)mutant lines (S) for *in-vivo* after 7 days for the treatment. a) no-NaCl b) 550 mM NaCl

2.4.2 Assessment of hydroxyproline resistance in leaf strips

Overall, leaf strips from both *in-vitro* and *in-vivo* plants exposed to hydroxyproline treatment showed a high degree of damage for the control population whilst the selected population showed a significant degree of resistance (Figure 11 Plate 7 & 8). Among the selected population there was a significant positive curvi-linear relationship between the *in-vitro* and *in-vivo* scores (Figure 12). As with NaCl resistance there was considerable variation between lines in the selected population (Figure 12) with selected lines S2, S12, S6 and S1 showing high levels of resistance.
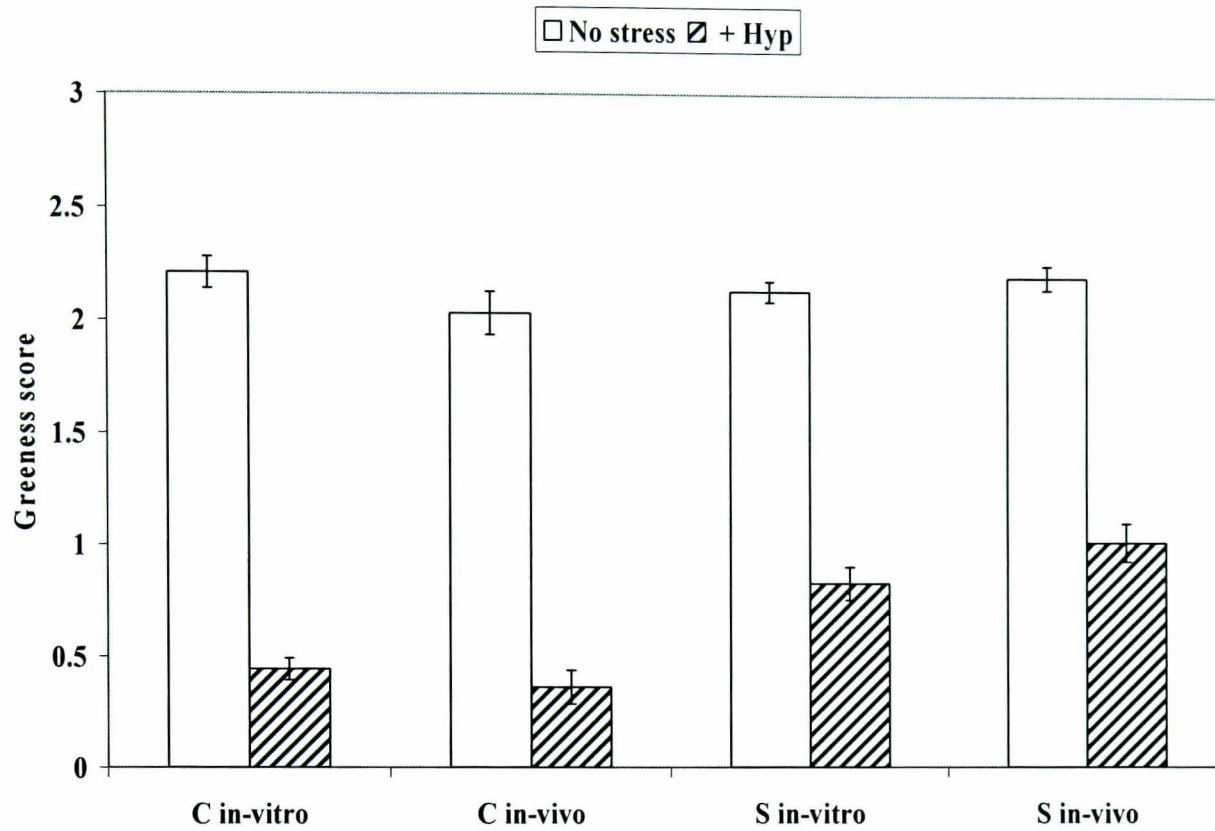
Figure 11. The effect of hydroxyproline (Hyp) on the greenness of leaf strips of control (C)and selected populations (S) of cauliflower lines for *in-vitro* and *in-vivo* plants. I bar = S.E & n=15.
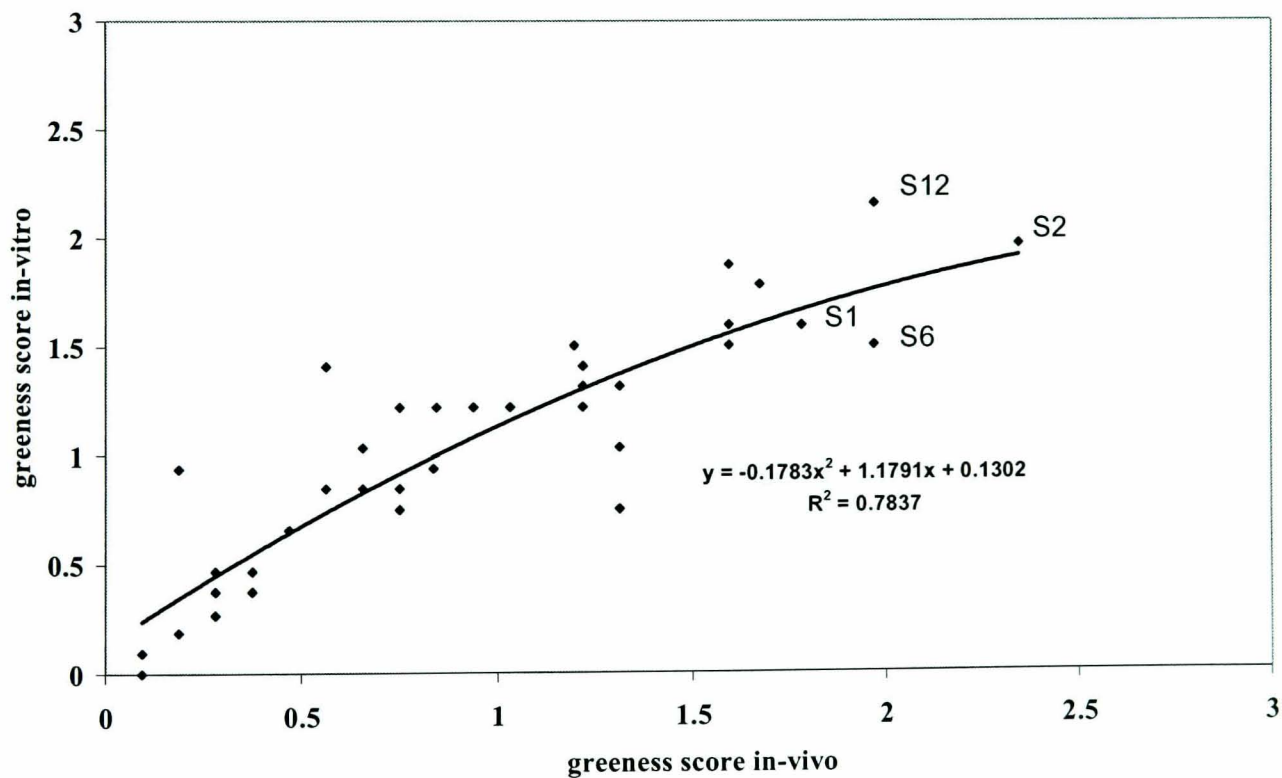
Figure 12. The relationship between *in-vitro* and *in-vivo* responses to the hydroxyproline treatment in the selected population (S).
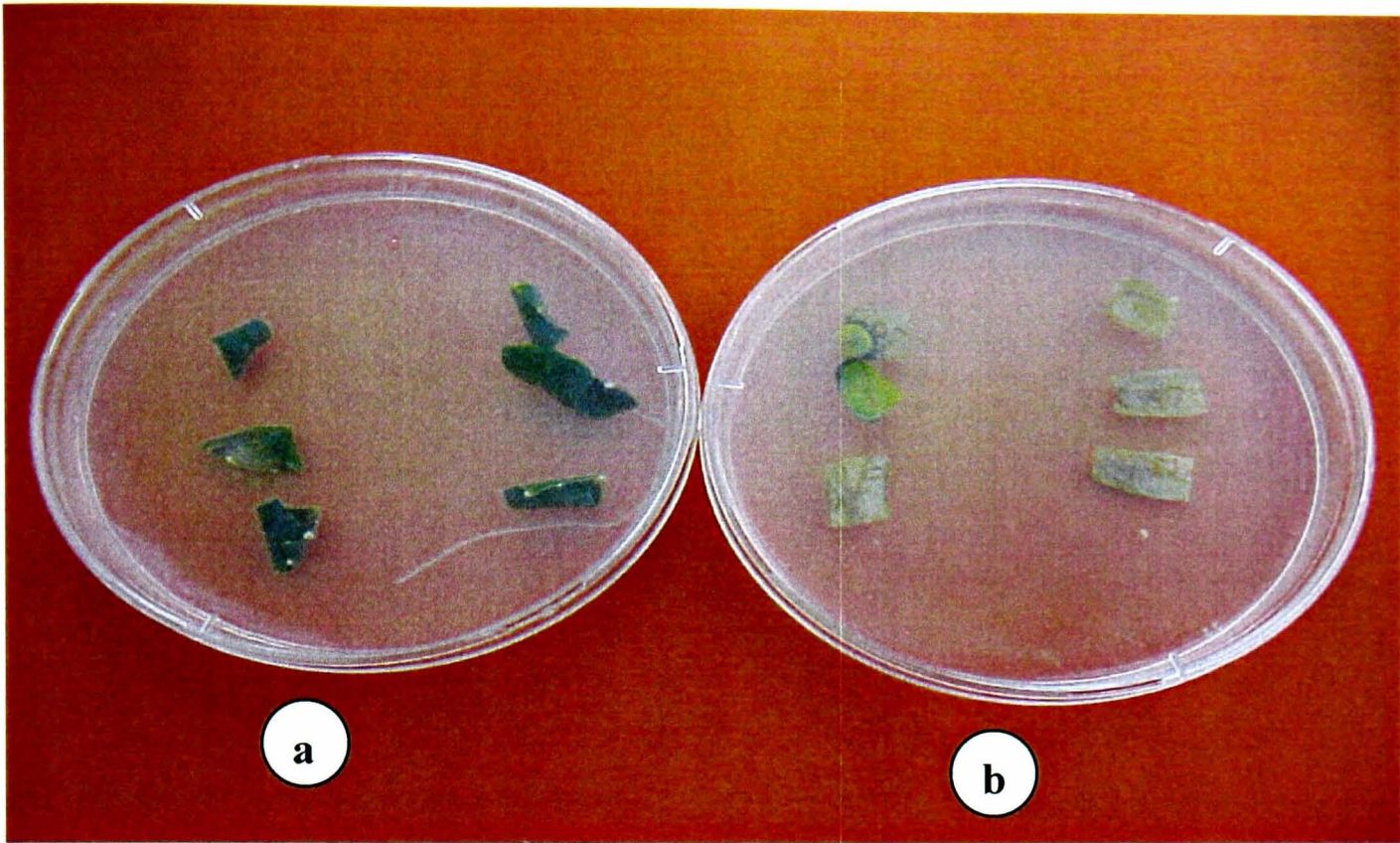
1)
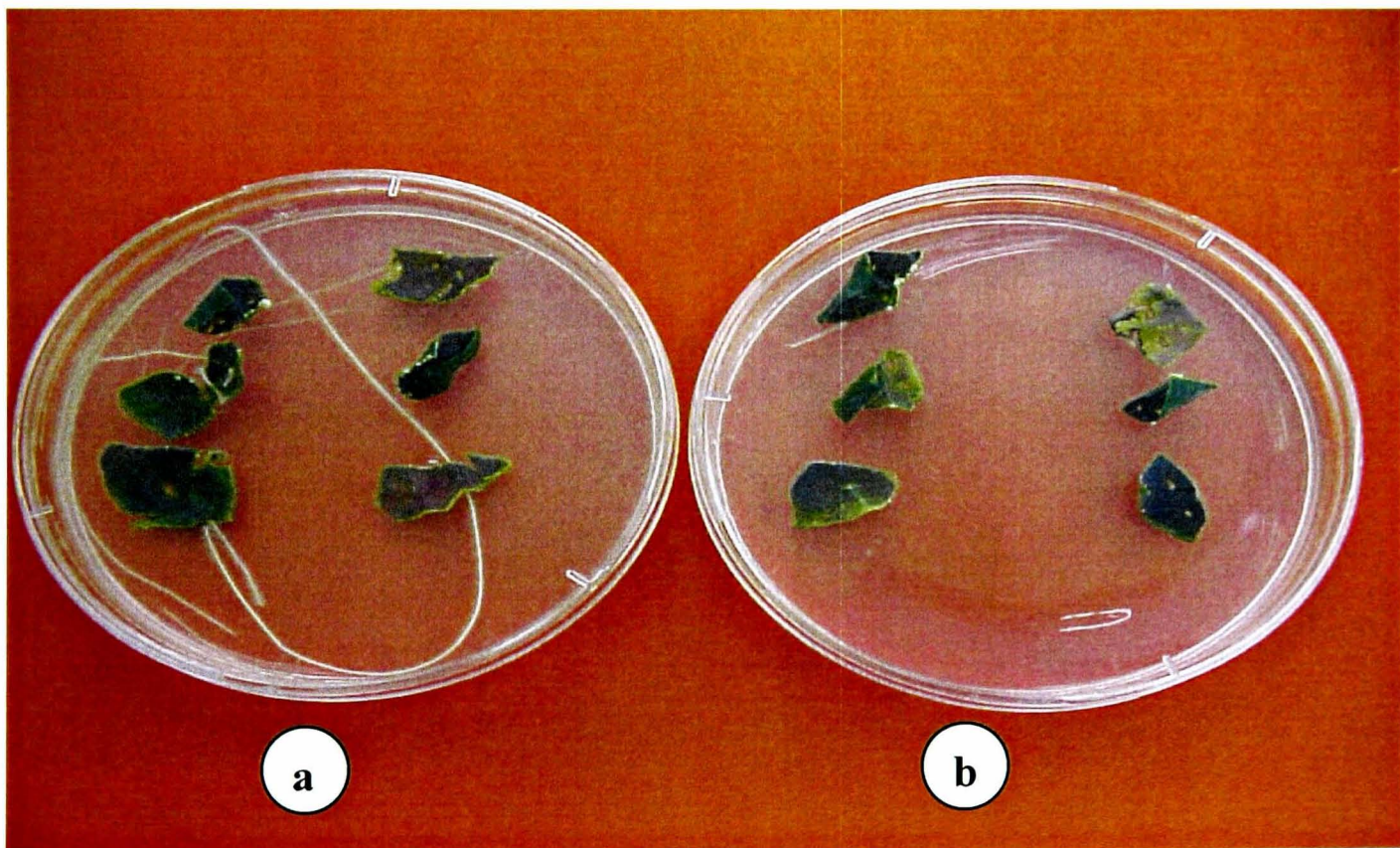
2)

Plate 7. The effect of hydroxyproline treatment on the leaf strips of cauliflower 1) control (C) and 2)mutant lines (S) for *in-vitro* after 4 weeks for the treatment. a) no-hyp b) 3 mM hyp
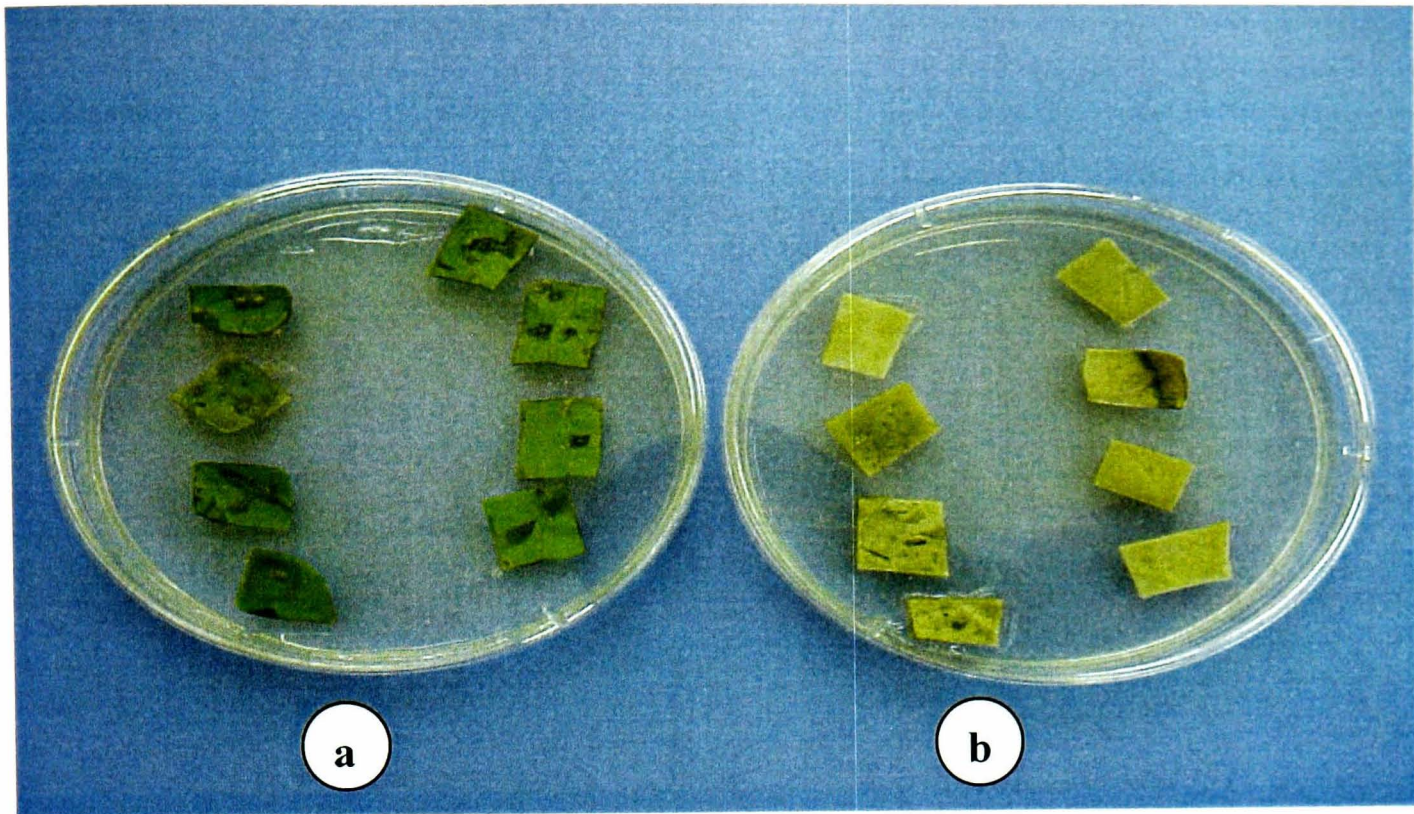
1)
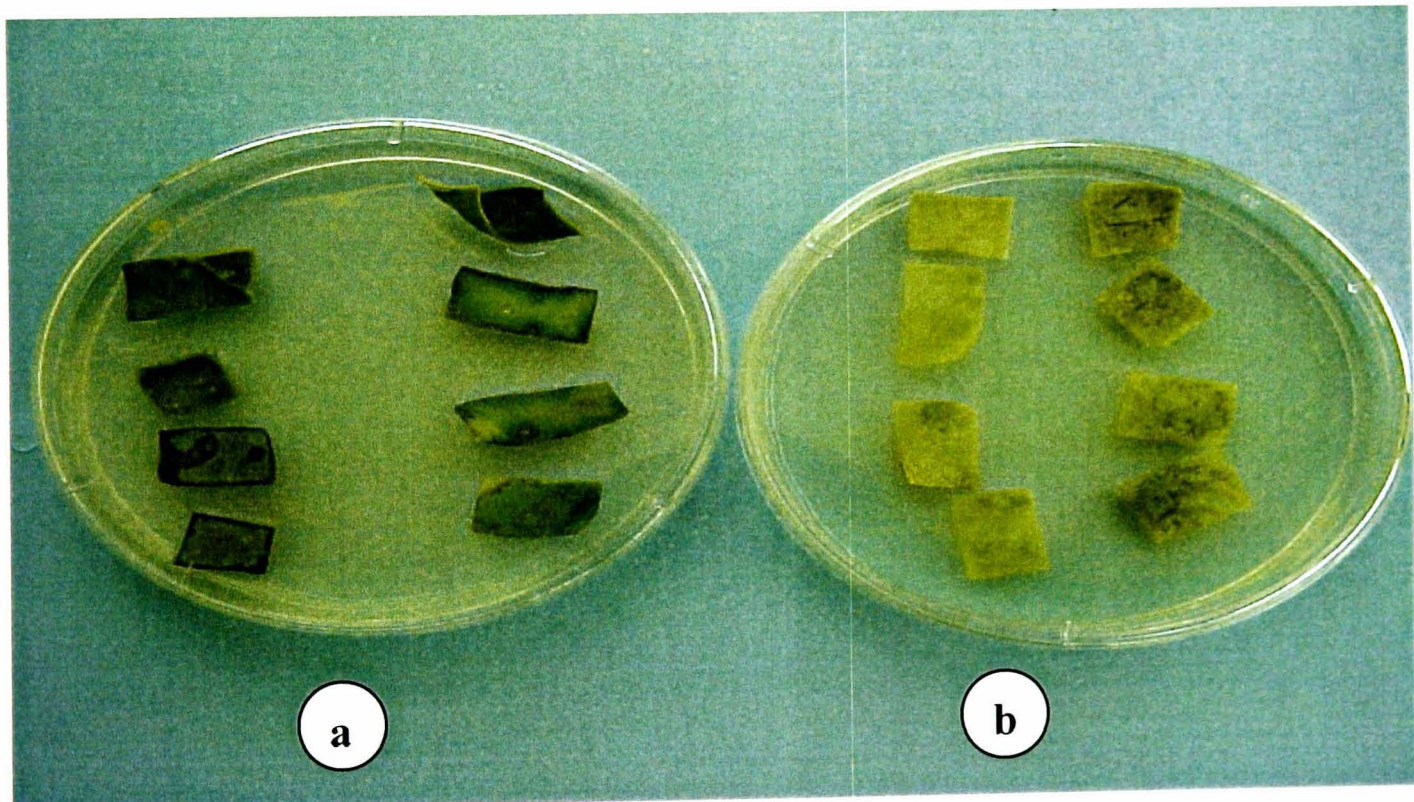
2)

Plate 8. The effect of hydroxyproline treatment on the leaf strips of cauliflower 1) control (C) and 2)mutant lines (S) for *in-vivo* after 4 weeks for the treatment. a) no-hyp b) 10 mM hyp

2.4.3 The effect of salinity and hydroxyproline on growth of cauliflower lines

Data for mean growth of cauliflower mutant lines shoots exposed to 350 mM NaCl or 3 mM hyp for 4 weeks in solid culture showed a slight decline in vigour at the fourth week probably indicating the need for subculturing while control shoots showed a fastest decline as exposed to same concentration for four weeks (Plate 9&10). Mutant line (S) had surviving clones at the NaCl (350 mM) and hydroxyproline (3 mM) concentration, while control lines (C) were unable to complete their growth under the same concentration of NaCl and hydroxyproline. By using (SRH), statistical test, significant different between the lines in their response to NaCl and hydroxyproline concentration was observed (Figure 13).
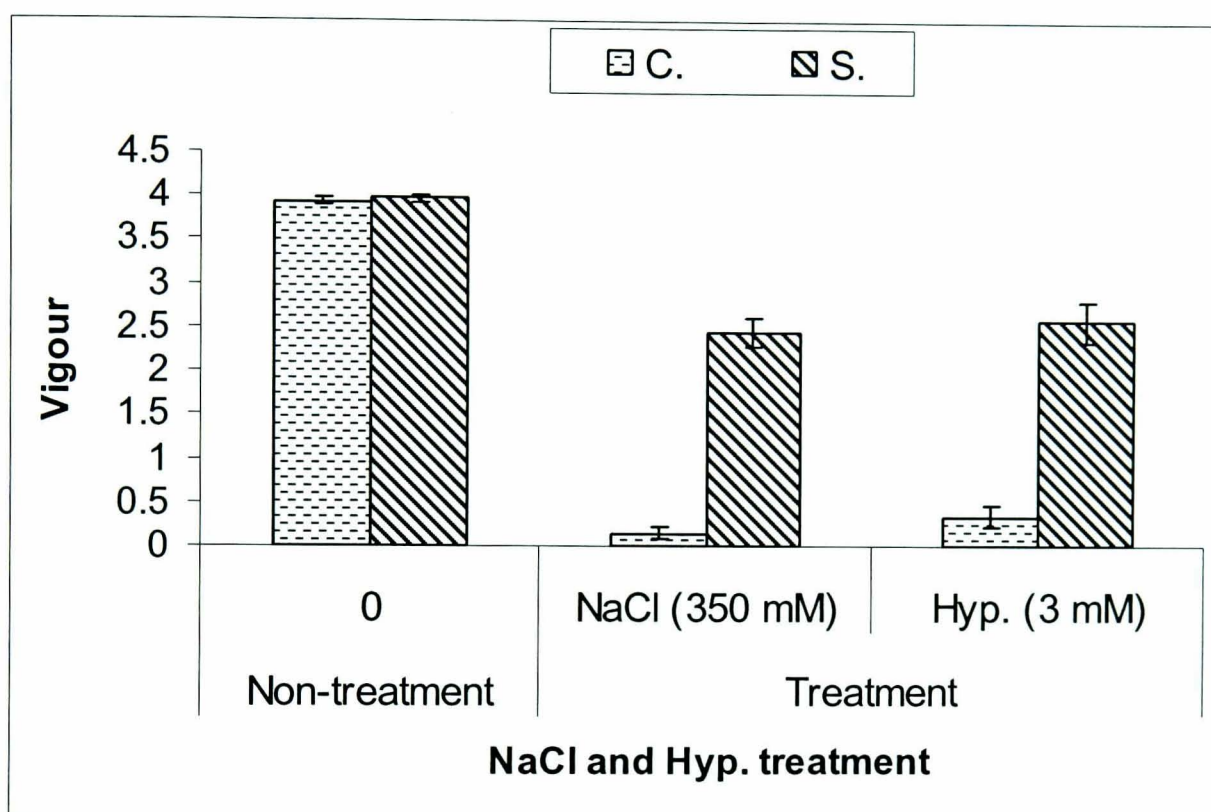
Figure 13. The effect of NaCl and Hydroxyproline (Hyp) on the growth of cauliflower control and cultured derived from *in vitro* mutant line after 4 week from subculture on S23 media. C= Control; S=selected lines. I bar=SE & n=15
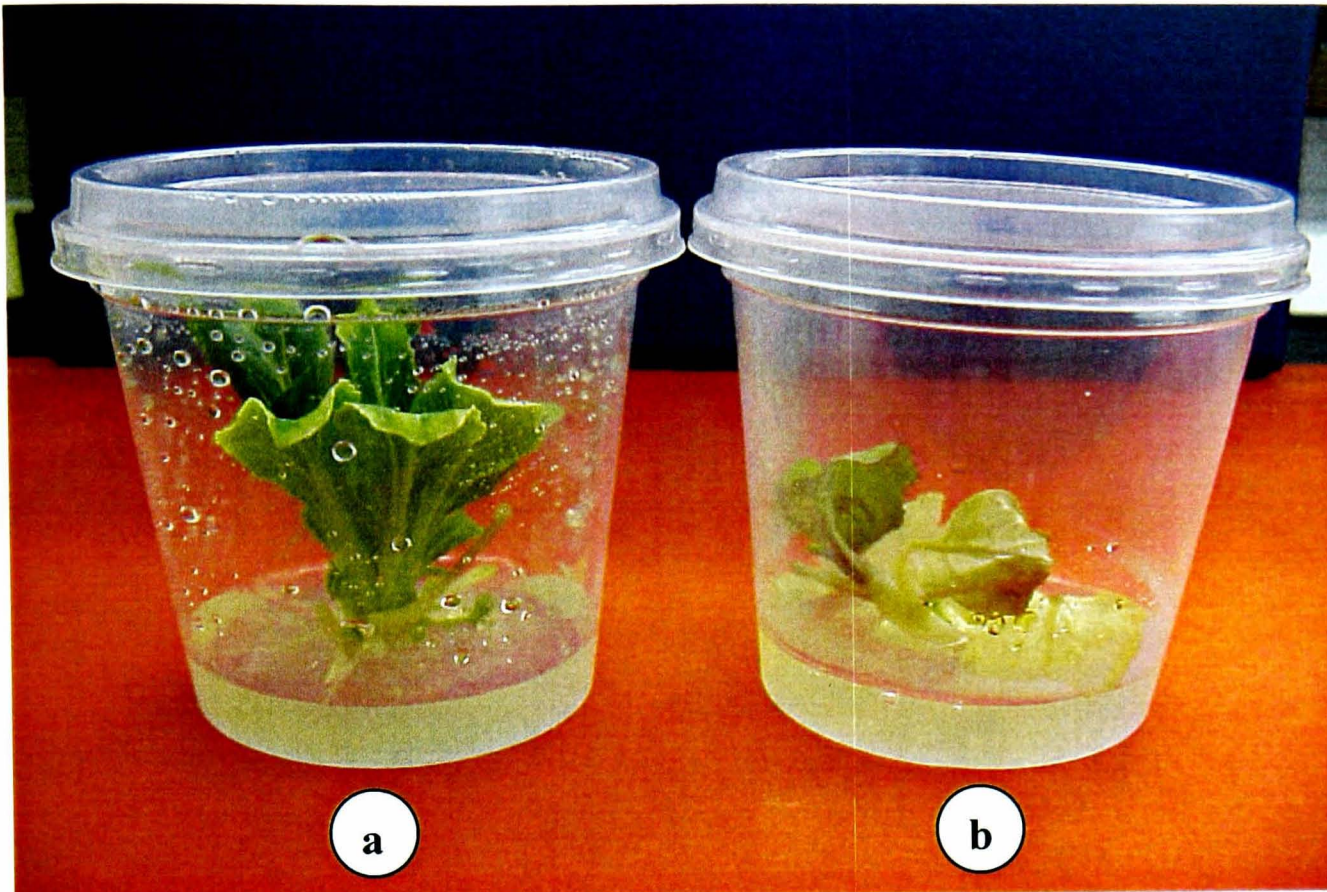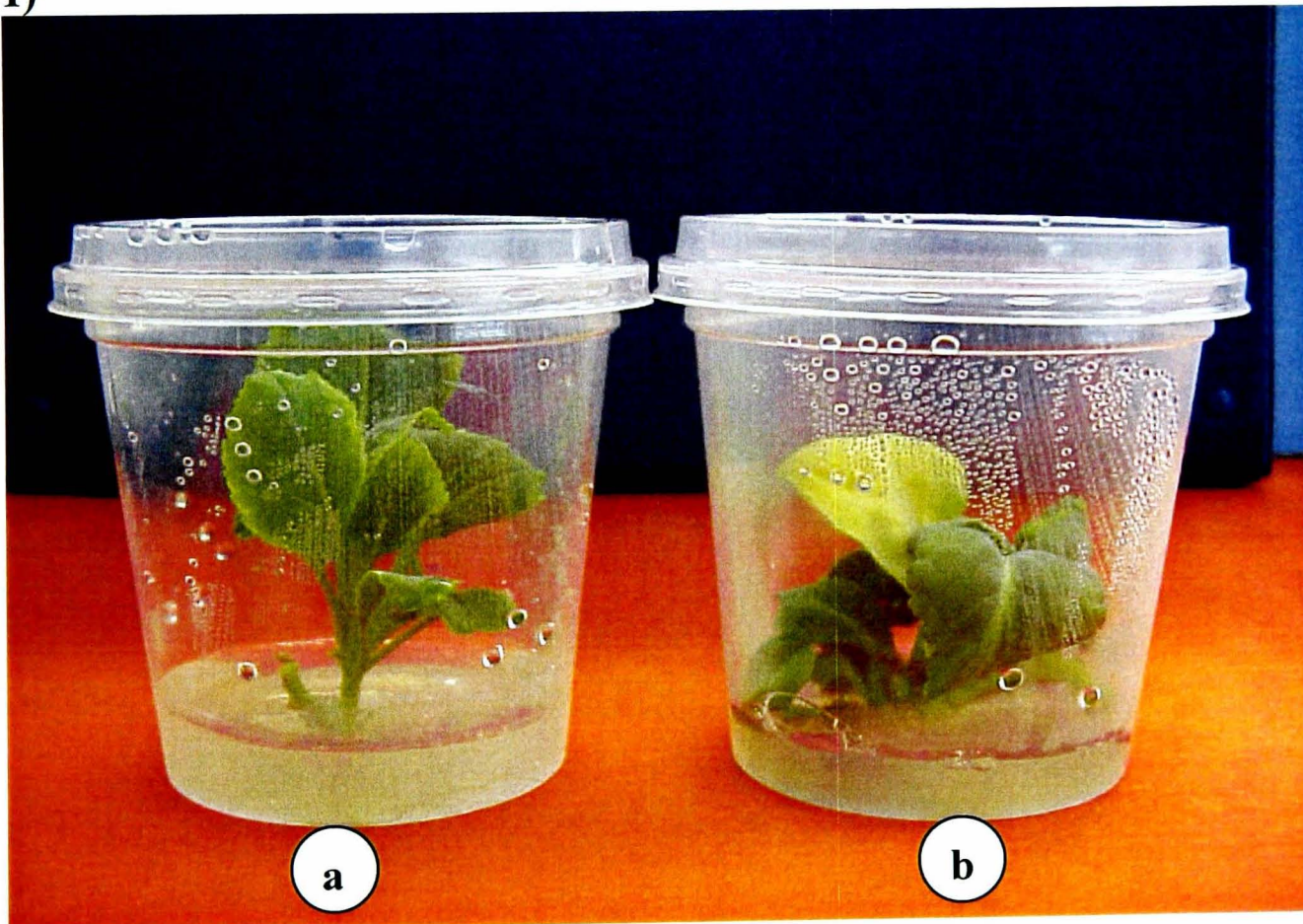
Plate 9. The effect of salinity on the growth of Cauliflower 1) control and 2) mutant line after 4 week from the subculture *in-vitro* on S23 media . a) control treatment (0.00) b) NaCl treatment (350 mM)
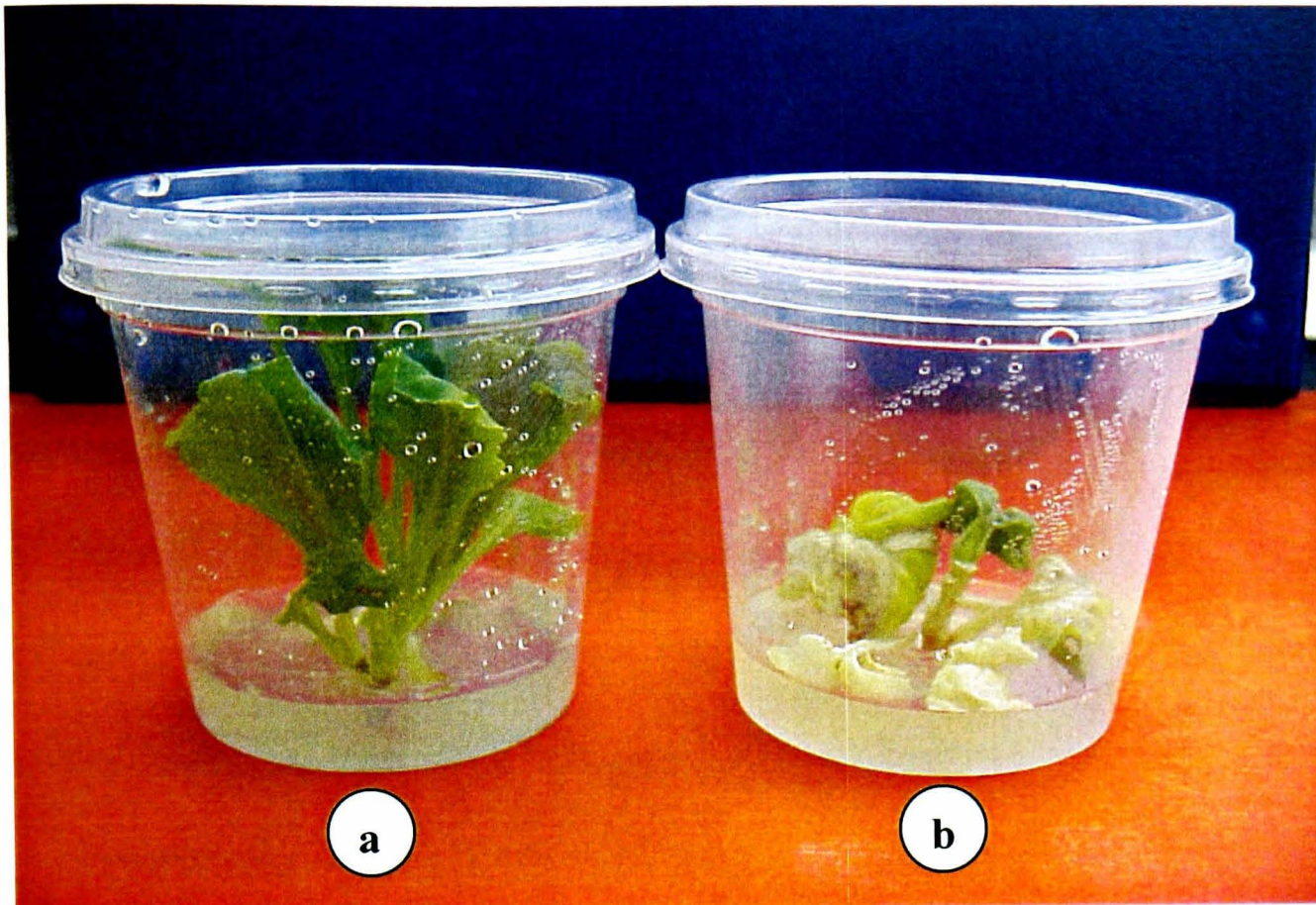
1)
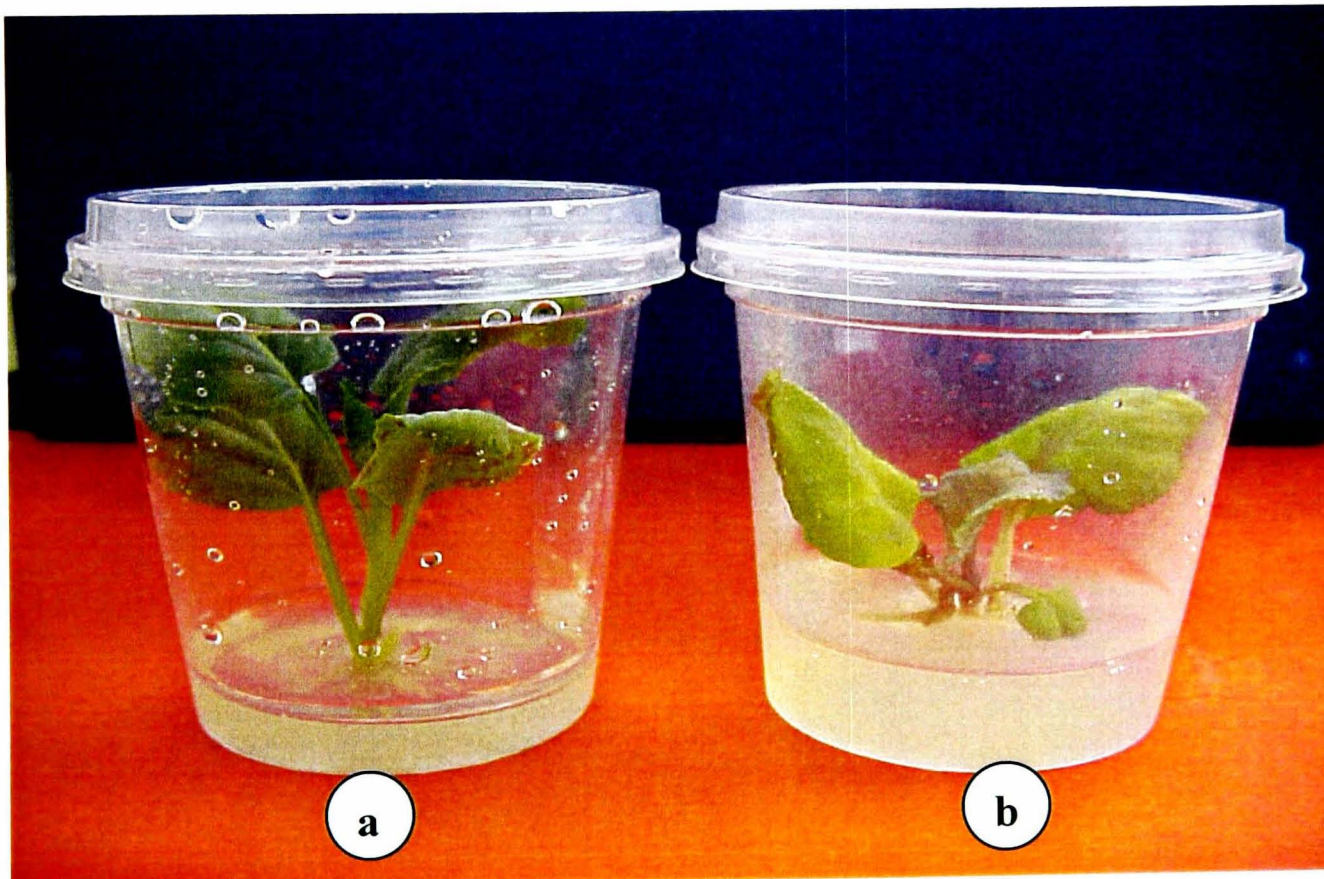
2)

Plate 10. The effect of hydroxyproline treatment on the growth of Cauliflower 1) control and 2) mutant line after 4 week from the subculture *in-vitro* on S23 media a) control treatment (0.00) b) hyp treatment (3 mM)

2.4.4 Assessment of leaf frost resistance

Frost damage increased at progressively lower freezing test temperatures with -7 °C leading to high levels of damage. Acclimation significantly improved resistance in both populations at all test temperatures but the improvement was generally only small with a 1 °C improvement due to acclimation. Significant linear correlations between test temperatures and between acclimated and non-acclimated responses of individuals demonstrated good reliability of the data (Figure 14). The selected population (S) had significantly improved frost resistance at all test temperatures compared to the control under both acclimated and non-acclimated conditions (Figure 15). In common with salinity and hydroxyproline stress the selected population demonstrated a wide range of frost resistance expression between lines with S2, S4, S1 and S21 showing the highest degree of frost resistance (Figure 14).
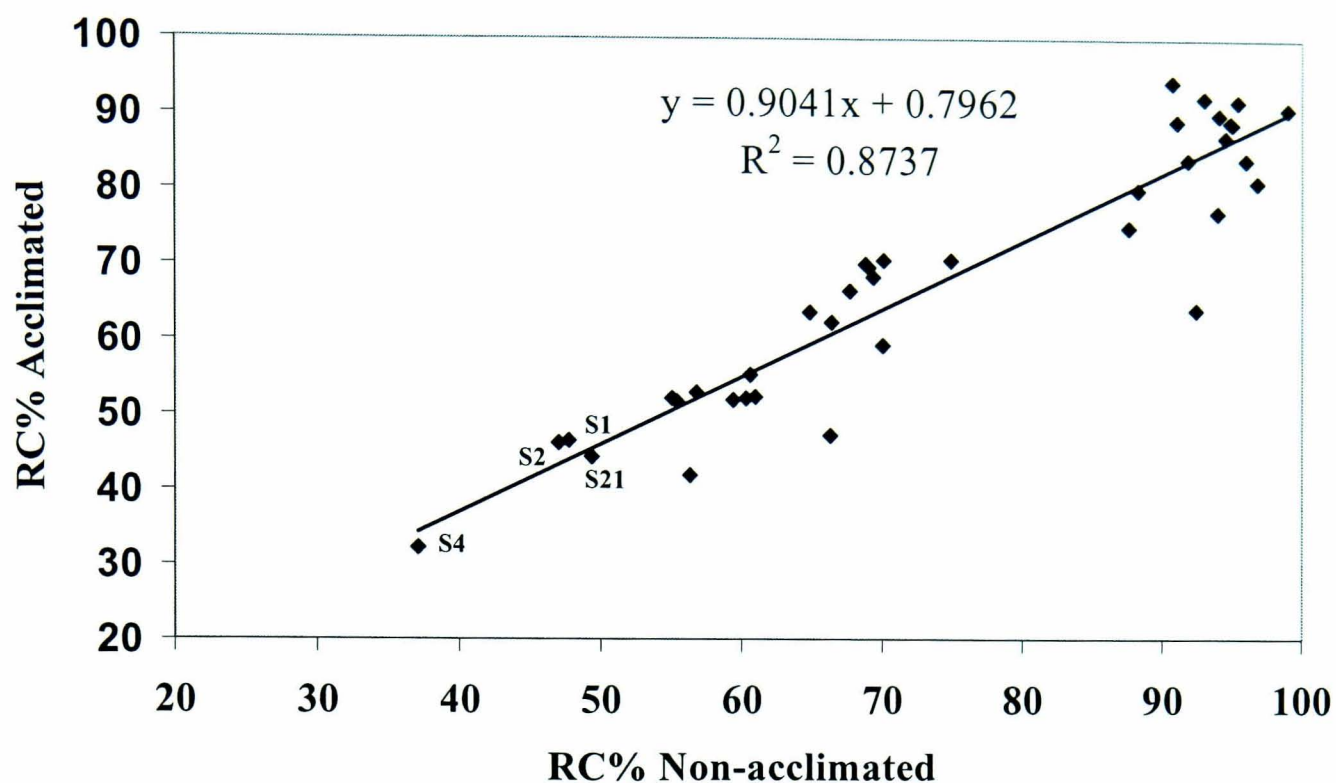
Figure 14. Correlation between acclimated and non-acclimated frost hardiness expression at -7 °C by using leaf disc (*in-vivo* plant) for the selected population (S) (n = 20).
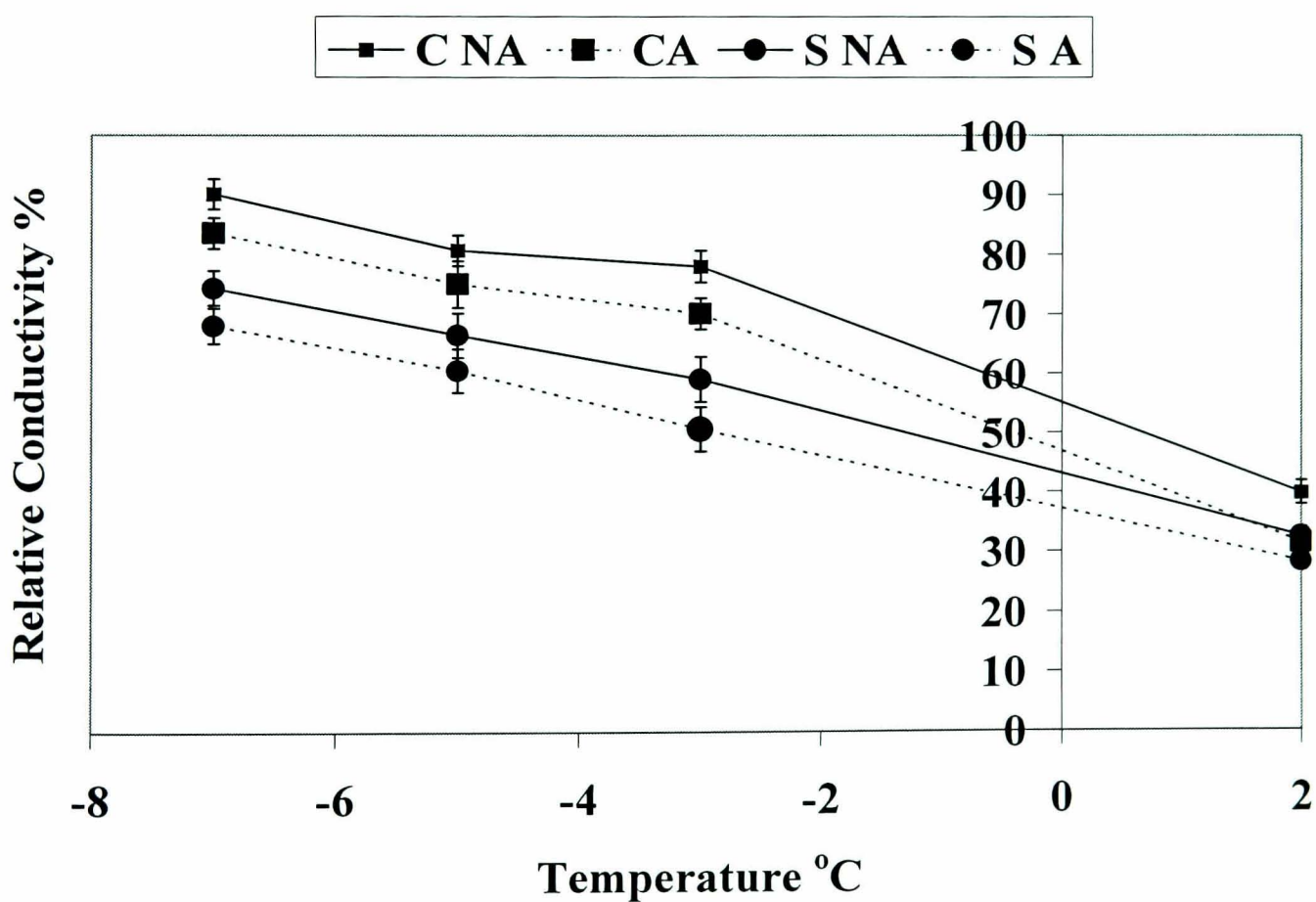
Figure 15. Effect of acclimation (A) and non-acclimation (NA) on frost resistance of *in-vivo* control (C) and selected (S) cauliflower populations

2.4.5 Proline accumulation as related to NaCl and hydroxyproline stresses

Proline contents of the control population averaged less than 10 µg mg^{-1} under both non-stress and stress (NaCl and hydroxyproline) conditions whereas with the selected population proline content was significantly higher under non-stress conditions and much higher under stress conditions (Figure 16). The greatest increase in proline content was when the selected population was stressed with NaCl with an overall average 17.5 fold increase over the control population.

Within the selected population there was considerable variation in the levels of proline expressed under stress with the highest responding genotype (S17) expressing 926 µg mg^{-1} under NaCl stress, nearly 100-fold increase over the control.

There was some correlation of proline levels with level of resistance although this was not strong ($R^2 = 0.3156$ for NaCl resistance and $R^2 = 0.1128$ for hydroxyproline resistance) and despite some high proline/high resistant lines such as S2, S17, S9 and S1 (Figures 17 and 18) there were also some high proline low resistant lines particularly under hydroxyproline stress eg S7 and S82 (Figure 18).

There was no overall correlation in the selected population under the two stresses (Figure 19). Thus, lines which were high proline expressors under NaCl stress (S1, S17, S6, S21, S64) tended to be low expressors under hydroxyproline stress and *vice versa* (S7, S82, S4). Only two genotypes (S9, S31) showed same level of high proline under both stresses.
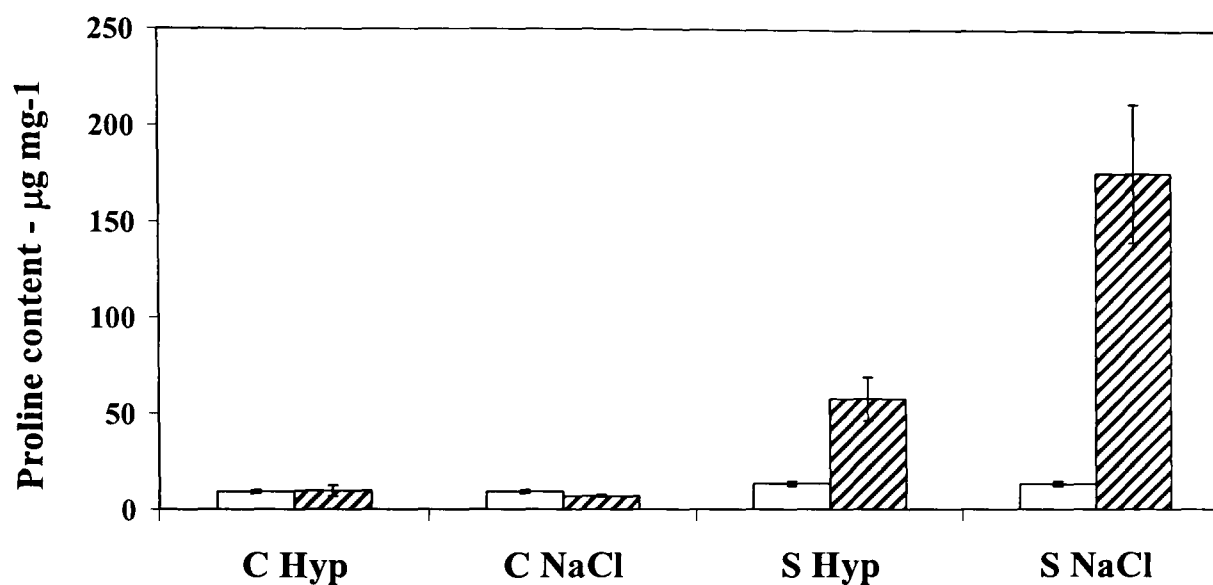
Figure 16. Mean proline content of *in-vitro* grown control (C) and selected (S) populations of cauliflower without (unshaded columns) and with (shaded columns) treatment with NaCl (350 mM) or Hydroxyproline (3 mM). I bar=SE and n=3
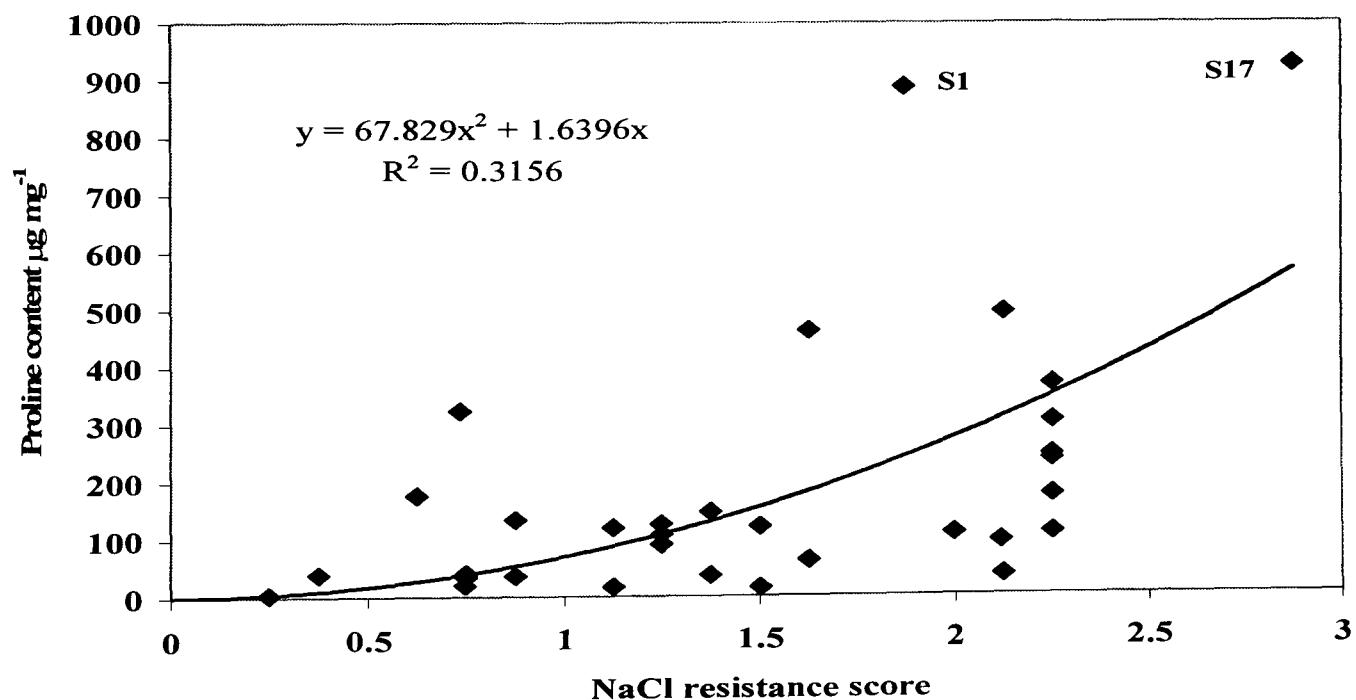
Figure 17. Scattergram of *in-vitro* NaCl resistance and Proline content under NaCl stress for genotypes from the selected population
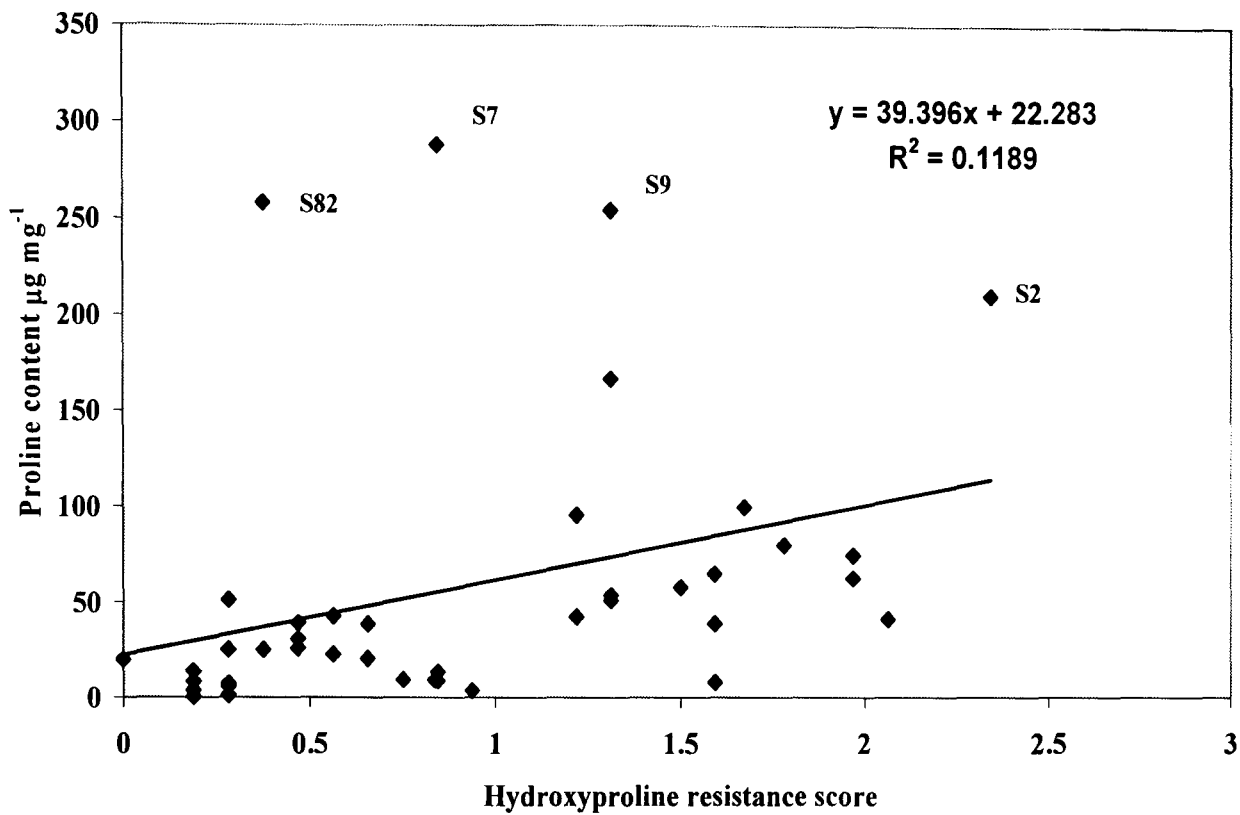
Figure 18. Scattergram of relative *in-vitro* hydroxyproline resistance and Proline content under hydroxyproline stress for genotypes from the Selected population (S).
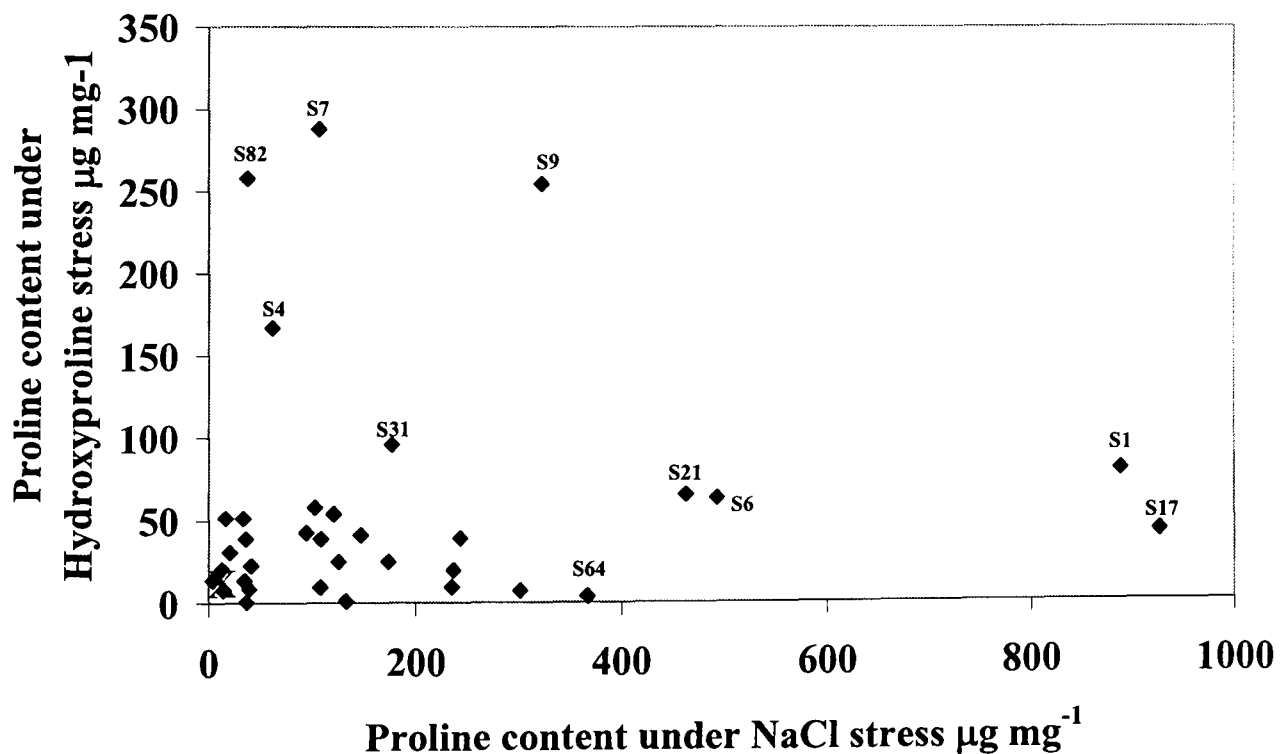
Figure 19. Scattergram of proline content of selected genotypes under hydroxyproline stress versus under NaCl stress. (Control population response is marked by X).

2.4.6 Summary of resistance data

The data for salt, hydroxyproline and frost resistance and proline accumulation were pooled to construct a profile of resistance expression for each line (Table 4) and illustrated a variety of complex effects. Only the lines (S2, S6, S16, S17, S21, S31, S84, S100 and S101) showed high triple resistance (salt, Hydroxyproline and frost) and high proline, five (S1, S17, S21, S100 and S101) showed high double resistance (salt and Hydroxyproline) and high proline and five selected lines (S7, S20, S45, S54 and S55) showed high single resistance with high proline, while the remaining lines showed varying lower degrees of resistance to the 3 stresses and variable levels of proline. It appears therefore that some of lines selected against one stress factor were linked with resistance to others, but many lines showed no linkage between the stresses. A few of the selected lines (S46, S59 and S73) demonstrated little or no improved resistance to abiotic stress and no significant change in levels of proline and these must be classified as escapes, revertants or lines with epigenetic effects which are lost during sub-culturing. One line (S82) had high proline but no appreciable improvement in stress resistance.

The relative resistance measured here correlated well with that recorded earlier for 12 of the selections by Fuller and Eed (2003) (Table 5). This clearly demonstrated the stability of the induced mutations over time and through many *in-vitro* sub-cultures and cold storage.

Table 4. Profiles of resistance expression of selected cauliflower lines.

Genotypes	NaCl		Hyp		Frost		Proline	
	In-vitro	*In-vivo*	*In-vitro*	*In-vivo*	Non-acc	Acc	NaCl stressed	Hyp. stressed
Lines with proline assessment								
S1	++	++	+	++	?	?	+++	+
S2	+++	+++	+++	++	+++	+++	?	+++
S4	++	+	+	-	+++	+++	+	+++
S6	++	+++	++	+	?	?	+++	+
S7	+	+	+	+	-	-	++	+++
S9	-	+	+	-	-	-	-	+++
S12	+	-	++	++	-	-	?	+
S13	+	+	-	-	++	++	+++	-
S16	+	+	+	+	+++	++	+++	+
S17	+++	+++	+	+	+++	++	+++	+
S20	++	++	+	+	?	?	++	+
S21	++	++	++	++	++	+++	+++	+
S31	++	++	+	+	++	++	+++	++
S42	+	++	++	+	++	+	-	-
S44	-	-	+	+	?	?	+	+
S45	+	++	-	-	++	++	+++	-
S46	-	?	-	?	?	?	-	-
S50	+	?	++	?	?	?	++	+
S53	+	?	++	?	?	?	++	+
S55	++	++	-	-	+	+	++	-
S58	-	+	-	-	-	-	-	+
S59	-	?	-	?	?	?	-	-
S64	++	+++	+	+	++	++	+++	-
S65	++	?	+	?	?	?	-	-
S67	++	?	-	?	?	?	+++	-
S68	++	?	-	?	?	?	+++	-
S70	+	?	-	?	?	?	-	+
S72	++	?	-	?	?	?	+++	-
S73	-	?	-	?	?	?	-	-
S77	++	++	+	+	++	+	+++	-
S79	+	++	-	+	-	-	-	+
S81	+	++	-	+	++	+	-	-
S82	-	-	-	-	?	?	-	+++
S83	+	+	-	-	-	+	++	-
S87	-	?	-	?	?	?	-	-
S98	-	?	-	?	?	?	-	-
S100	++	+++	++	++	++	++	++	+
S101	++	++	++	++	++	++	?	++
S105	-	?	-	?	?	?	-	-
S109	-	+	-	+	-	-	+	-
S120	+	+	+	+	-	-	+++	?
S125	++	++	+	+	-	-	+++	-
Lines with no proline assessment								
S19	?	?	+	+	?	?	?	?
S22	?	?	++	+	-	-	?	?
S48	++	?	-	?	?	?	?	?
S51	+	++	-	-	-	-	?	?
S52	+	?	-	?	?	?	?	?

S54	+	++	-	-	++	+	?	?
S56	++	++	+	+	++	++	?	?
S62	+	?	-	?	?	?	?	?
S63	+	?	-	?	?	?	?	?
S69	+	+	-	-	++	++	?	?
S71	+	+	-	+	++	+	?	?
S74	+	?	-	?	?	?	?	?
S76	++	?	+	?	?	?	?	?
S84	++	++	-	-	++	++	?	?
S85	-	+	-	-	-	-	?	?
S86	+	?	++	?	?	?	++	?
S97	+	?	-	?	?	?	?	?
S102	++	?	+	?	?	?	?	?
S104	+	?	-	?	-	-	?	?
S106	+	+	-	-	+	+	?	?
S107	-	-	-	+	-	-	?	?
S110	+	-	-	-	-	-	?	?
S111	+	+	-	-	++	+	?	?
S114	-	?	-	?	?	?	?	?
S116	-	?	-	?	?	?	?	?
S118	-	-	-	-	-	-	?	?
S119	+	?	-	?	?	?	?	?

a) NaCl and Hydroxyproline according to percentage greenness of the leaf discs and leaf strips:

Resistance score
Highly Resistant (+++)
Resistant (++)
Moderate (+)
Sensitive (-)

b) Frost resistance according to relative conductivity (%) after freezing to -7 °C:

Relative Conductivity (%) Acclimated and Non-acclimated	Resistant Rating
< 45 Acc; <55 NA	Highly Resistant (+++)
45 – 60 Acc; 55-70 NA	Resistant (++)
60 – 75 Acc; 70-85 NA	Moderate (+)
>75 + Acc (Con = 83); >85+ NA (Con= 90)	Sensitive (-)

c) Proline content after stressing to NaCl and Hydroxyproline (μg mg^{-1} in leaf)

Proline value (mg/g)	Content Rating
>120	Very High (+++)
81 - 120	High (++)
41 - 80	Moderately High (+)
< 40 (Control means < 10)	Low (-)

? = insufficient material to test.

Table 5 . Profile of resistance expression in each of the selections. (after Eed 2001).

Groups	Selected plant	Salt	Frost	Hyp	Proline/ In-vitro	Proline/ In-vivo
Triple Resistance	S2	+++	+++	++	+++	++
	S6	+++	+++	+++	++	+
Double Resistance	S1	+++	-	++	+++	++
	S4	++	+++	-	++	+
	S5	+++	-	+++	++	+
	S7	++	-	++	++	++
	S8	+++	-	+++	+++	++
	S9	+++	+++	-	++	+
	S11	+++	-	+++	++	+
	S12	+	-	++	+++	++
Single Resistance	S3	++	-	-	++	+
	S10	-	+	-	++	+

Code: +++ strong; ++ moderate; + weak; -same as control.

The responses of the selected lines were investigated by correlation for relationships between the responses to the 3 abiotic stresses. There was a weak negative correlation between frost and salt resistance, a weak positive correlation between salt and hydroxyproline resistance and no relationship between frost and hydroxyproline (Figure 20).

(a)

(b)
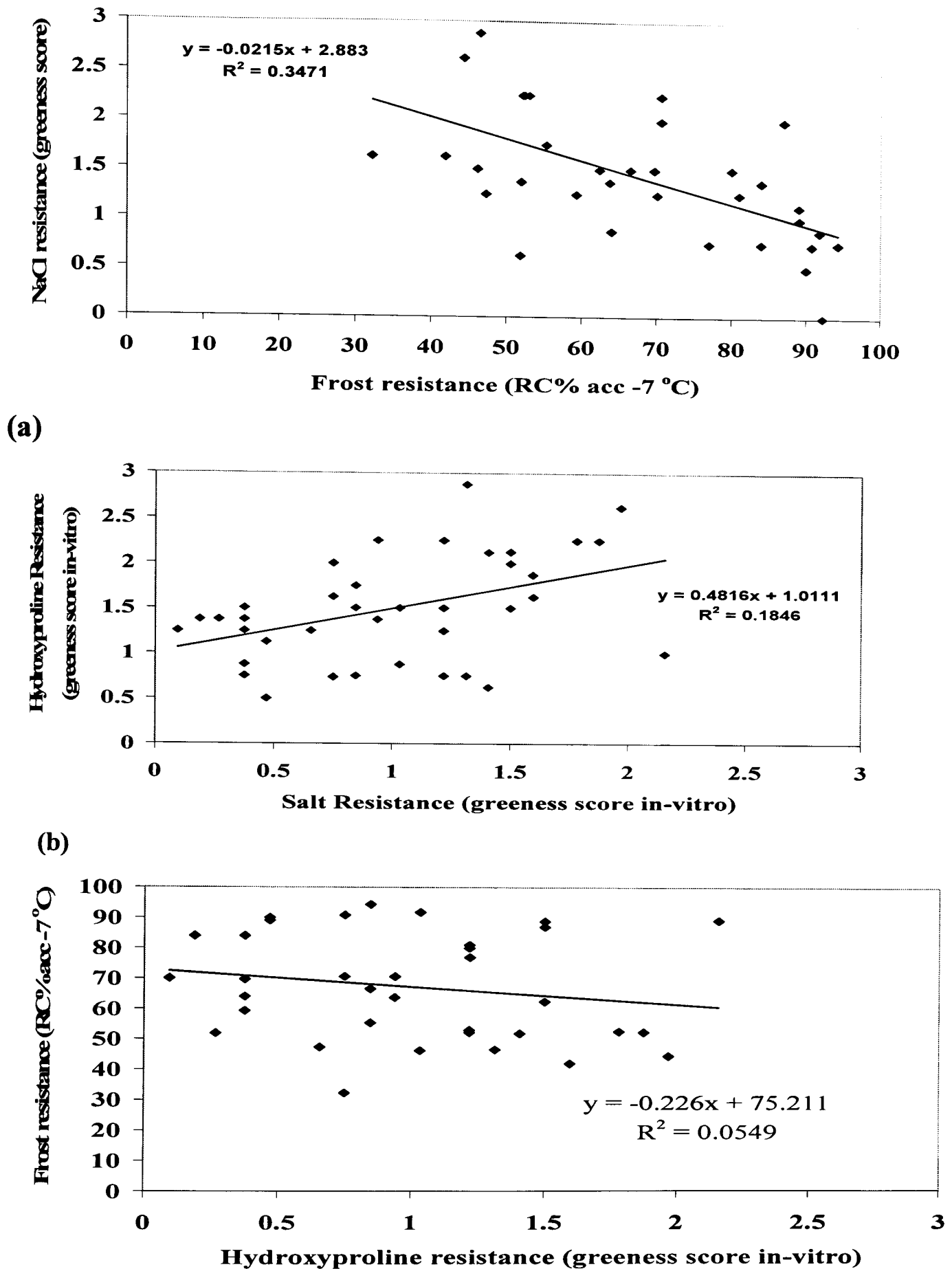
(c)

Figure 20. **The relative relationships of the selected lines to three abiotic stresses. (a) Frost resistance and salt resistance (b) Hydroxyproline resistance and salt resistance (c) Frost resistance and hydroxyproline resistance.**

2.5 Discussion

The results presented here clearly show that the lines created by Eed (2001) by NEU and NMU mutagenesis retained abiotic stress resistance through many *in-vitro* sub-cultures over a 2-3 year period and after prolonged storage at low temperature. Furthermore, when *in-vivo* plants were weaned from these lines they demonstrated the same resistance as determined 2 years previously and this broadly correlated with the *in-vitro* assessments of resistance indicating a stability of the traits. The short selection process with relatively high selection pressure that was used in this investigation clearly demonstrated that this kind of selection is useful in cauliflower to create genotypes resistant to abiotic stress as is the case in other Brassica species (Ashraf and Harris 2004; Ashraf and McNeilly 2004).

Hydroxyproline is generally toxic and impairs plant cells; resistant cells and plants were selected from a large population by growing them in the presence of an inhibitory concentration of hyp and this resistance was retained in some selections when weaned *in-vivo*. The resistance to hydroxyproline shown by selected leaf strips from mutagenesis might occur by a number of pathways, e.g. relaxed control of the proline biosynthesis pathway or decreased uptake or selective discrimination against hydroxyproline. As for hydroxyproline selection the result indicated a marked declined in leaf survival at 3 mM and 10 mM for *in-vitro* and *in-vivo* plants, respectively.

Most of the lines showed the same degree of resistance for *in-vitro* and *in-vivo* plants, this could be due to the stability of the genome when cultured *in-vitro* with no loss of genes responsible for salinity and hydroxyproline resistance (Karp and Bright 1985). Control plants showed no resistance to 3 mM and 10 mM hydroxyproline with severe bleaching effects. It is possible that hydroxyproline leads to a lack of maintenance of chlorophyll proteins including chlorophyll-binding protein with subsequent loss of chlorophyll and the bleaching. Alternatively disruption of chloroplast proteins may be leading to high ROS level leading to photo bleaching. The greater sensitivity of *in-vitro* leaves probably reflects a thinner cuticle which would increase hydroxyproline uptake to lethal levels at lower concentrations. The resistance to hyp shown by the selected leaf strips from mutagenesis may be due to mutation resulting in feedback insensitivity allowing the naturally occurring proline to accumulate to levels at which it can successfully compete with hydroxyproline.

The leaf disc assay was able to discriminate between control and salt resistance plants after only 7 days incubation. The results showed that control plants had no resistance and that selected plants had varying degrees of resistance. Leaf discs of control plants lost their chlorophyll resulting in a bleaching effect under salt stress. This may suggest that leaf discs of control plants lost the chlorophyll as a symptom of salt stress injury or that the

plasmalemna is damaged, the cell contents leak out and the cell dies. However, Gibon *et al.,* (2000) hypothesised that the loss of chlorophyll was a result of stress induced senescence or could contribute to avoidance of photo-inhibitory damage under stress.

The decline in shoot vigour of control shoots grown on NaCl or hyp containing media probably result from the irretrievable competition for a proline–binding site thus reducing uptake of proline leading to protein synthesis inhibition and subsequent plant death. Such perturbation of protein synthesis could also lead to proteins which have altered properties, which could be detrimental to the growth of the cell (Singh and Widholm 1975 Widholm, 1976). A similar observation has been reported by Hasegaw and Inoue (1983), who demonstrated that retardation of rice seedling growth was detected in culture at concentrations above 5 mM hyp. Furthermore, rice callus exhibited a reduction in dry weight gain under the influence of 10 mM hyp (Chauhan and Prathapasenan 2000).

Data for the mean growth of the cauliflower lines showed that increased NaCl concentration reduced the percentage of growth of the lines. The reduction in growth results from excessive salt uptake inducing nutritional imbalances or deficiencies between K and Na which has effects on enzyme proteins and membranes. Yang *et al.,* (1990) demonstrated that the reduction in growth

may reflect the metabolic energy cost associated with salt adaptation, reduced photosynthetic rates, reduced C gain, salt injury to tissue and attainment of maximum salt concentration tolerated by fully expanded leaves. Control lines were killed at 350 mM which might result from a rapid rise in salt concentration in the cell walls or cytoplasm when the vacuoles could no longer sequester incoming salt which causes plant death. Another explanation by Huang and Redman (1995) proposed that the death of leaves due to the build up of salt in tissues would prevent the supply of nutrients to emerging leaves, leading eventually to the death of the plant. Kingsbury *et al.,* (1984) found that sensitive species were more impaired by salt stress than resistance ones, due to a greater osmotic shock, reduced photosynthesis and C gain resulting in lower growth in these species.

Munns (2002) observed that salinity reduces the ability of plants to take up water, and this quickly causes reductions in growth rate. The initial reduction in shoot growth is probably due to hormonal signals generated by the roots. Vijayan *et al.,* (2003) indicated that NaCl has been found to inhibit the growth and development of mulberry shoot and root *in-vitro.*

The experiments presented here give no definitive indication as to the mechanism(s) of abiotic resistance in the tested mutants. The selection method of using hydroxyproline has assumed that resistance arises through an

influence on the proline biosynthesis pathway such as decreased uptake of hydroxyproline (Widholm, 1976) or preferential incorporation of the naturally occurring proline (Negrutiu *et al.,* 1978) or a result of feedback insensitivity allowing proline to accumulate to levels at which it can successfully compete with hydroxyproline.

S2, S4, S17 and S21 showed frost resistance accompanied by salt resistance or resistance of both salt and hydroxyproline with increased proline. Kueh and Bright (1981) and Dix *et al.,* (1984) succeeded to select mutant cell lines resistant to hydroxyproline and found that these mutant cell lines overproduced proline. The positive correlation between frost, salt and hydroxyproline resistance agrees with many other reports (Tantau and Dorffling 1991; Dorffling *et al.* 1993; Guy, 2003). The positive correlation confirms the suggestion of Zhu *et al.,* (1997) who reported that many genes regulated by salt stress are also responsive to freezing stress. Dorffling *et al.,* (1993) showed that it is possible to select stable wheat variants with an increased frost tolerance by means of *in-vitro* selection of hydroxyproline-resistant cell lines.

The plants under the freezing treatments -3, -5 & -7 °C were affected by freezing stress, the damage varied between the treatments, and plants under treatment -7 °C recorded higher frost damage compared with -3 °C and -5 °C

treatment. S2, S4, S16 and S17 showed resistance, or moderate resistance to frost, more than other lines under acclimated and non- acclimated treatment, accompanied by a higher leaf proline content which might indicate that proline is involved protection mechanisms against freezing injury. Laurich and Dorffling (1994) mention that proline is causally involved in the cold hardening process. Winkel (1989) used proline accumulation as a biochemical marker for increased frost tolerance in winter barley. Thus selection of 'high proline' genotypes may yield improved frost tolerance.

In this investigation the frost hardening treatment developed freezing tolerance, and induced an acclimation response in both the control plant and the mutant lines, in agreement with Laurich and Dorffling (1994). Whilst cauliflower is known to acclimate in the field (Fuller *et al.*, 1989), other workers have reported difficulties in inducing hardening in this species (Deane *et al.*, 1995). Guy (2003) found that the ability of higher plants to acclimate and tolerate freezing stress is a complex quantitative trait and the product of the activities of not one but a sizable suite of genes (200 – 2000). It is likely therefore that the mutants produced here carry the same or similar mutations and this will only be revealed by differential gene screening methods.

Chapter 3

Efficient protocol to integration APX and SOD gene into cauliflower by using *Agrobacterium* mediated transformation.

3.1 Introduction

Cauliflower (*Brassica oleracea var botrytis L.*) is known as one of the most responsive species for tissue culture but one of the recalcitrant species for tissue culture and genetic transformation (Passelegue and Kelam 1996; Puddephat *et al.,* 1996). There fore the he pr-request of the presented investigation was to establish optimization an efficient protocol to tissue culture was the first goal for this investigation. While the second goal was to establishment transformation protocol to integration APX and SOD genes into cauliflower and test the putative transgenic plants under salt stress.

3.1.1 Develop an efficient and repeatable protocol for cauliflower regeneration

To identify optimal conditions for obtaining a high growth of healthy and friable callus with shoot regeneration, several factors were considered as follows:

First, the source of explant is important; generally healthy tissues are preferred for growth and *in vitro.* Regeneration provided it is free of any contaminating micro-organisms which compete adversely with the plant material. In these research two different explants, hypocotyls and cotyledons were employed to study the optimization conditions for shoot regeneration.

Second, plant hormones participate in both genetic and environmental control of growth and differentiation. The pattern of distribution of growth hormones

in the plant is controlled by interactions between the environment and genetic factors in the plant. They may be either growth inhibitors or promoters depending on the site of action and concentration of the substance. Plant growth regulators need to be added to the culture medium in most circumstances. There are several recognized classes of plant growth substance, though only three growth regulators will be considered here: Auxin, Cytokinin and Gibberellins.

Auxin controls various processes such as cell growth and elongation. Auxin controls cell growth by affecting protein synthesis, possibly by modulating transcription (Beran and Northeate 1981) and secretion of the hydrogen ions into and through the cell wall, this acidification of the cell wall increases its ability to expand. Two different types of auxin (2,4.D and NAA) were used in this research. Effective concentrations of each auxin will vary, and need to be adjusted for the type of plant material, and stage of culture. The second class of compounds are cytokinins. In tissue culture, cytokinins are necessary for cell division (Ogawa *et al.,* 2003). Cytokinins have been shown to activate RNA synthesis and stimulate protein synthesis and enzyme activity (Kulaeva 1982 and Nehal *et al.,* 2005). In this research Kinetin and BAP were used at different concentration to study the effect of different concentration of cytokinin on shoot regeneration. Gibberellins are a group of naturally occurring plant hormones that affect cell enlargement and division which

leads to internode elongation in stems. Gibberellins are generally not necessary for the induction of growth and differentiation in plant tissue cultures. High concentration of GA3 (1 – 8 mg/l) induced growth of undifferentiated callus cells (Adnane *et al.,* 2001 and Mohan Jan 2001). GA_3 has promoted the growth of callus and elongation of the shoot with auxin and low rates of cytokinin. This research tested the effect of different concentration of GA_3 on shoot elongation.

Poor shoot elongation response may be associated with ethylene produced by cultured cells or tissues (Chi and Pua 1989 and Bisbis *et al.,* 2000). Silver nitrate ($AgNO_3$) is used in *in vitro* cultivation as an inhibitor of ethylene production (Purnhauser *et al.,* 1987 and Biddington 1991). $AgNO_3$ is believed to inhibit ethylene action by competing with ethylene for bidding sites located predominantly at the intracellular membrane (Beyer, 1976; Veen and Overbeek 1989). In the other side $AgNO_3$ as also been shown to significantly enhance both the percentage shoot regeneration and shoot elongation (Palmer 1992 and Aylin *et al.,* 2005).This research examined the effect of different concentrations of $AgNO_3$ on Shoot elongation. Two concentration 0.5 and 3.5 mg/l were tested because many researches have indicated that a high concentration of $AgNO_3$ led to partial necrosis of turnip rape explants during long culture periods (Kuvshinov *et al.,* 1999).

3.1.2 Examination of hypocotyls and cotyledon regeneration under antibiotic conditions

One of the main objectives of this work was to obtain plant transformation using the Kanamycin, Gentomycin and Tetracyclin resistance gene as selectable marker. Before attempting plant transformation it was necessary to determine that the antibiotics used and their respective concentrations for selection of transformed plant culture were effective with the plant material and that the inhibitors (Carbenicillin and cefotaxime) used to eliminate *Agrobacterium* after infection were not phytotoxic. Experiments concerning the effect of different concentrations of antibiotics on non-transformed cultures of cauliflower are presented in this chapter. Plant materials at various stages of differentiation were exposed to the antibiotics to observe possible variations in their growth responses. Induction and growth of callus and shoot regeneration were evaluated. These control experiments were carried out either preliminary to or during the subsequent transformation procedures.

3.1.3 Efficient transformation protocol for the production of transgenic cauliflower plants

One of the aims of the work described in this chapter was the development of reliable transformation protocols for cauliflower dependent on a high transformation rate, effective selection from transformed plants and the establishment of efficient shoot regeneration procedures. This can be obtained by the inter play of a large number of factor including plant genotypes (Han *et*

al., 2000), type of explant (Anne and Elizabeth 1996), bacteria strains (Melanie *et al.,* 1991), age of explant (Laurie, 1994), the external conditions during pre-culture of *Agrobacterium* and plant material during co-cultivation (Gheysen *et al.,* 1998), pre-incubation period and Co-cultivation period (WU *et al.,* 2003). In this chapter more details about the effect of type of explant (hypocotyl & cotyledons), Seedling age (4 and 8 and 12 days), bacteria strains (APX, SA and TA),bacteria density at OD_{600} (0.5, 1:10 and1:20 dilution), pre-incubation period (2 and 4 days) and Co-cultivation periods (1, 2, 3 and 4 days) were examined.

Also one of the most common a approaches to increase *Agrobacterium transformation* frequency is the use of certain metabolites such as acetosyringone (Carigo *et al.,* 2005). Acetosyringone has been identified as specific inducer substances act through a sensoe/regulator system (Leroux *et al.,* 1987). Induction of the virulence (Vir) system is achieved via a two-component (VirA/VirG) singal transduction system , VirA is an inner membrane protein that functions as a receptor to sense the acetosyringone released from the plant wound, which senses exudates of wounded plant cells in conjunction with the periplasmic sugar-binding protein ChvE(O) (Figure 21).
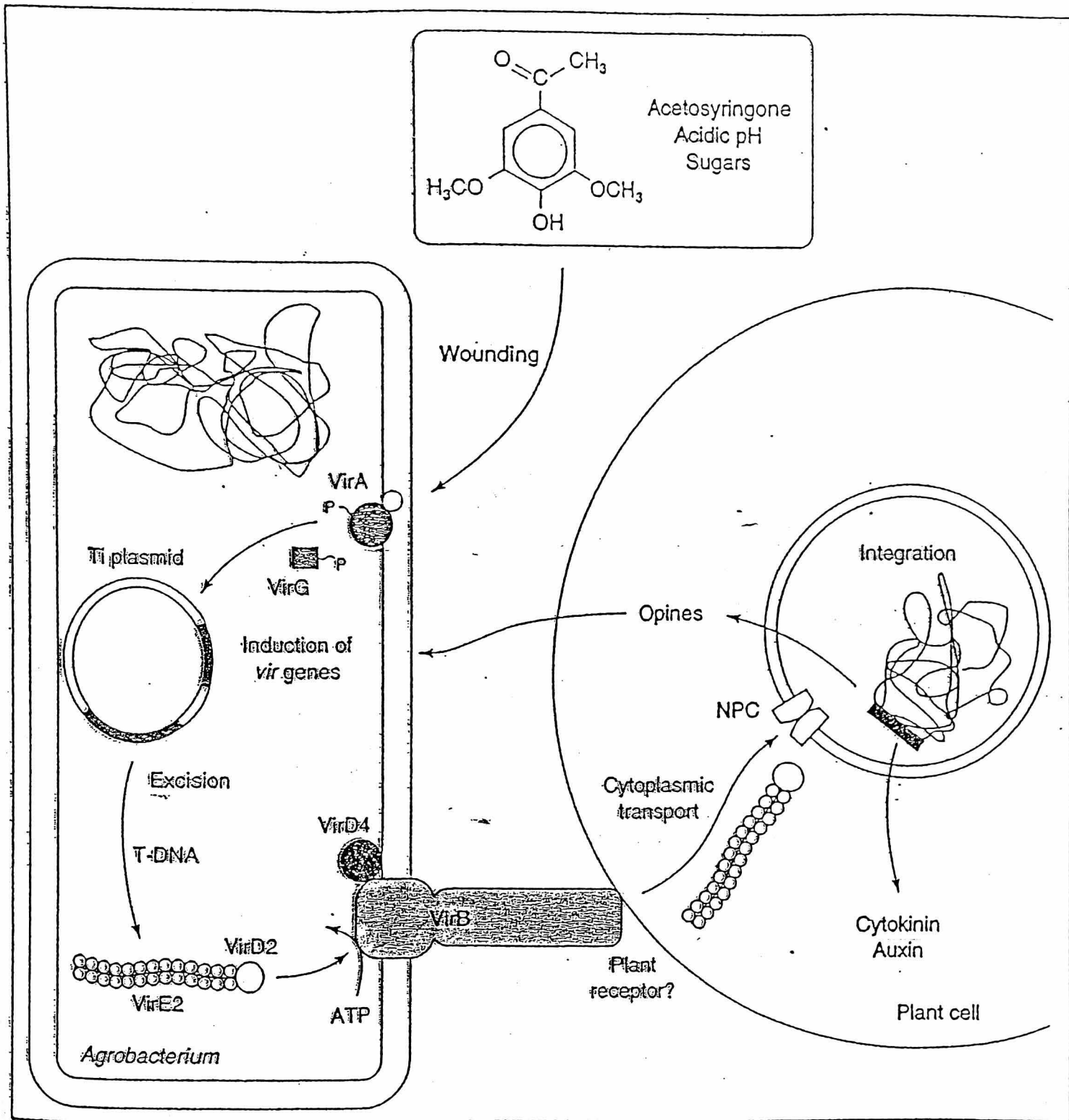
Figure 21: The role of acetosyringone in transfer T-DNA from *Agrobacterium tumefaciens* to plant cell. Source (Baron and Zambryski 1995).

3.1.4 Identify gene constructs in *Agrobacterium* vectors

One limiting step in the procedure of constructing plant gene expression vectors and *Agrobacterium transformation* is the verification process that confirms the integrity of the binary vector while it is harboured in the *Agrobacterium* strain. It is imperative that no structural alternations have occurred that would mitigate the function of the vector for transformation (An *et al.,* 1985 and 1988). Recently, techniques have been developed to detect the presence, size and orientation of inserts in recombinant plasmids by Polymerase Chain Reaction (PCR) amplification of DNA isolated from individual colonies of *E.Coli* (Gussow and Clackson, 1989; Pampfer, 1993) and *Agrobacterium* (Hass *et al.,* 1995). Simplified the procedures for template isolation from *Agrobacterium* are described in this chapter. Principally, the technique was abridged by elimination of boiling and centrifugation steps and this helped minimize the cross contamination among different samples.

3.1.5 Analysis for stable integration of transgene into the plant genome.

Analysis for stable integration of transgene can be performed on regenerated plants or progenies (Wijayanto and Mchughen 1999) from putative transformed tissue using the following methods:

First; Polymerase Chain Reaction with Suitable primer of the transgene (APX and SOD) can be used to detect the presences or absence of the transgene in

the plant genome (Klimaszewska *et al.,* 1997; Walter *et al.,* 1999 and Kery *et al.,* 2005). The primer will amplify the transgene and this is then analyzed on an electrophoresis gel.

Second; leaf disc assay was used as diagnostic marker to detect the presence or absence of foreign gene in transformation experiments (Palmgren *et al.,* 1993 and Chakrabarty *et al.,* 2002). The leaf disc assay used to detect APX and SOD expression in this study has advantages as it is rapid, simple, requires minimal use of chemicals and plant tissues, and causes no permanent damage to the plant.

Third; the marker or operator gene GUS encoding the beta-glucuronidase enzyme. B-glucuronidase encoded by the Vidx locus (Novel and Novel 1973), is a hydrolase that catalyse the cleavage of wide variety of B- glcuronidase. The natural substrate is probably B. glcuronidase linkages in mucopolysaccharides and various biological glucuromides such as those of steroid hormones (Stoeber, 1961).

The B- glcuronidase enzyme has a number of features ideal for use in transformation experiment. Firstly, the enzyme is not normally present in plant tissue, and secondly; expression of the enzyme can easily be assayed for providing clear and conclusive results (Bronwyn *et al.,* 2002 and Parasharami

et al., 2006). The beta- glucuronidase enzyme assay can be by histochemical or fluometric which gives a blue precipitate at the site of enzyme activity. The markers gene APX, SOD and GUS were used as selectable marker in the transformation experiments. The use of the genes encoding B- glucuronidase, Superoxidase dismutase and Ascorbate peroxidase enzymes as a marker of transformation in cauliflower and the binary vectors in which they are carried will be described in (Figure 25).

3.1.6 Effect of Salinity Stress on Transgenic Plant.

There are a large number of goals to which the new genetic engineering techniques can apply. One of these goals is creating transgenic plant tolerant to abiotic stresses such as drought, salinity and frost.

In this research APX and SOD gene were employment as stress genes to abiotic stresses. One of the main aims in this experiment was to test the transgenic plant under salt stress conditions by measurement of shoot fresh weight as a growth character influenced by salt treatment. Five weeks of salinity treatment had a strong impact on shoot growth, with both fresh and dry weight significantly $(P = 0.05)$ reduced (Chen, 2005). Lopez and Satti (1996) showed that addition of 50 mM NaCl to nutrient solution significantly reduced fresh weight.

Proline content was also used in this experiment as a biochemical marker to distinguish between transformed and non-transformed plant. Camara *et al.,* (2000) observed an increase in proline in maize calli subjected to NaCl concentrations higher than 100mmol l^{-1}. Ghoulam *et al.,* 2002 ; Carter *et al.,* (2006) concluded that salt tolerance in sugar beet, *Melaleuca cuticularis* and *Casuarina obesa* is associated with high content of proline.

Last part of this investigation was concerned with the measurement the Sodium, Potassium, Calcium and Magnesium content in non-transformed and transformed plant under salinity stress. There is evidence that a cell's ability to retain K$^+$ is at least as important for plant salt tolerance as its ability to exclude or compartmentalize toxic Na$^+$ (Shabala, 2000; Shabala *et al.,* 2003). Flowers *et al.,* (1977) suggested that a preference for K at high salt concentrations is a useful attribute in salinity treatment and is provides a quick and reliable screening test on seedlings that will save field space and time. Wyn *et al.,* (1984) showed a positive correlation between salt tolerance and exclusion of Na in wheat and suggested that this is an efficient selection criterion. Chen (2005) found that very strong negative correlation between the magnitude of K$^+$ efflux from the root and salt tolerance of a particular barley cultivar and K$^+$ efflux from the mature root zone of intact 3-day-old seedlings following 40 min pre-treatment with 80 m M NaCl was found to be a reliable screening indicator for salinity tolerance in barley. Sharma and Gill (1994)

concluded that salt tolerance in *B. juncea* is associated with relatively lower Na and higher potassium accumulation in leaves. Despite this, that Yeo and Flowers (1983) showed Na exclusion alone should not be used as an indicator of salt resistance. Also Lopez and Satti (1996) showed that addition of 50 mM NaCl to nutrient solution significantly reduced calcium and potassium. Eric (2005) demonstrated that salt treatments increased Na^+ content , decreased K^+ and Ca^+ while Mg+ was unaffected in leaves of *P. euphratica*. Combined analysis based on two salinity levels revealed significant salinity-level effects for shoot dry weight, plant height, root length, and ions in shoot tissues, including potassium (K), sodium (Na), Ca/Na, and K/Na while Salinity had no significant effect on shoot calcium (Ca) concentration (Rameeh *et al.,* 2004).

3.2 Aim and objectives

Aim

The aim of this investigation was to establish an efficient and reproducible transformation protocol for the production of transgenic cauliflower plants and an expedient and reliable method to confirm integration of resistance genes into the plants.

Objectives

1- Develop an efficient and repeatable protocol for cauliflower regeneration and use the regeneration protocol in transformation of cauliflower tissues.

2- Examine the effect of different antibiotic concentrations on cauliflower hypocotyls and cotyledons.

3- Test the effect of Carbenicillin and Cefotaxime antibiotic on regeneration and *Agrobacterium* elimination and regeneration of cauliflower tissues.

4- Investigate the susceptibility of cauliflower cotyledons and hypocotyls to different *Agrobacterium tumefaciens* strains.

5- Examine the effect of seedling age, explant type, pre-incubation and co- incubation period on the efficiency of cauliflower transformation.

6- Determine whether the addition of acetosyringone to the bacteria incubation medium and plant growth medium would increase the transformation efficiency of the *Agrobacterium tumefaciens* strains.

7-Investigate the possibility of transformation of cauliflower using curd explants.

8- Conduct molecular analysis of *Agrobacterium* plasmid using PCR.

9- Evaluate the integration of resistance genes (APX and SOD) in cauliflower, this can confer resistance against the Abiotic stresses, by using:

a- Gus gene assay (histochemical and fluorogenic assay).

b- Leaf disc assay.

c- DNA analysis (PCR).

10- Examine the effect of salt treatments on a growth character (shoot fresh weight) and biochemical characters (proline, Na, K, Ca and Mg content) in transgenic and non-transgenic plants.

3.3 Materials and Methods

3.3.1 Plant materials source

The F1 hybrid cauliflower Medallion (Elsoms Seeds Ltd) was employed in plant regeneration and transformation studies. Seeds were stored at 4°C (dark) prior to use. The advantage of using F_1 hybrid seed include the low percentage of genetic variation between individuals resulting in uniformly affected regeneration capacity, uniform germination percentage and vigour giving uniform explants.

3.3.2 Chemical materials source

All chemical, equipment and instrument used in the laboratory were supplied by Sigma, Aldrich and Fisher Company. For making solutions, chemicals were weighed either on 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 stock solution of NaOH and HCl (Appendix 1a & 1b) with concentrations ranging from 1 to 2 M. Before adjusting the pH of solutions, the pH meter was always calibrated with standards (pH=4.0 and 7.0).

3.3.3 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°C 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.

3.3.4 Sterile techniques 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 °C for 20 minutes. Media were sterilized in an autoclave, with a standard sterilisation cycle of 20 m at 121°C. Growth regulaters, AgNO$_3$ and antibiotics were sterilised using a Millex-Gv 0.22 µM filter unit and added after media autoclaving.

3.3.5 Surface sterilization and germination of seeds

Cauliflower seeds were surface sterilised for 30 second in 70% (v/v) ethanol followed by continuous shaking for 5 minutes in 10% (v/v) commercial un-thickened bleach solution) in a laminar flow cabinet and finally washed three

times in sterilised distilled water (Figure 22). Germination medium was M&S (Murashige and Skoog 1962) (Appendix 2), pH 5.7, and 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 25±1 °C, 16h light, 8h dark with a radiant light intensity of 50μmol^{-1}m^{-2}s^{-1} for 8 days.

3.3.6 Culture cabinet system

Seed sterilization, seed sowing and explant culture on different medium were prepared using aseptic technique by using a horizontal laminar air flow cabinet. 30 minutes before starting any culture, air flow was switched on and swabbed with 70 % ethanol prior to use. After culture the explants (in the plastic pots) were maintained in a growth chamber at 25±1 °C, 16h light, 8h dark with a radiant light intensity of 50μmol^{-1}m^{-2}s^{-1} for 8 days.
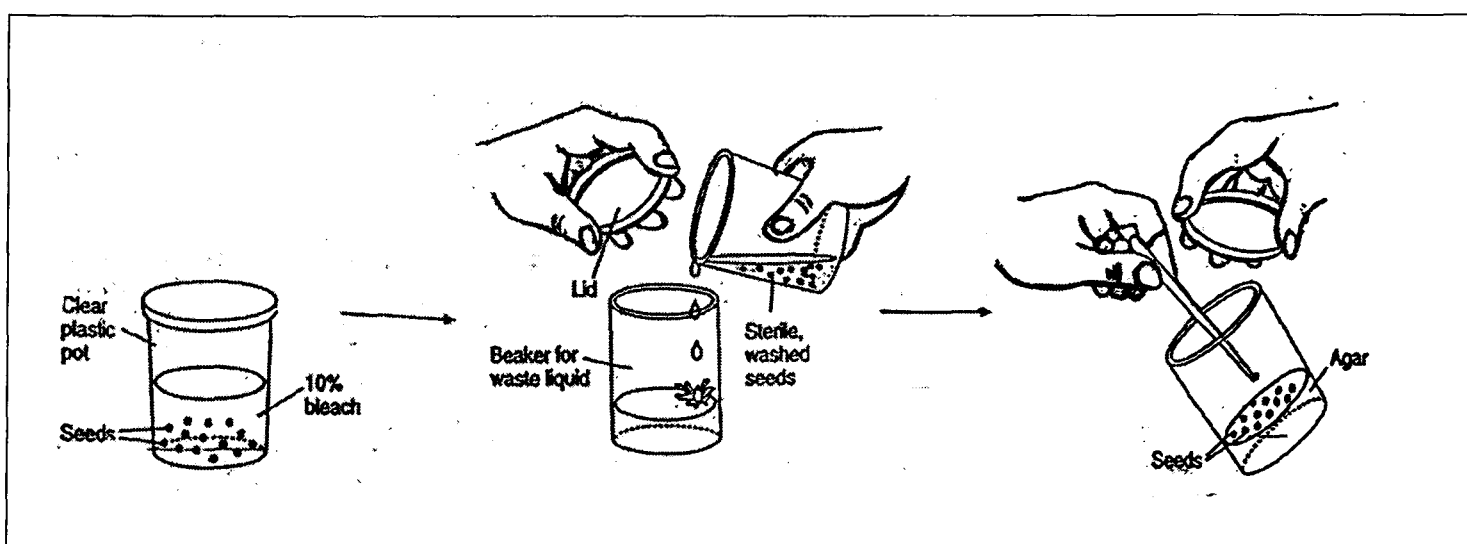
Figure 22. Seed sterilization and sowing

3.3.7 Experiments

3.3.7.1 Tissue culture experiments

The potential production of a large number of plants via tissue culture is a pre-requisite for gene transfer, since the levels of transgenic expression in plants are generally low and variable. The primary aim of these experiments was the development of an efficient protocol for the regeneration of plants from cotyledons and hypocotyls and reproducible *in- vitro* plant regeneration. Several factors have been reported to affect regeneration *in- vitro* such as culture medium, explants source and growth regulators. Each of these factors and their relationship to regeneration were tested in these experiments.

3.3.7.1. a Experiment 1

Culture media for callus induction and regeneration

The media used was S23 medium, which was the same as the initial germination medium with the addition of Sucrose 30g l^{-1}, Thiamine 0.0004 g l^{-1}, Adenine 0.080 g l^{-1}, Sodium Phosphate 0.170 g l^{-1} and supplemented with 2 mg l^{-1} 2,4-D (2.4-dichlorophenoxyacetic acid) (Appendix 3)as the best concentration to encourage callus induction for cauliflower explants (Eed 2001) . The pots were kept as described in 3.3.6.

The 8 days old seedlings were removed from the pots and dissected in a sterile laminar flow cabinet to obtain cotyledons and hypocotyls (Figure 23). Hypocotyl and cotyledon segments were then placed in Petri dishes (20 explants per petri dish) containing 15 ml of S23 medium and Petri dishes sealed with parafilm. After one week the explants were subcultured on regeneration medium S23a (Appendix 5), S23 without 2,4-D, and supplemented with 20 hormone combinations of Auxin [0.0, 0.05, 0.1 and 0.2 mg l^{-1} NAA (Naphthalene acetic acid)] and cytokinin [0.0, 1.0 and 3.0 mg l^{-1} Kinetin or BAP (6-benzyl aminopurine)] (Table 6). Stock solutions of auxin and cytokinin was prepared (Appendix 4).

The experiment was laid out as a completely randomized design and each treatment was based on 27 explants (3 replicate of 9 plants/each). The pots were kept as described in 3.3.6 but for 4 weeks.

The explants were incubated for 4 weeks prior to recording regeneration frequency. Regeneration frequency was recorded as a mean percentage of explants with shoot induction (number of regenerated explants / total number of explants * 100).

Table 6. Various combinations of auxin and cytokinin

Code	Auxin Concentration mg l^{-1}	Cytokinin Concentration mg l^{-1}
T1	0.0 NAA	0.0 K or BAP
T2	005 NAA	0.0 K or BAP
T3	0.1 NAA	0.0 K or BAP
T4	0.2 NAA	0.0 K or BAP
T5	0.0 NAA	1.0 K
T6	005 NAA	1.0 K
T7	0.1 NAA	1.0 K
T8	0.2 NAA	1.0 K
T9	0.0 NAA	3.0 K
T10	005 NAA	3.0 K
T11	0.1 NAA	3.0 K
T12	0.2 NAA	3.0 K
T13	0.0 NAA	1.0 BAP
T14	005 NAA	1.0 BAP
T15	0.1 NAA	1.0 BAP
T16	0.2 NAA	1.0 BAP
T17	0.0 NAA	3.0 BAP
T18	005 NAA	3.0 BAP
T19	0.1 NAA	3.0 BAP
T20	0.2 NAA	3.0 BAP
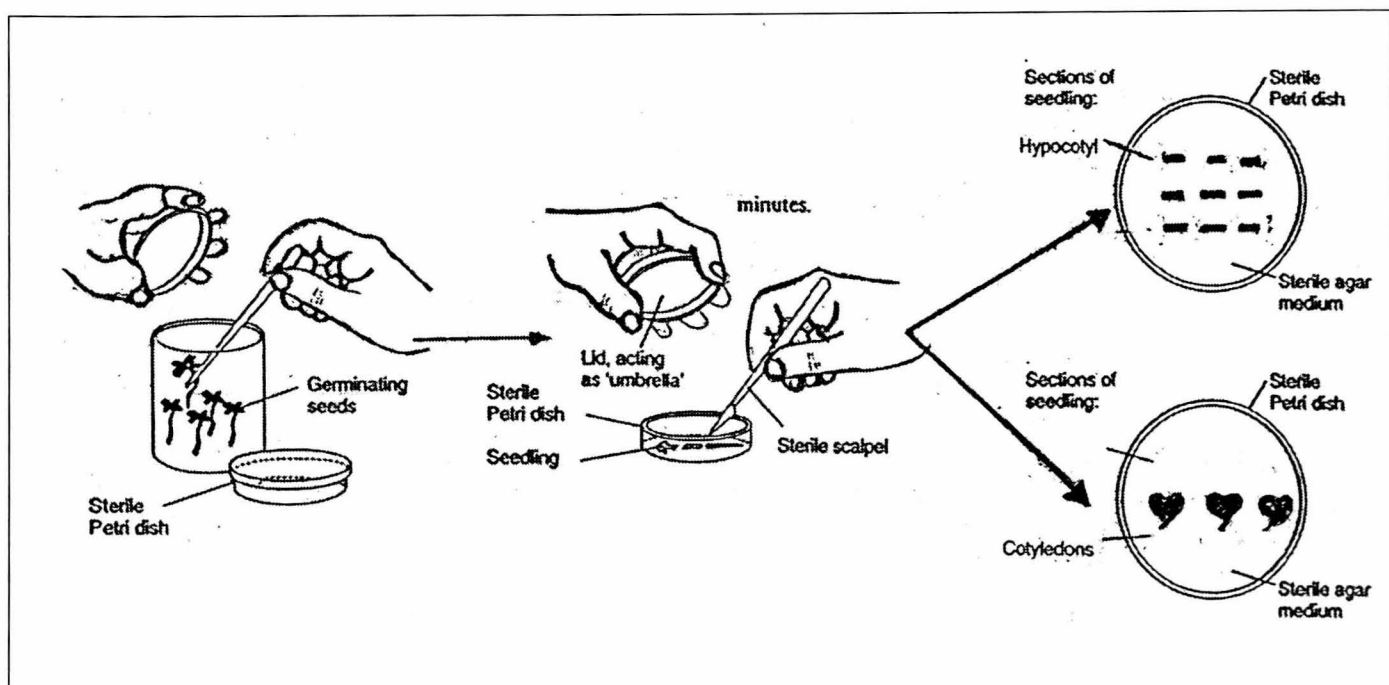
Figure 23. Culture the explant on callus induction medium

3.3.7.1. b Experiment 2

Culture media for root induction

The media were prepared as previously described in experiment 1. Regenerated shoots on S23a media were cultured on different root induction media contain different combinations of auxin and cytokinin as described forshoot regeneration. Three replicates of 27 shoots (9 shoots for each replicate) were used and incubated as described in 3.3.6. After 2 weeks from incubation the root formation was recorded as a mean percentage of explants with root induction (number of rooting plants/total number of explants * 100).

3.3.7.1. c Experiment 3

Culture media for shoot elongation

This experiment was designed to study the effect of $AgNO_3$ (silver nitrate) and GA_3 (gibberellic acid) on shoot elongation in cauliflower.

Three different levels of $AgNO_3$ (0.0, 0.5 and 3.5 mg l^{-1}) and two different level of GA_3 (0.0 and 0.01 mg l^{-1}) examined the effectiveness on shoot elongation of 8 days old explants. Explants were transferred to pots containing 25 ml S23b (Appendix 6). The experiment was replicated 3 times. Each replicate had 9 plants. The pots were kept in the growth chamber for 4 weeks.

The scoring system is regardless the length (cm) between the first node and the last node as follows:

0.0 cm= no elongation (-); 0.1 – 2.0 cm = low elongation; 2.1– 4.0 cm = middle elongation; 4.1– 6.0 cm= high elongation and >6.0 = very high elongation.

3.3.7.2 Antibiotic experiments

In order to study the formation and growth of callus and shoots on cauliflower explants exposed to antibiotic, the following experiment was carried out. All explants used in this experiment were theoretically susceptible to kanamycin, gentomycin, tetracyclin, carbenicillin and cefotaxime but above a critical dose level. The experiments were designed to determine the critical level of antibiotic required to inhibit regeneration during transformation screening experiments.

3.3.7.2. a Experiment 1

Effect of different combinations of antibiotic on callus induction and plant regeneration

Seeds of cauliflower were sterilised and germinated aseptically (as described in 3.3.5). Cotyledons with 1-2 mm petioles and hypocotyl sections of 2-3 mm in length are cut from 8 day old germinated seedlings. 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 (Figure 23). For callus induction, the culture medium employed was S23c medium containing S23 medium supplemented with a combination of antibiotic (Table 7), while for shoot regeneration S23d (Appendix 8) medium was employed with the same combination of antibiotic as the S23c medium .

Table 7. Different combination of antibiotic

code	For study the effect of kanamycin and gentamycin	For study the effect of kanamycin and tetracyclin	For study the effect of tetracycline and gentamycin
T1	0.0 mg/l K + 0.0 mg/l G	0.0 mg/l K + 0.0 mg/l T	0.0 mg/l T + 0.0 mg/l G
T2	2.5 mg/l K + 1.0 mg/l G	2.5 mg/l K + 1.0 mg/l T	1.0 mg/l T + 1.0 mg/l G
T3	5.0 mg/l K + 2.0 mg/l G	5.0 mg/l K + 3.0 mg/l T	3.0 mg/l T + 2.0 mg/l G
T4	15 mg/l K + 4.0mg/l G	15 mg/l K + 6.0mg/l T	6.0 mg/l T + 4.0 mg/l G
T5	25 mg/l K + 6.0 mg/l G	25 mg/l K + 9.0 mg/l T	9.0 mg/l T + 6.0 mg/l G
T6	35 mg/l K + 8.0 mg/l G	35 mg/l K + 12.0 mg/l T	12.0 mg/l T + 8.0 mg/l G

Stock solutions of antibiotics were prepared (Appendix 9) then filter sterilised, as described in 3.3.4, and added to cooled (but still liquid) sterile callus or regeneration media before being poured in to pots; then the pots were incubated in growth chamber for 4 weeks.

The experiments were based on 3 replicates of 27 explants for each treatment. Callus induction was recorded as percentage (number of explant formatted callus / total number of explant * 100)

Shoot regeneration frequency was recorded as described in 3.3.7.1.a.

3.3.7.2. b Experiment 2

Effect of different concentrations of cefotaxime and carbenicillin on plant regeneration and *Agrobacterium* elimination on cotyledons and hypocotyls

Cefotaxime and Carbenicillin antibiotic are used to kill the *Agrobacterium tumefaciens* after co-incubation mainly due to their low plant toxicity. The experiment was conducted to determine the effect of Cefotaxime and Carbenicillin on plant regeneration and disinfection. Non-transformed (control) explants were cultured, as described before, on S23d media supplemented with different concentrations of cefotaxime or Carbenicillin (0.0, 100, 200, 300, 400, 500 and 600 mg l^{-1}). The antibiotic was filter-sterilised prior to the addition to the medium. The pots were incubated in growth chamber for 4 weeks and then scored for plant regeneration as in experiment 1, while elimination was scored as follows: 0 = no elimination (visible bacteria growth and culture death), 1= elimination (no sign of bacteria growth, explant green and growing).

This experiment was conducted on 3 replicates of 15 explants for each treatment.

3.3.7.3 Transformation experiments by using hypocotyl and cotyledon explants

The main purpose of this study was to establish a protocol for cauliflower transformation using *Agrobacterium tumefaciens*. Several aspects of this research were investigated independently and are described separately below. They include preparing the *Agrobacterium* and host plant and techniques for selection of transformed cells. Three *Agrobacterium tumefaciens* strains were kindly sent from United States Department of Agriculture, and have been used in this study to investigate the biology of *Agrobacterium tumefaciens* vectored cauliflower transformation. The virulent *Agrobacterium tumefaciens* strain used were APX, SA and TA. APX and TA strains used pRTL2 and pCGN1578 as a vector while SA strain used pRTL2 and pBiN+ARS as a vector (Figure 24). Schematic presentation of strains APX, SA and TA are given in Figure 25.

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-glucronidase (GUS) operator gene under the transcriptional control of cauliflower mosaic virus 35S promoter. APX and TA, SA contains APX and SOD stress gene, respectively. Both TA and SA contain tetracyclin resistance genes. Three *Agrobacterium* strain APX, TA and SA were able to grow in rich media including (kanamycin + gentamycin), (tetracyclin + gentamycin) and (kanamycin + tetracyclin) respectively
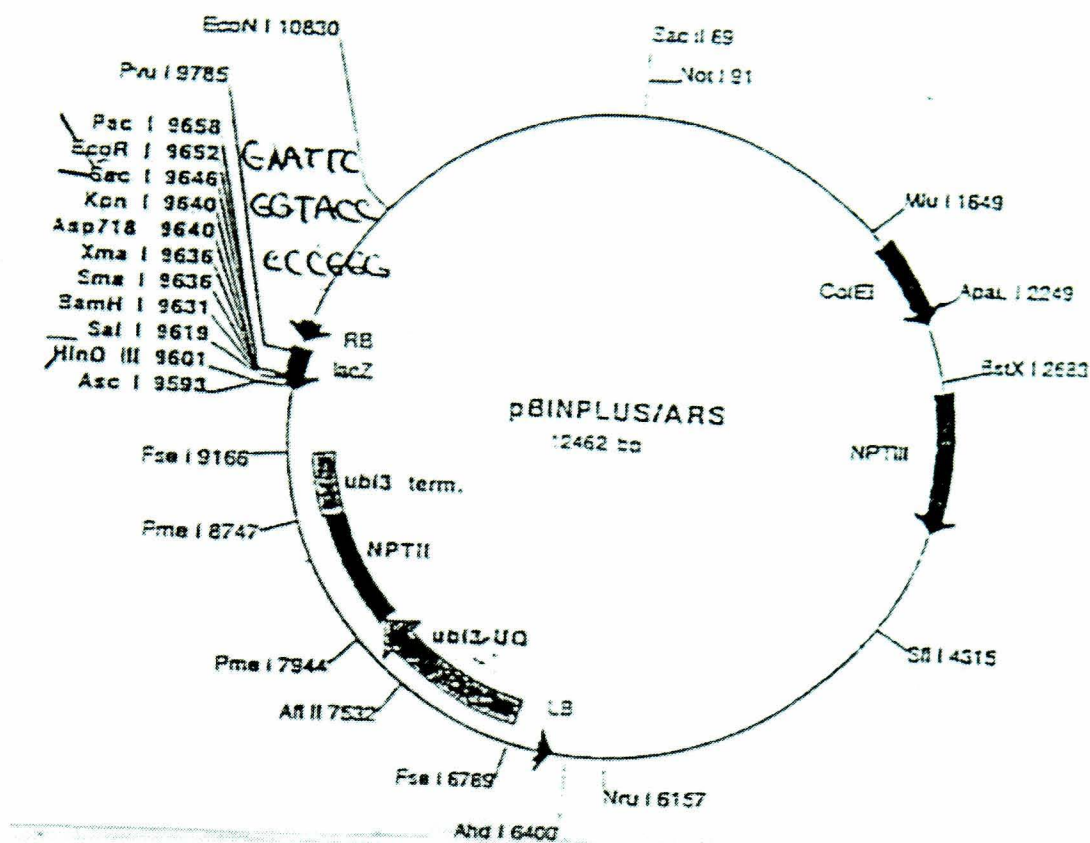
(a)
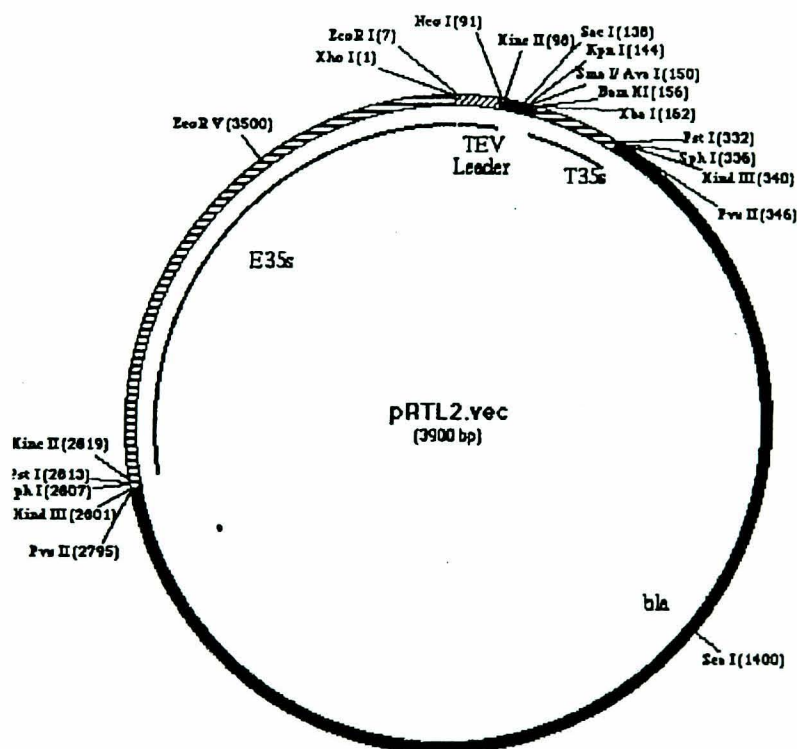
(b)

Figure 24: a) structure of plasmid P$^{BIN+ARS}$ and b) Structure of plasmid P^{RTL2} · Source : United State Department of Agriculture, Washington, USA. www.usda.gov
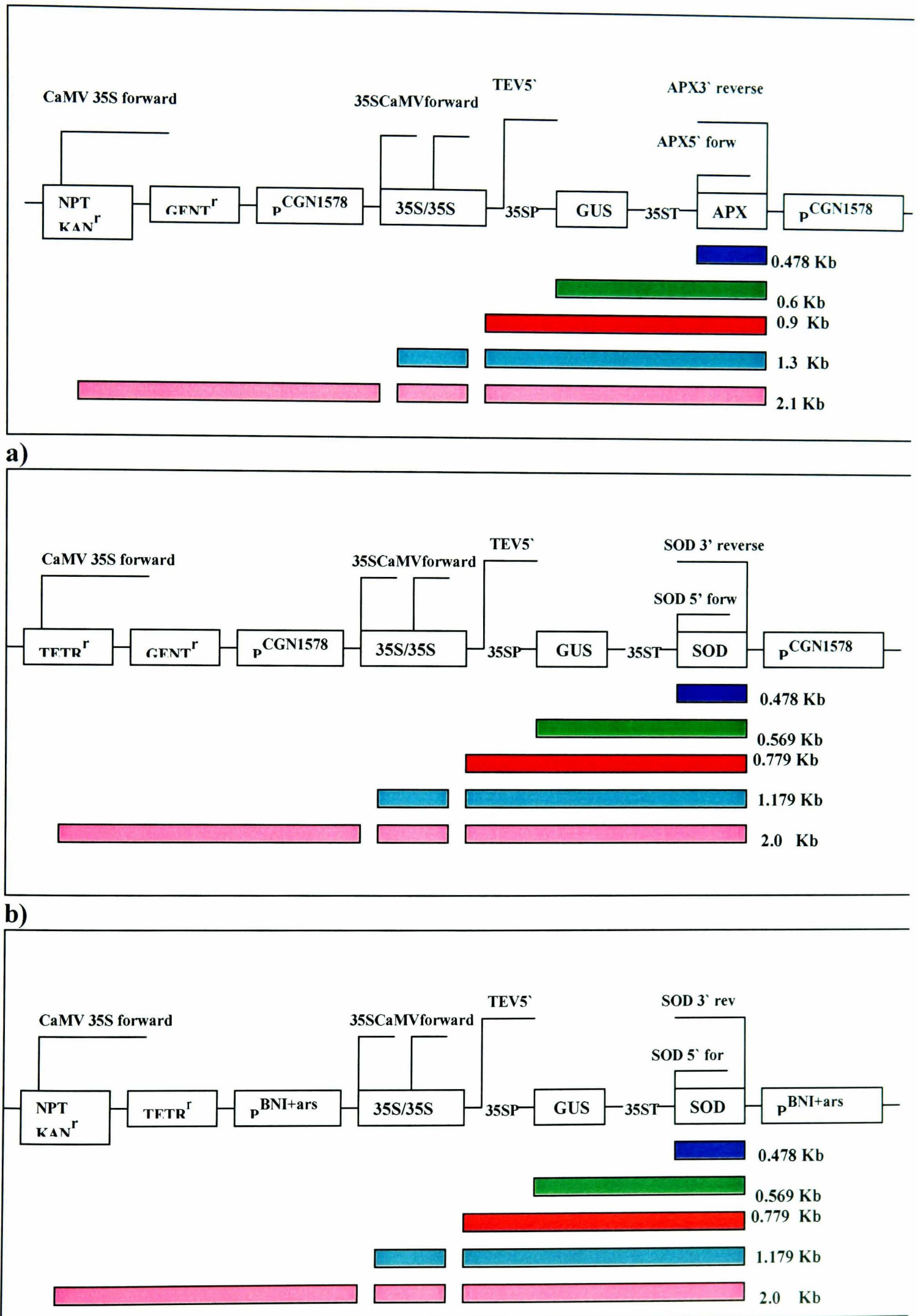
Figure 25. Schematic presentation of a) APX, b) TA and c) SA strain.

Determination of optimum culture conditions for *Agrobacterium tumefaciens*

Optimum culture conditions for *Agrobacterium tumefaciens* strains APX, SA and TA were determined by comparing growth in Luria-Broth solid medium (Appendix 7a) and YEB solid medium (Appendix 7c) supplemented with 75 μM acetosyringone and different combinations of antibiotic for each strain (Table 8). One ml of culture suspension for each strain was added to 9 ml of medium, thus making a 10^{-1} dilution (Figure 26).

Using a 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, serial dilution were making . This procedure was repeated six times until the original sample had been diluted to 10^{-6}. Subsequently, 0.1 ml of the final dilution was transferred to 3 Petri dish either containing 20 ml LB or YBE solid medium supplemented with the appropriate antibiotic concentration for each strain. The bacteria droplet was spread on LB and YBE agar plates with a glass spreader and incubated inverted at 27°C for 32 hours.

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

Agrobacterium **strain maintenance**

Dependent on the results of the previous experiment, for all the transformation experiments the strains were maintained by sub culturing single colonies on LB solid or in liquid medium (Appendix7a or 7b) using flow aseptic technique (Figure 27), supplemented with different combinations of antibiotic (Table 8) and 70 μM acetosyringone. The antibiotics used were 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 (Figure 28) and incubated at 26±1 ℃ in the dark for 24 to 48 hours. After colonies had grown sufficiently to be visible on the agar medium (Plate 11), the plates were sealed with parafilm and placed at 4℃ to act as a working plate.

Liquid Culture

The strains were grow in liquid culture using a shake bench at 50 rpm in the growth room at 27 ℃, suitable culture vessels included conical flasks and sterile red topped 60 ml plastic pots. Growth of the strains usually proceeded quiet well after 48 hours except if it had been inoculated directly from a cold stored culture when the lag phase was extended.

Preservation of *Agrobacterium tumefaciens* strains

Short term Storage

Colonies of the three strains of *Agrobacterium tumefaciens* strains were maintained for periods of a few weeks on the surface of agar media with the plates tightly wrapped in parafilm and stored inverted at 4°C.

Long term Storage

Long term storage of the *Agrobacterium tumefaciens* strains was carriedout by first pipetting 0.85 ml of on overnight growth culture to 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°C.

Table 8. Combinations of antibiotics used to maintain the *Agrobacterium* strains

Agrobacterium strains	Antibiotic concentration mg/l
APX	50 mg/l kanamycin + 10 mg/l gentamycin
SA	50 mg/l kanamycin + 12 mg/l tetracyclin
TA	10 mg/l gentamycin + 12 mg/l tetracyclin
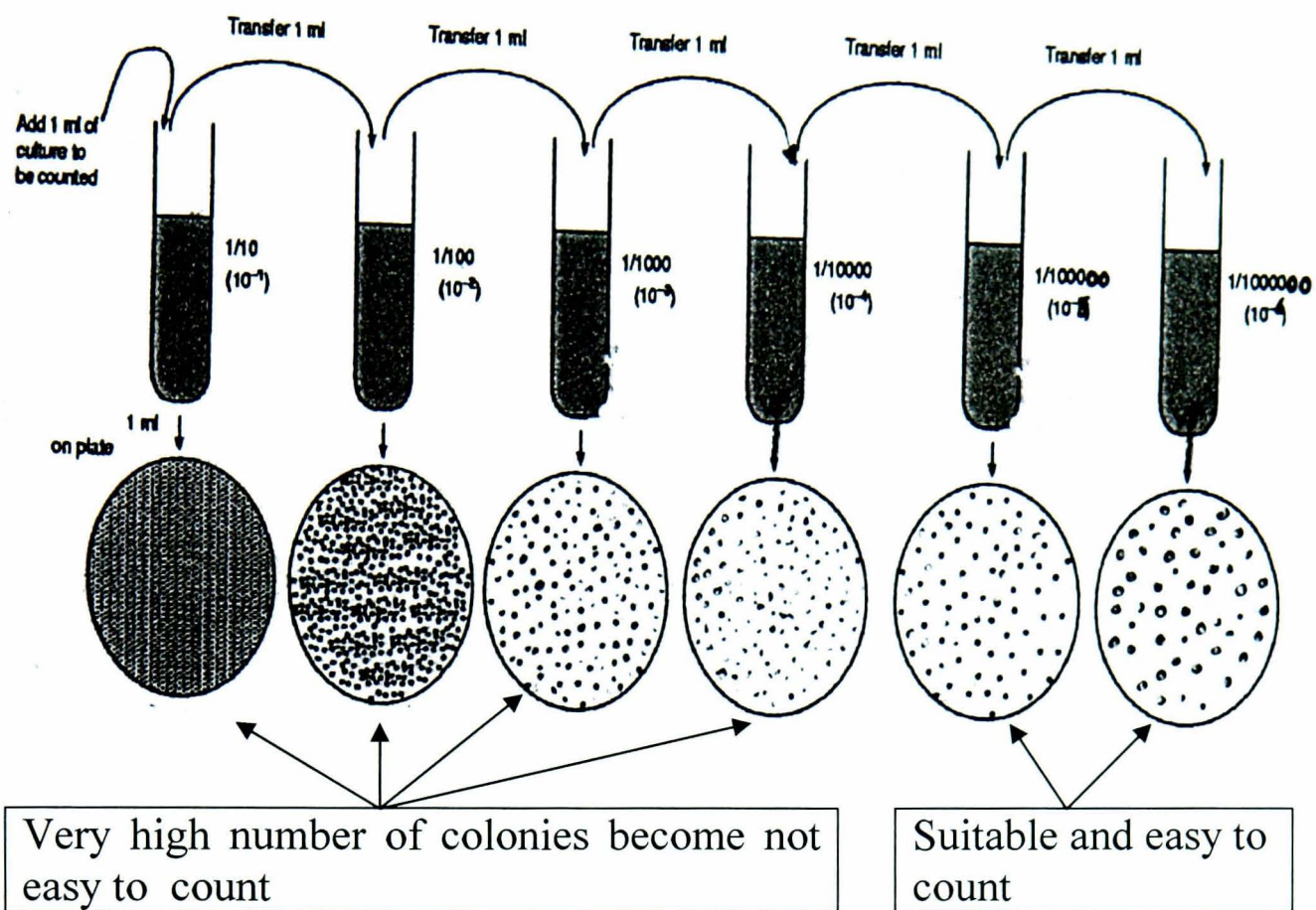
Figure 26. Serial dilution steps for *Agrobacterium* account.

Plate 11. *Agrobacterium* colonies after incubation at 28 °C for 48 hours on LB medium.
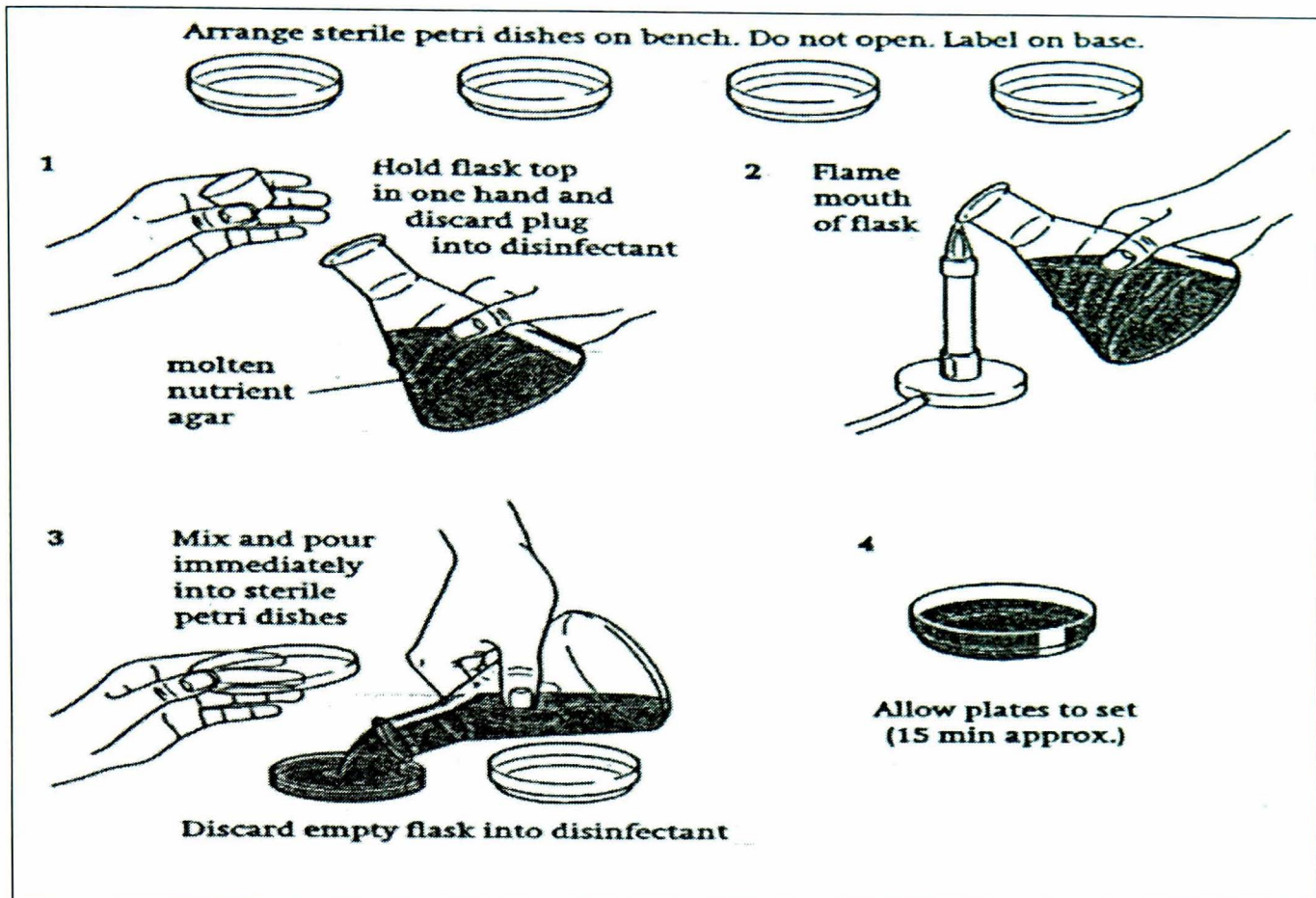
Figure 27. **Aseptic technique for pouring the media in petri dishes.**

Source : Pauline and Susan, 2000.
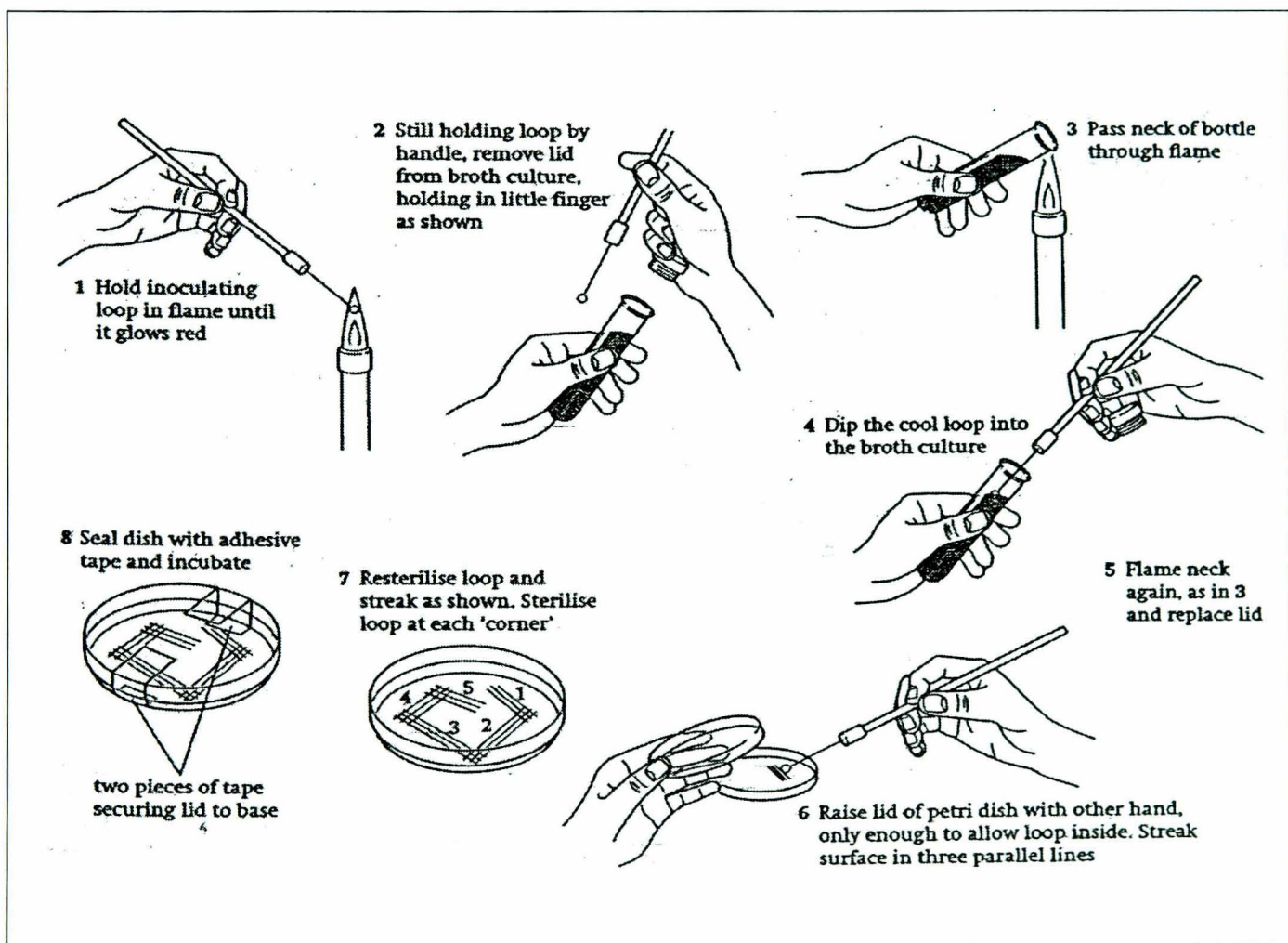
Figure 28. Quarter striking technique for *Agrobacterium*.

Source: Pauline and Susan, 2000.

3.3.7.3. b Experiment 2

Determination of a growth curve for *Agrobacterium tumefaciens* strains

For transformation experiments it is recommended that the cultures are in the log growth phase with on optical density at 600 nm of about 0.5. The following experiment was conducted to determine the log phase stage for each strain.

The growth curve were obtained from cultures inoculated from actively growing liquid culture (0.1 ml culture in 25 ml LB media) supplemented with the appropriate combination of antibiotic as described earlier. The OD was recorded after 8, 16, 24, 32, 40 and 48 hours. The absorbance was monitored by using a Helios Epsilon spectrophotometer at 600 nm.

3.3.7.3.c Experiment 3

An expedient and reliable method to identify APX and SOD gene constructs in *Agrobacterium* vectors

This experiment was carried out to develop a procedure for detection of presence of insert DNA in recombinant plasmids in individual *Agrobacterium tumefaciens* colonies without the necessity of separate procedures for DNA isolation and purification.

Step 1: Bacterial colony template preparation

A. tumefaciens (strains APX, SA and TA) were grown in solid or liquid LB medium supplemented with different combinations of antibiotic selection, , APX (50 mg l^{-1} kanamycin + 10 mg l^{-1} gentomycin) , SA (50 mg l^{-1} kanamycin + 12 mg l^{-1} tetracycline) and TA (10 mg l^{-1} gentomycin + 12 mg l^{-1} tetracycline for strain TA). Subsequently, all cultures were incubated at 26 ± 1 °C in the dark for 24 to 48 hours. After *Agrobacterium* colonies had grown sufficiently to be visible on the agar medium, as in plate 12, a sample from each colony was obtained using a sterile wooden toothpick.

A single colony was resuspended in sterile double deionised water (20 μl) until no clumps were visible. *Agrobacterium* suspension was heated at 95 °C for 20 minutes then centrifuged (13000 rpm for 10 second) briefly to pellet debris. The majority of the sample was then directly placed into 25μl a microfuge tube.

Step 2. PCR Reaction

The primers 5'-TTTCGGAACAATTAAGCACCAA-3' and 5'-AAGAGGGCGGAATACAGAGTCAGT-3' were used for the PCR reaction of APX strain and 5'-CAACATGGGAAAGGCTGTGGTTG-3' and 5'-GTTGGAAAGCACAACTTAACC-3' for SA and TA strains. Each PCR reaction mixture was 25μl PCR mixture, 0.2 μl of each primer, 5.6 μl

template and 19 μl deioinzed autoclaved water. Before starting the first PCR

cycle, the thermocycler (Gene Amp® PCR system 9700) was heated to 95°C

for 30 min, and then the sample was heated to 94°C for 2 min. This was

followed by 30 cycles of 55°C for 1 min, 75°C for 1 min. In the final cycle, the

reaction period at 75°C was extended to 10 min.

Step 3. Preparation of agarose gel

8% agarose and 100 ml of 1x TBE 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 in to an agarose gel tray (gel tray volume

of 50 ml). The gel was kept for 10-15 minutes until solid, then the tray was

put into position in the gel tank and enough electrophoresis buffers (1xTBE)

added to cover the gel top a depth of 1-2 mm. and the gel comb was carefully

removed so as not to damage the sample wells.

Step 4. Preparation samples and loading

After the PCR processes finished, PCR tubes were taken and 5μl from each

tube was transferred to a new microfuge tube after adding 1μl of loading

buffer and the 6 μl sample loaded into the sample wells slowly.

Step 5. Running and visualization

After loading the lid was placed carefully on the gel tank then the power cables were connected to the power supply and switched on. The gel was run at 100 Volts for approximately 45 minutes, and viewed by illumination with UV light using the transilluminator/gel documentation system (UVItec)

3.3.7.3. d Experiment 4

Optimization of parameters enhancing transformation efficiency

The high frequency of shoot regeneration from cotyledons and hypocotyl 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 the *Agrobacterium* strains (APX, SA and TA). Parameters were tested, one at a time, in sequential order: density of bacterial culture (0.5 OD_{600} and its dilution (1:10 and 1:20); explant types (cotyledons and hypocotyls); explant age (4, 8 and 12 days old seedling); virulence inducer($0.0\mu M$, $35\mu M$, $70\mu M$ and $105\mu M$ acetosyringone); pre - culture (0, 2 and 4 days); and co-cultivation (0, 1, 2, 3 and 4 days). These parameters were evaluated on the basis of fluorometric GUS activity coupled with regeneration efficiency.

Co-cultivation of explants with *Agrobacterium*

1.1 Plant materials and culture conditions

Explants were prepared as previously described in (3.3.5)

2. 1 Bacterial inoculation of the explants

A. tumefaciens cultures of APX, SA and TA strains were prepared from fresh colonies grown on Luria Broth plates and kept at 4 °C. One colony was transferred to 20 ml LB liquid medium and grown overnight at 28 °C. All bacterial cultures were used to inoculate explants when O.D. (at 600 nm) was 0.5 and 1:10 and 1:20 dilutions were employed with M&S medium without growth regulators or antibiotics.

For each transformation experiment 50-100 explants from cotyledons (with 1-2 mm petioles) and hypocotyls (sections of 3 mm in length), cut from 8 day old germinated seedlings, were divided into groups. Each group was pre-cultured for 0, 2 and 4 days on medium (S23 supplemented with 70 μM acetosyringone). Explants were immersed for 5 minutes in an *Agrobacterium tumefaciens* suspension and gentle shaking was used to ensure good explant bacterial solution contact.

3.3.7.3.d.1 Effect of *Agrobacterium* dilution

Agrobacterium tumefaciens strains APX, SA and TA were used for transformation of the cotyledons or hypocotyls. The transformation procedure was as described in 3.3.7.4. *Agrobacterium* was used at two different

concentrations; undiluted OD_{600}=0.5 and diluted in 1:10 and 1:20 with liquid M&S medium.

3.3.7.3.d.2 The effect of explant age

The important of seedling age in the transformation experiments was evaluated by using three different seedling ages (4, 8 & 12 days). The transformation procedure was as described in 3.3.7.4.

3.3.7.3.d.3 The effect of acetosyringone

This experiment was designed to determine whether the addition of acetosyringone (3, 5-dimethoxy 4-hydroxy acetophenone) to the incubation medium would increase the transformation efficiency of the *Agrobacterium tumefaciens* strains APX, SA and TA. Different concentrations of acetosyringone were applied (0 μM, 35μM, 70μM and 105μM) during the transformation steps.

3.3.7.3.d.4 Effect of pre-culture period of explants prior to transformation

In this experiment the cotyledon and hypocotyl explants were first pre-cultured (0, 2 and 4 days) on S23 medium prior to inoculation with *Agrobacterium*. The transformation procedure was as described in 3.3.7.4.

3.3.7.3.d.5 Effect of co-cultivation period

The importance of the duration of the co-cultivation period of plant materials with *Agrobacterium tumefaciens* was recorded using five different co-cultivation periods (0, 1, 2, 3 and 4 days).

3.3.7.4 Selection of plant transformation

The explants co-incubated with *Agrobacterium* were washed for 30 secs with 250 mg l^{-1} Cefotaxime to inhibit *Agrobacterium* growth, followed by three washes with sterilized distilled water. After 5 days of growth in S23b (S23a medium with 250 mgl^{-1} Cefotaxime), transformation was assessed using GUS assay. Explants then transferred to S23d medium with different concentrations of the appropriate antibiotic dependant on the *Agrobcaterium* strains, APX strain (25 mg l^{-1} kanamycin + 6 mg l^{-1} gentamycin), SA strain (15 mg l^{-1} kanamycin + 6 mg l^{-1} Tetracycline) and TA strain (6 mg l^{-1} gentamycin + 9 mg l^{-1} tetracycline for strain TA). The leaf disc assay was applied to detect transformation during this step.

3.3.7.5 Analysis of transformation

Three different assays were employed to detect the putative transgenic plant as following:

4.3.7.5.1 Leaf disc assay

Leaves from green shoots were removed from shoot and cut into small sections, the midrib and other main veins were removed and discarded. The leaf discs were carefully pressed into S23 supplemented with different combination of antibiotic (15 mg l^{-1} Kanamycin + 6 mg l^{-1} Tetracyclin; 25 mg l^{-1} Kanamycin + gentamycin 6 mg l^{-1}; 6 mg l^{-1} Tetracyclin + 9 mg l^{-1} gentamycin). Leaf discs from non-transformed tissue culture regenerated plantlets were used as controls. The plates were sealed with parafilm and incubated for 7-14 days.

Data were recorded using a scale as follows: 0 = white leaf disc without callus induction (non-transformed); 1 =green leaf disc with callus induction (transgenic plants).

4.3.7.5.2 GUS Assay

The detection of GUS gene activity was performed using the Gus assay (Jefferson *et al.,* 1987) in cauliflower plantlets after 3 days of co-cultivation. Two types of GUS assay, histochemical and fluorogenic, were applied in this study as follows:

4.3.7.5.2.a Histochemical assay

By this assay qualitative data concerning the specificity of GUS gene expression in tissues were obtained. GUS histochemical assay buffer solution

was prepared by dissolving 5 mg X-gluc in 1.0 ml dimethyl sulfoxide (DMSO) and then adding 10 ml of 50 mM sodium phosphate (Na_2HPO_4, NaH_2PO_4) . The explants were incubated in the GUS assay buffer overnight at 37 °C. After staining and before microscopic analysis the GUS assay buffer was removed and replaced with 70 % ethanol for at least 5 min. The intensity and localization of blue staining was quantified using a stereomicroscope.

4.3.7.5.2.b Fluorogenic assay

The GUS fluorogenic assay is a sensitive fluorescence procedure (Jefferson, 1987) which uses MUG (4-methyl umbelliferyl beta-D-glucuronide) as a substrate to verify the activity of the GUS gene in transformed plant tissues. For all the transformation experiments, testing different parameters, the fluorometric GUS assay was performed.

Plant tissue (100 mg) was ground in 100 µl GUS lysis buffer consisting of 50 mM NaPO4 at pH 7.0, 10 mM Beta-mercaptoethanol, 1 mM Na_2EDTA, 0.1% Triton X-100, 0.1% L-lauroylsarcosine sodium salt. The mixture was centrifuged in a microfuge for 5 min at 4 °C at 15000 rpm, and the supernatant was collected and added to the substrate (1 mM MUG in extraction buffer). The reaction mixture was incubated at 37 °C, and the reaction terminated after 24 hours, by the addition of 1 ml of 0.2 M Na_2CO_3. Results were obtained by placing the eppendorf tubes on a UV transilluminator and photographed. Non transformed plants were used as control.

3.3.7.5.3 DNA analysis (PCR)

The PCR technique was applied to identify the integration of foreign genes into the plant. This technique depends on the isolation of genomic DNA from plant tissue and molecular analysis of transgenic plants as follows:

1. Genomic DNA analysis

About 1-3 g plant material was collected (transformed and non transformed plants) and homogenised in liquid nitrogen using a pestle and mortar and then transferred into 50 ml centrifuge tube. The ground plant material was mixed with 10 ml 2 x CTAB buffer (Appendix 10) and 20μl ß-mercaptoethanol.

After that the tubes were incubated at 65 ^{0}C for 1 h and centrifuged for 20 minutes at 4000 rpm at room temperature (20 ^{0}C).

The supernatant was then transferred to a new tube and RNAse 1: 1000 dilution of RNAse stock (100 mg ml^{-1}) was added and kept at 37 ^{0}C for 30 minutes (when RNA activity is not so high then this is not needed).

To each sample 1 volume of chloroform/isoamylalcohol (24:1) was added and centrifuged for 30 minutes at 4000 rpm at room temperature (20 ^{0}C).

For DNA precipitation, the supernatant was transferred to a new tube and mixed with the same volume of 2-propanol and incubated overnight at 4 ^{0}C.

The sample was centrifuged at 10000 rpm for 30 minutes at room temperature and the supernatant discarded.

The pellet, was washed with 2 ml of 70 % (v/v) ethanol was added and centrifuged again for 10 minutes at 10000 rpm at room temperature (20 ^{0}C).

The supernatant was removed again and pellet was drying by passing air over for about 20 minutes or at room temperature for one hour.

The pellet was resolved with 100 μl autoclaved double distilled water and incubated at 50 ^{0}C for 15 minutes, then centrifugation for 5 minutes at 10,000 rpm at room temperature. Finally the solution was collected in a new eppendorf.

A known weight of lambda DNA was loaded to compare DNA concentrations.

2. Removing RNA from DNA extract

This procedure was used to remove RNA from the genomic DNA sample since RNA activity can be found to be very high in agarose gels.

2 μl RNAase was added to the 50 μl DNA extract in a new eppendorf and kept overnight at room temperature. subsequently 50μl of 4M lithium chloride and 400μl of 100% cold ethanol were added, stored at –20 ^{0}C for 2 hours and centrifuged for 10 minutes at 10,000rpm at 4 ^{0}C.

The supernatant was removed from the eppendorf and the pellet was washed with 500μl of 70% ethanol and centrifuged again for 10 minutes at 10,000 rpm at room temperature.

Again the supernatant was removed and dried by passing air over for about 20 minutes. The DNA pellet was dissolved in 50 μl autoclaved DD water and incubated at 50^0C for 15 minutes.

The sample was centrifuged for 5 minutes at 1000 rpm at room temperature and after that the solution was transferred to new eppendorf tube.

3. Polymerase Chain Reaction

DNA from transformed (positive GUS assay) and randomly selected non transformed plants was extracted according to the previously described method. Detection of the APX and SOD genes in the sample was conducted using PCR with the specific primers as described earlier (3.3.7.3.c).

The PCR reaction was performed in a 25 μl by preparation of Master Mix for PCR reaction as follows:

10X buffer (sigma)	2.5μl
2.5% dNTP	0.5μl
Primer1	1μl
Primer 2	1μl
Taq	1μl
Template	1μl
0.025M $MgCl_2$	1.5μl

H_2O to complete the volume of 25μl

DNA was amplified by using the following program using the DNA thermal cycle: 1 cycle of 94^0C for 4min; 42 cycle of $94 \ ^0C$ for 30 sec; $36 \ ^0C$ for 30 sec; $72 \ ^0C$ for 2 min; and 1 cycle of $72 \ ^0C$ for 2 min.

4. Preparation of agarose gel, sample preparation, loading and running and visualization

As described previously in 3.3.7.3.c

3.3.7.6 Transformation experiments by using curd explants

3.3.7.6.1 Curd explants preparation and sterilization

Cauliflower Medallion curds were harvested and stored at 4 ° C for a duration not exceeding 7 days. Cauliflower was taken and cut into florets. To maintain the aseptic procedures the florets were surface sterilized, this removed any contamination, which might be on the cauliflower surface. To sterilize the cauliflower it was immersed in 10 % bleach for 15 minutes, shaking it occasionally. After 15 minutes the bleach was poured away and the floret was rinsed with sterile distilled water. This was repeated twice to remove all the bleach. The floret was then removed to a sterile Petri dish

using sterile forceps. The shavings were then cut into very small pieces.

3.3.7.6.2 Curd infection with *Agrobacterium* strains

Small pieces from curd were treated with *Agrobacterium* strains as described before in 3.3.7.3. d. (Experiment 4)

3.3.7.7 Expression of tolerant genes (APX and SOD) in the putative transgenic plant under salt stress

This experiment was conducted to determine the expression of the APX and SOD genes after integration in the plant genome. Stress was applied in this experiment to test the effect of salt stress in the putative transgenic plants compared with non-transformed plants as a control. Shoot tips of transformed and control plants *in-vitro* culture were aseptically subcultured onto solid medium supplemented with 300 mM NaCl and the pots were incubated in the growth chamber. After 4 weeks incubation on salt and control media the following parameter were measured

3.3.7.7.1 Shoot fresh weight

After four weeks shoot fresh weight was measured (mg)

3.3.7.7.2 Proline content

Determined as described in Chapter 3.

3.3.7.7.3 Sodium, potassium, calcium and magnesium content

Dried shoots of each treatment (control and 300 mM NaCl) were freeze dried for three days to obtain dried samples. 2.5 ml of nitric acid (HNO_3 conc.) was

added to 0.07 g of dry sample and the mixture boiled for 60 – 90 mins, then transferred to a beaker and the volume adjusted to about 40 ml with distilled water and boiled for 10 minutes. This was cooled and then filtered through glass wool into a flask and beaker rinsed with distilled water into a 50 ml volumetric flask. The concentration of Na and K were determined by flame emission spectrophotometry while Ca and Mg concentration were determined by flame absorption spectrophotometer using a GBC Model 902 AAS-AES (Plate 12)
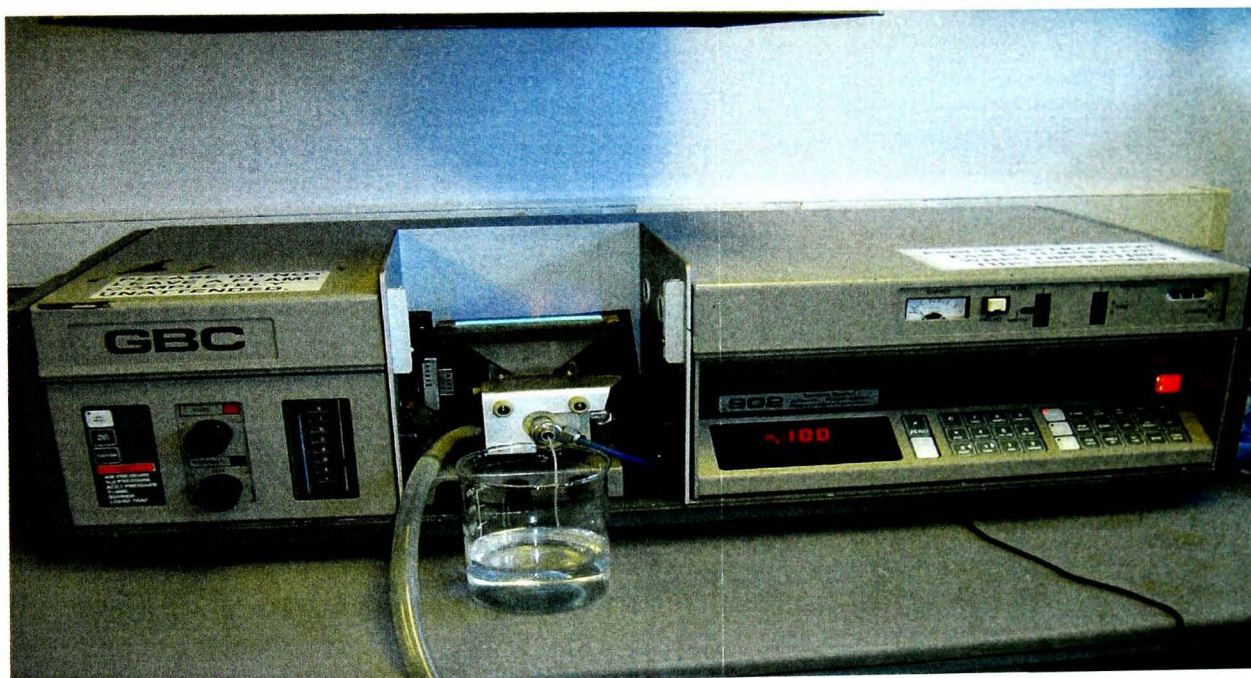
Plate 12. Flame absorption spectrophotometer (GBC Model 902 AAS-AES)

3.3.8 Statistical analysis

Statistical analysis was performed with the software package Minitab. A standard analysis of variance (ANOVA) was used, the post-hoc Tukey-Honstely significant difference test (Tukey-HSD), at a 95% significant level, was used to identify any significant differences between the treatments.

3. 4 Results

3.4.1 Determination of optimum culture conditions for *Agrobacterium tumefaciens* strains

On the basis of CFU (colony-forming units), *Agrobacterium tumefaciens* strains (APX, SA and TA) grew slightly faster, but non significantly, in LB growth medium than YEB medium. The total mean average of high concentration of cell suspension (5.3 x 10^8 cfu/ml) was recorded for *Agrobacterium tumefaciens* strains growing on LB medium (Figure 29). Significant differences were recorded between the strains at P<0.001.

Agrobacterium tumefaciens strain SA carrying the plasmid pRTL2 and pBIN+ARS as a vector grew faster and as a more homogeneous suspension in LB medium. Culture grown in LB medium had 5.8 x 10^8 cell/ml while YEB culture after 24 hours growth had a lower concentration of cell suspension, which was mainly grouped in clumps. The results showed that *Agrobacterium tumefaciens* strain SA recorded a high count of cell/ml either in LB or YBE medium followed by APX strain then TA strain. These results showed that LB medium was a more suitable medium for the growth of the different *Agrobacterium tumefaciens* strains. LB medium was used there after in all transformation experiments.
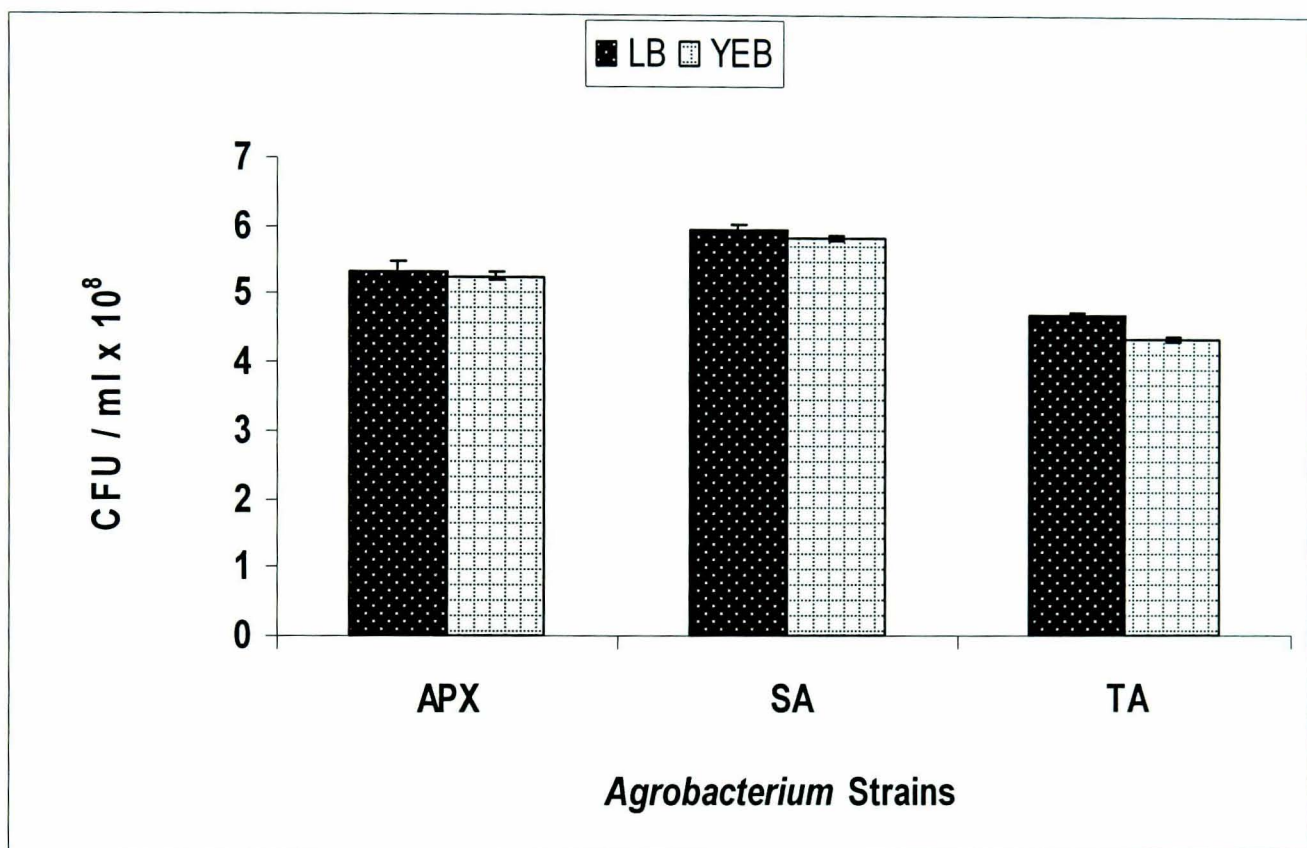
Figure 29. Effect of LB and YEB media on the growth of different *Agrobacterium tumefaciens* strain. I bar = SE

3.4.2 Determination of *Agrobacterium tumefaciens* growth on liquid LB medium

Figure 30 show that, when quantifying bacteria growth via optical density, the maximum growth of APX strain (1.12); and SA strain (1.41) was obtained after 70 hours while the maximum growth for TA strain (1.33) was obtained after 60 hours.

A classic sigmoid growth curve was not obtained for any of the strains and the log phase of growth (OD_{600} of about 0.5) varies between the strains. For *A. tumefaciens* strain SA and TA log phase was detected after 30 hours while for APX strain it was detected after 40 hours.

For transformation experiments its preferable that the culture are still in log phase and therefore a 30 hours and 40 hours grown culture would appear to be ideal for the SA , TA and APX strain, respectively.
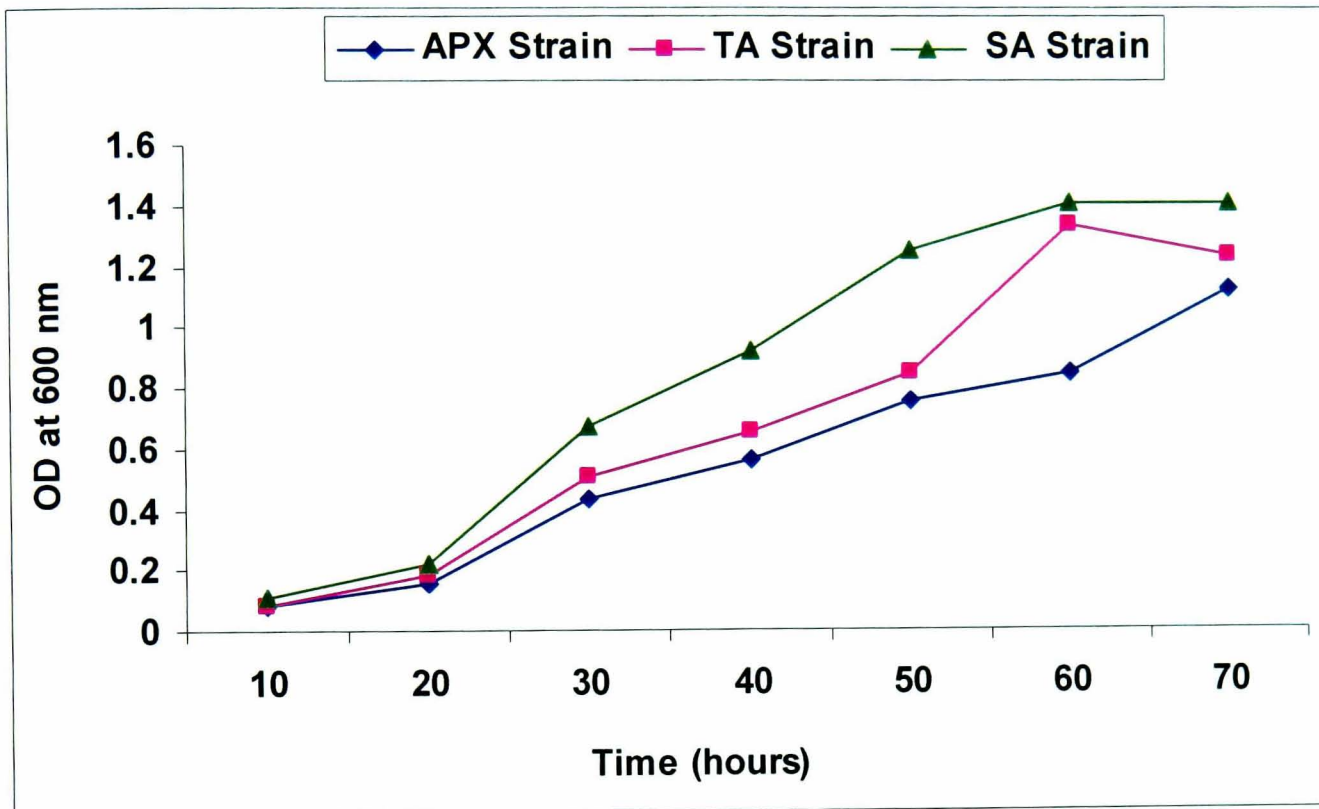
Figure 30. *Agrobacterium tumefaciens* growth on liquid LB medium

3.4.3 Establishment of tissue culture of cauliflower from hypocotyl and cotyledon explants

3.4.3.1 Effect of different combination of growth regulators on shoot regeneration

Observation of shoot regeneration was made during the first four weeks of culture (Figure 31 and Plate 13). Data showed that shoot regeneration was possible for all tested media using hypocotyls and cotyledons. Culture of hypocotyls on S23 supplemented with 3 mg l^{-1} BAP combined with 0.2 mg l^{-1} NAA achieved the highest shoot regeneration (79.6 and 76.8 %) from both hypocotyls and cotyledons, respectively.

The interaction between cytokinin types and concentration employed affected shoot regeneration percentage. In this experiment BAP 3 mg l^{-1} significantly increased shoot regeneration more than Kinetin at the same concentration (Figure 30). In general, cytokinins are absolutely necessary for shoot regeneration from hypocotyls and cotyledons. The regeneration potential for hypocotyls was significant better than cotyledons at the same level of cytokinin.

3.4.3.2 Effect of different combination of growth regulators on root formation

The results indicated that the medium containing 2.0 mg l^{-1} NAA appeared to stimulate the highest rooting frequency (86.1 and 73.1 %) for both hypocotyl and cotyledon explants, respectively (Figure 32 and plate 13). Furthermore, high levels of cytokinin (Kinetin or BAP) with different concentrations of NAA reduced root formation. S23 medium supplemented with 3 mg l^{-1} kinetin and 0.05 mg l^{-1} NAA recorded the lowest percentage of root formation (29.6 %).

The amount of auxin (NAA) and cytokinin (Kinetin or BAP) in the media was also varied to establish the concentration of the growth regulators required for maximum root formation. It was found that NAA at concentration of 0.2 mg l^{-1} was optimum for root formation.
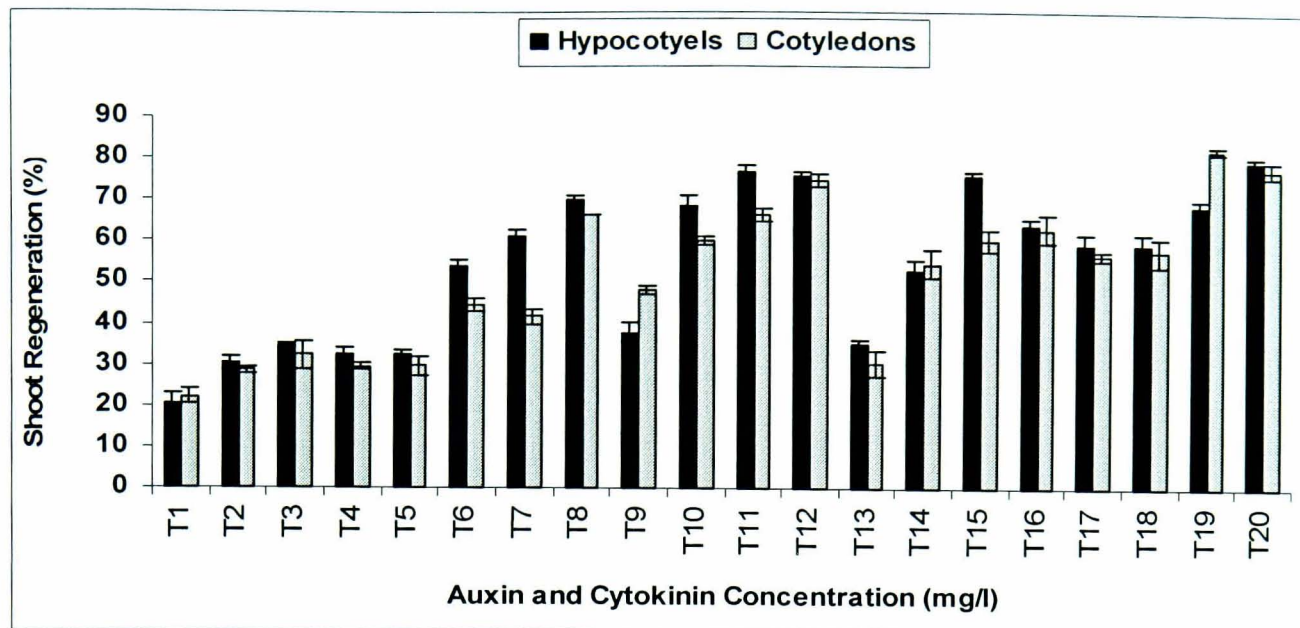
Figure 31. Effect of different combination of auxin and cytokinin on shoot regeneration derived from non-transgenic plants. I bar=SE & n=27
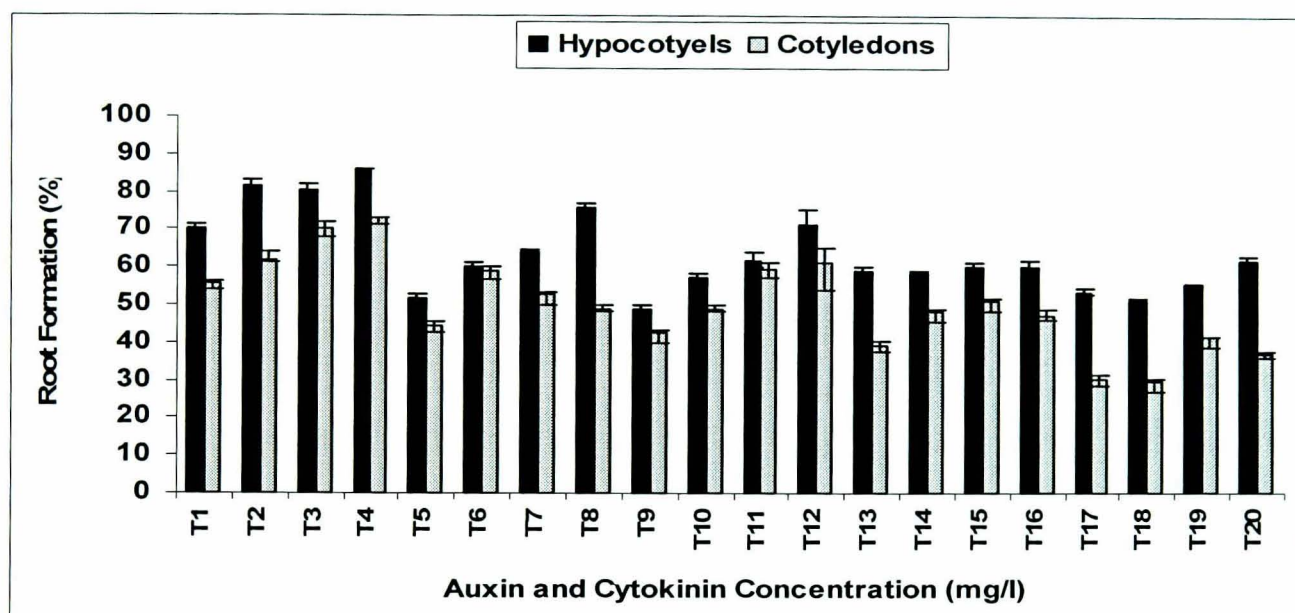
Figure 32. Effect of different concentrations of auxin and cytokinin on root formation. I bar=SE & n=27

Code	Auxin	Cytokinin	Code	Auxin	Cytokinin
T1	0.0 NAA	0.0 Cytok.	T11	0.1 NAA	3.0 Kinetin
T2	0.05 NAA	0.0 Cytok.	T12	0.2 NAA	3.0 Kinetin
T3	0.1 NAA	0.0 Cytok.	T13	0.0 NAA	1.0 BAP
T4	0.2 NAA	0.0 Cytok.	T14	0.05 NAA	1.0 BAP
T5	0.0 NAA	1.0 Kinetin	T15	0.1 NAA	1.0 BAP
T6	0.05 NAA	1.0 Kinetin	T16	0.2 NAA	1.0 BAP
T7	0.1 NAA	1.0 Kinetin	T17	0.0 NAA	3.0 BAP
T8	0.2 NAA	1.0 Kinetin	T18	0.05 NAA	3.0 BAP
T9	0.0 NAA	3.0 Kinetin	T19	0.1 NAA	3.0 BAP
T10	0.05 NAA	3.0 Kinetin	T20	0.2 NAA	3.0 BAP

a) **Regeneration steps from hypo.**   b) **Regeneration steps from coty.**

c) **Plant regeneration from hypo.**   d) **Plant regeneration from coty.**

Plate 13 . Shoot initiation, root formation and plant regeneration from hypocotyls and cotyledons

3.4.3.3 Effect of AgNO₃ and GA₃ on shoot elongation

In regeneration experiments it was found that the addition of $AgNO_3$ had no effect on shoot number but increased the number of elongate, shoots on the explants. In some explants most of shoots failed to elongate, or shoot elongation was delayed, when the morphogenic callus remained attached to the hypocotyl or cotyledon explants.

In an attempt to elongate the regenerated shoot of hypocotyls or cotyledons, different combination of $AgNO_3$ and GA_3 were investigated. Analysis of the results in Figure 33 showed that no-elongation response was obtained in regenerated shoots of both explants while S23 supplemented with different concentration of AgNO3 and GA_3 showed different results. Lowest elongation appeared when using S23 medium without $AgNO_3$ and GA3 while the highest shoot elongation response (4.9 cm and 4.1 cm) on S23 medium supplemented with 3.5 mg l^{-1} $AgNO_3$ with 0.01 mg l^{-1} GA_3 for hypocotyls and cotyledons explants, respectively. Highly significant differences at $p < 0.001$ was detected between the treatments.
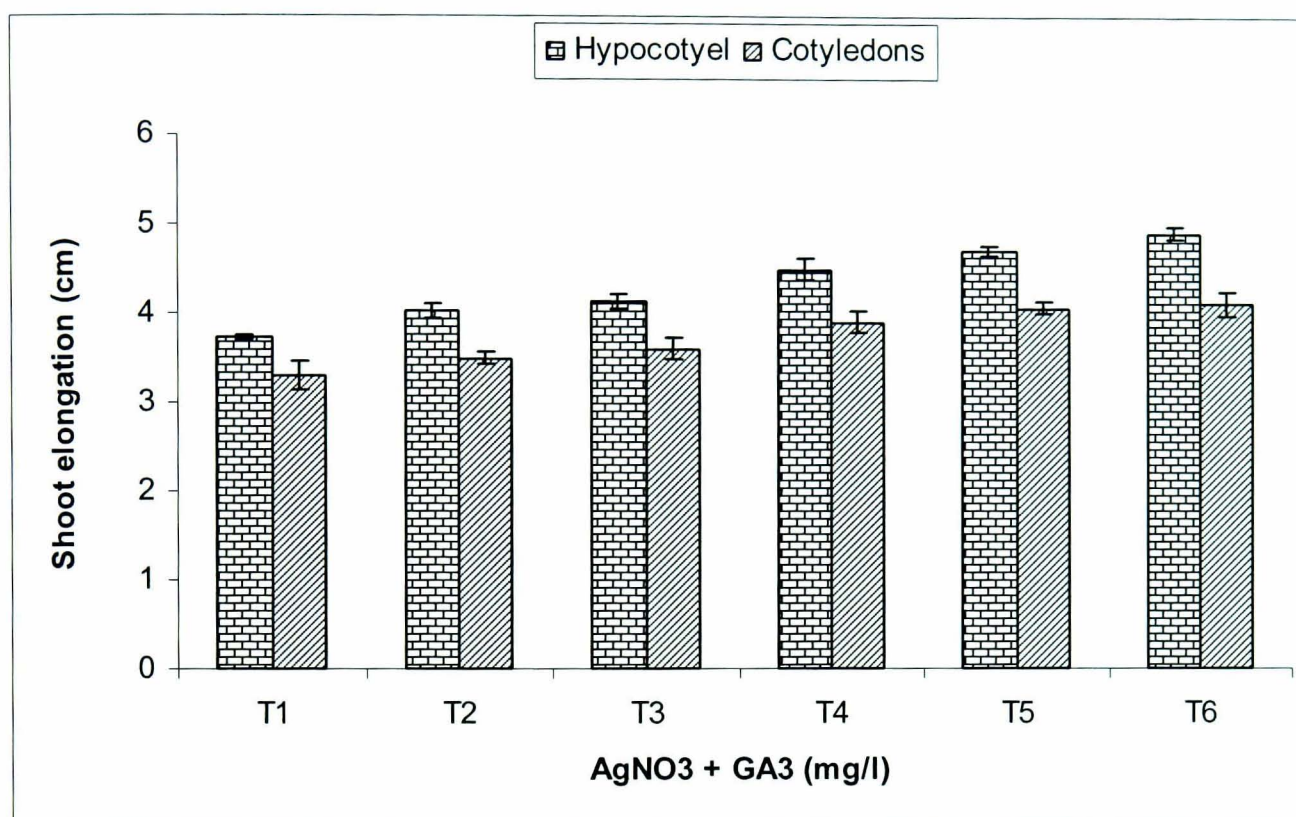
Figure 33. Effect of different concentration of AgNO$_3$ and GA$_3$ on Shoot

elongation .   (T1 = 0.0 AgNO$_3$ + 0.0 GA$_3$; T2 = 0.5 AgNO$_3$ + 0.0 GA$_3$;

T3 = 3.5 AgNO$_3$ + 0.0 GA$_3$; T4 = 0.0 AgNO$_3$ + 0.01 GA, T5 = 0.5 AgNO$_3$ +

0.01 GA$_3$; T6 = 3.5 AgNO$_3$ + 0.01 GA$_3$). I bar = SE & n=27

3.4.4 Effect of carbenicillin and cefotaxime on shoot regeneration

Prior to transformation experiments cefotaxime and carbenicillin, which are widely used antibiotics for *Agrobacterium tumefaciens* elimination after co-cultivation, were tested for their effect at different concentrations on shoot regeneration. The hypocotyls of non-transformed seedlings were arranged horizontally and gently pressed into the surface of the medium, and cotyledons were placed upright with the cut end embedded in the medium.

Experimental design was 9 explants per treatment, and each treatment was repeated at least 3 times at various concentrations of carbenicillin or cefotaxime (0, 100, 200, 300, 400,500 and 600 mg l^{-1}) were added to the shoot regeneration medium.

The data presented in Figure 31 showed that inhibition of shoot regeneration was significant for carbenicillin above 200 mg l^{-1} and above 400 mgl^{-1} for cefotaxime with severe inhibition occurring at 500 mg l^{-1}, concentrations commonly used in the literature. Results also showed marginally higher shoot-regeneration rates for hypocotyls compared to cotyledons (Figure 34).

3.4.5 Effect of carbenicillin and cefotaxime on elimination of *Agrobacterium* after co-cultivation

Elimination of *Agrobacterium* increased linearly with increased carbenicillin and cefotaxime concentration. The highest elimination (86.6 %) was recorded for cotyledon explants treated with carbenicillin concentration at 600 mg l^{-1} whilst was (80%) for cefotaxime treatment at 500 mg l^{-1} (Figure 35)

Carbincillin was slightly more efficacious than cefotaxime but neither antibiotic was able to eliminate *Agrobacterium* completely at the concentration tested. When the results of this experiment are examined with the previous experiment, it is clear that a compromise between *Agrobacterium* elimination and shoot regeneration must be made. Whilst 500 mg l^{-1} would be better for elimination, it was decided to use a concentration 250 mgl^{-1} cefotaxime so as not to inhibit shoot regeneration unduly.
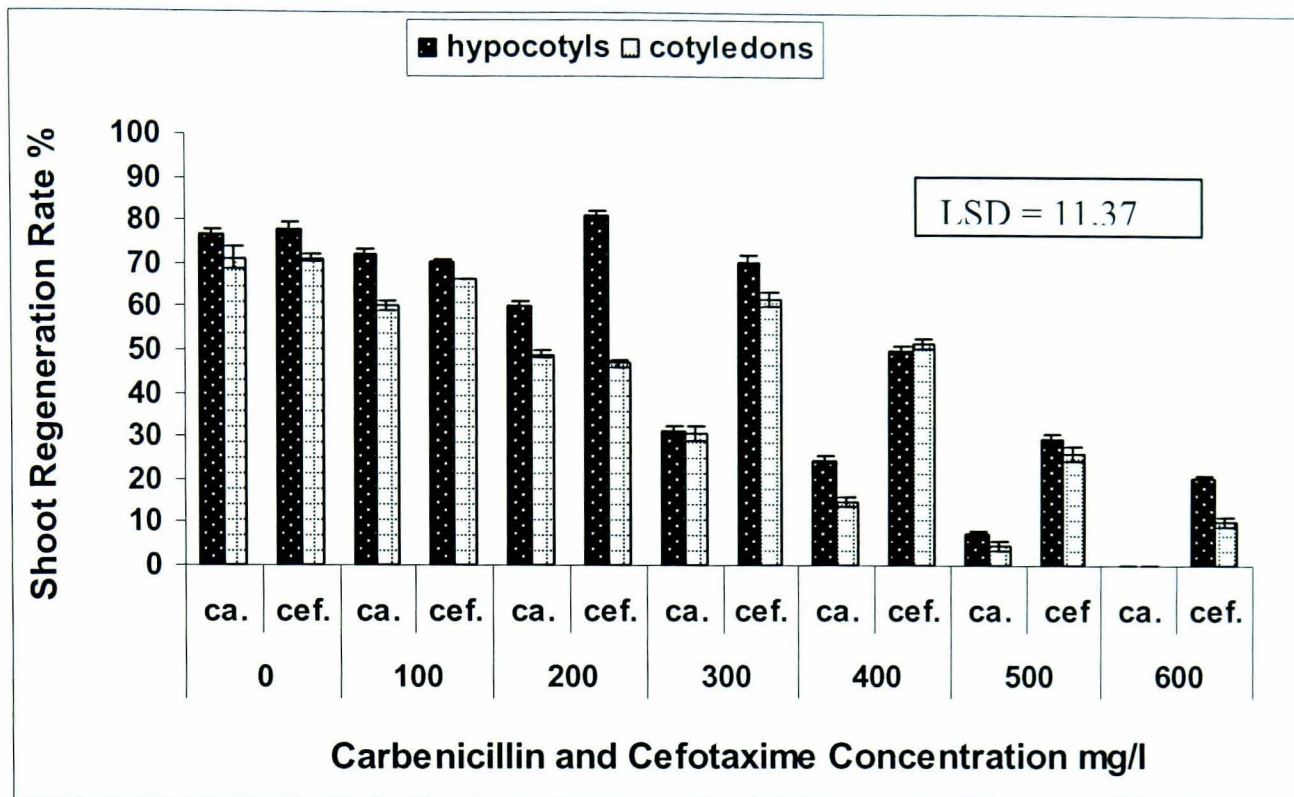
Figure 34. Effect of carbenicillin or cefotaxime on shoot regeneration. I=SE and n=15.
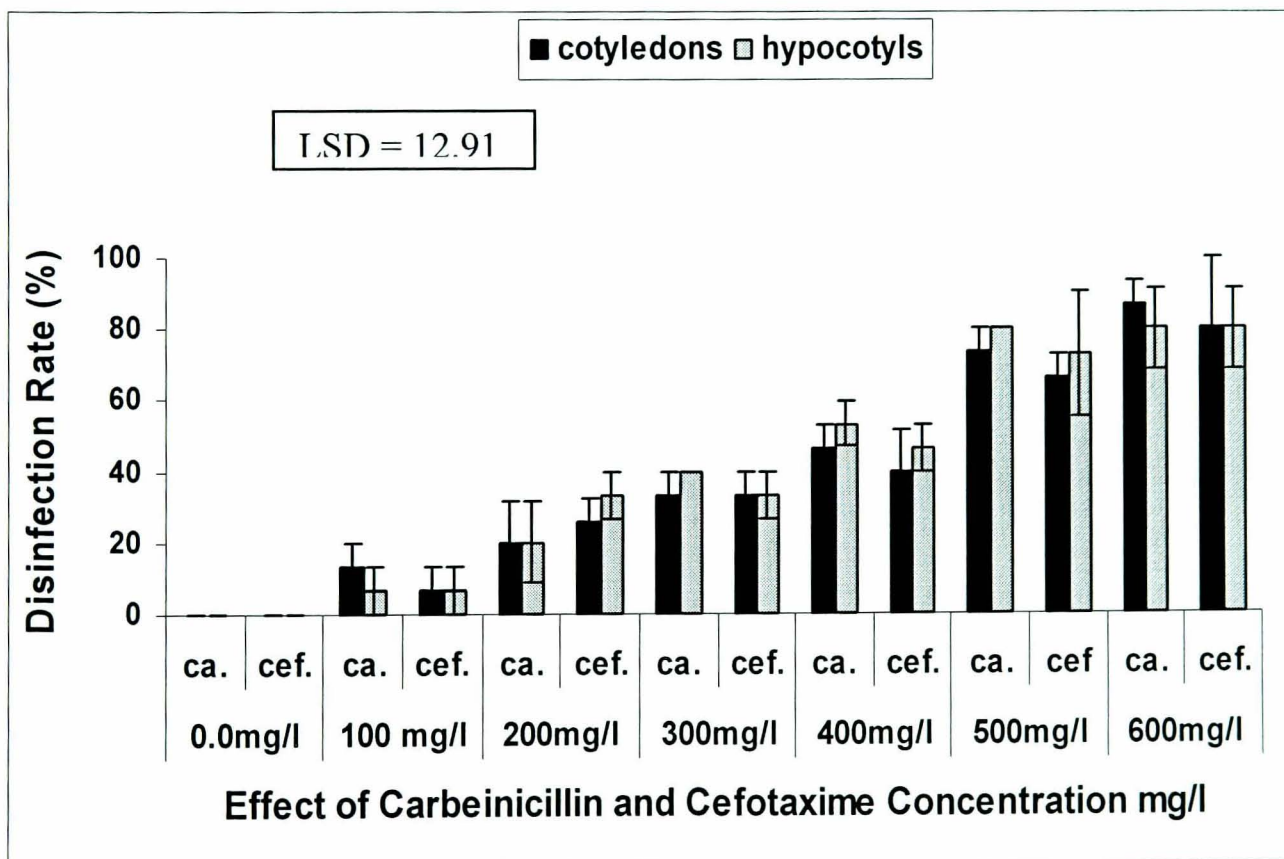
Figure 35. Effect of carbenicillin and cefotaxime on elimination of *Agrobacterium.* **I=SE and n=15.**

3.4.6 Sensitivity to kanamycin, tetracycline and gentamycin

3.4.6.1 Effect on callus and shoot regeneration

The selection antibiotics kanamycin, tetracyclin and gentomycin 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 callus growth and shoot induction was determined prior to transformation experiments. Previous experiments indicated that callus was induced on medium supplemented with 2 mg l^{-1} 2, 4-D (Plate 14 a & b) and shoot and root formation was produced on S23 medium supplemented with 0.2 NAA + 3.00 BAP and 20.2 NAA mg l^{-1}, respectively (Plate 14 c & d).

3.4.6.1.a Effect of kanamycin and tetracyclin

Various concentrations of kan. and tet. (Table 7), in addition to 250 mg l^{-1} cefotaxime were added to callus induction and shoot regeneration medium. Significant inhibition of callus induction and shoot regeneration was observed for the explants at concentration higher than kanamycin 5 mg l^{-1} + tetracyclin 3 mg l^{-1} (Figure 36 and Plate 15). The result showed that shoot regeneration from hypocotyl explants was less effective than cotyledons under all the treatments. As a result of this experiment the kanamycin 15 mg l^{-1} + Tetracyclin 6 mg l^{-1} was selected as an effective concentration for selection between the transgenic and non-transgenic plants

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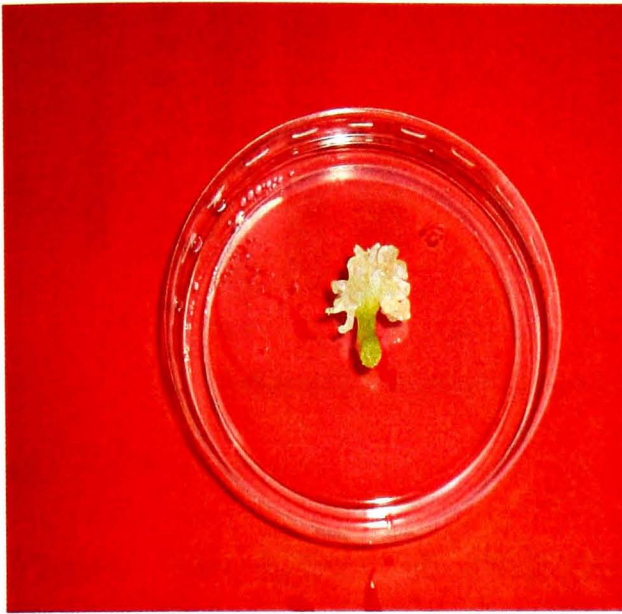
a)

b)

c)

d)

Plate 14. Callus induction from a) hypocotyl and b) cotyledon explant c) the start of shoot regeneration from cotyledon d) complete shoot regeneration and root initiation under control treatment
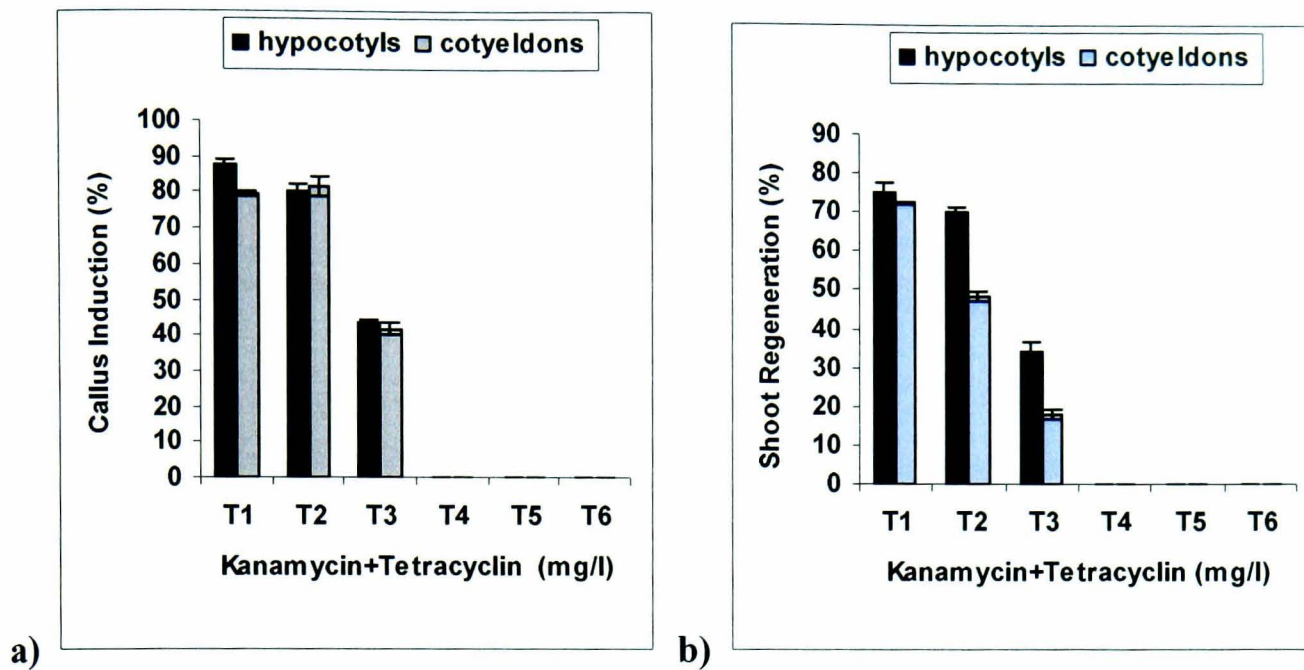
Figure 36: Effect of different combinations of kanamycin and tetracyclin (mg l^{-1}) on a) callus induction and b) shoot regeneration. I bar=SE & n=27.

Plate 15. Effect of different concentrations of kanamycin and tetracyclin on shoot regeneration {T1=0.0 Kan. + 0.0 Tet.; T2= 2.5 Kan. + 1.0 Tet.;T3=5.0 Kan. + 3.0 Tet.; T4=15 Kan. + 6 Tet.; T5= 25 Kan. + 9.0 Tet.; T6= 35 Kan. + 12 Tet. (mg l^{-1})}

3.4.6.1. b Effect of kanamycin and gentamycin

A combination of different concentrations of different two antibiotic (kanamycin and gentamycin) was added to callus induction and shoot regeneration media in the presence of 250 mg l^{-1} cefotaxime (Table 7). Observation of the development of cultures after four weeks are presented in Figure 36 and Plate 16.

The presence of 25 mg l^{-1} kan., + 6 mg l^{-1} gent., in the culture medium reduced but did not completely inhibit callus but no shoot regeneration was observed (Figure 37). No callus or shoot formation occurred on explants incubated on media containing 35 mg l^{-1} kan., + 8 mg l^{-1} gent.

For selection experiments concentration of kanamycin 25 mg l^{-1} + gentamycin 6 mg l^{-1} was used as effective selection concentrations for transgenic plants.
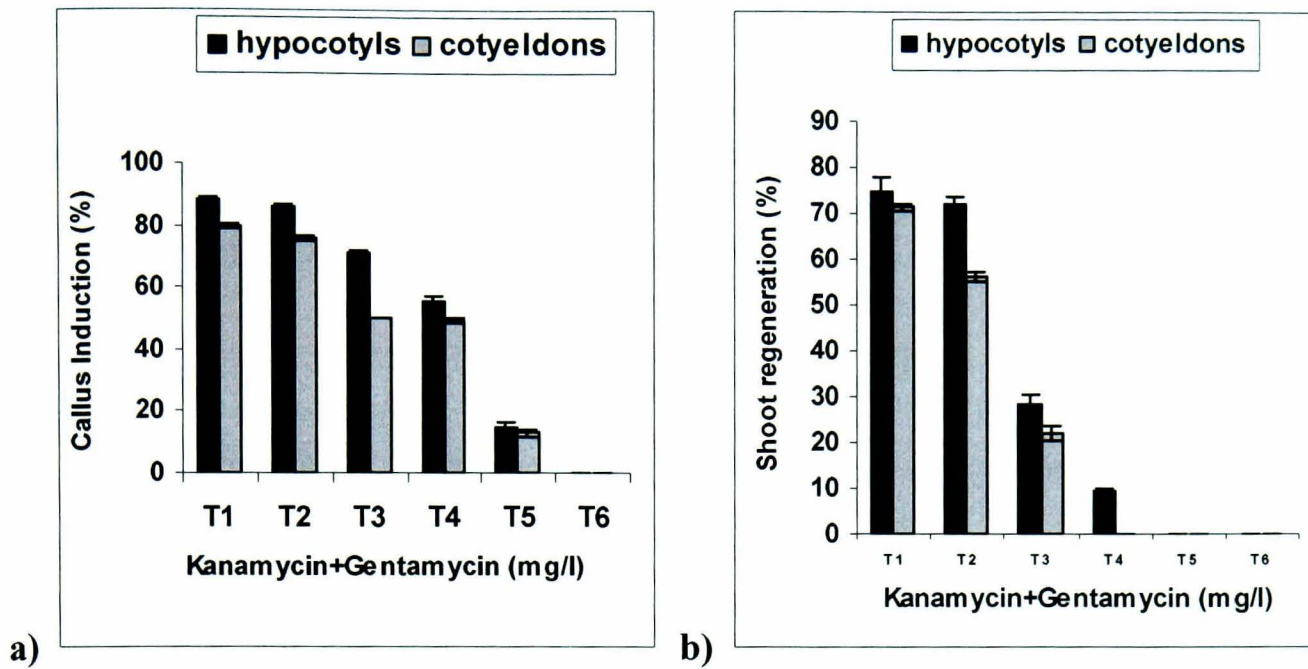
Figure 37. Effect of different combination of kanamycin and gentamycin on a) callus induction and b) shoot regeneration. I bar=SE & n=27

Plate 16. Effect of different concentration of kanamycin and gentamycin on shoot regeneration. {T1=0.0 Kan. + 0.0 Gent.; T2= 2.5 Kan. + 1.0 Gent.; T3=5.0 Kan. + 2.0 Gent.; T4=15 Kan. + 4 Gent.; T5= 25 Kan. + 6 Gent.; T6= 35 Kan. + 8 Gent. (mg l^{-1})}

3.4.6.1. c Effect of tetracycline and gentamycin

Different combinations of Tetracycline and Gentomycin were applied in this experiment to select the effective selection concentration between transgenic and non-transgenic plants (Table 7). The results showed that there was an inhibitory effect and a gradual decrease in green colour as antibiotic concentrations increased.

Tetracyclin, at 4 mg l^{-1} combined with Gentomycin at 6 mg l^{-1} , significantly reduced callus induction and shoot regeneration (Figure 38 and Plate 17).Very small numbers of explants on the above media produced callus clumps or shoots. Most of these calli and shoots went dark at the end of the third week in culture and the majority were totally necrosed by the fourth week. Callus induction and shoot regeneration were completely inhibited and there was no growth at concentration of tetracyclin 6 mg l^{-1} + gentamycin 9 mg l^{-1} or greater.

The 6 mg l^{-1} kanamycin + 9 mg l^{-1} gentamycin combination was chosen to select between transformed and non-transformed plants.
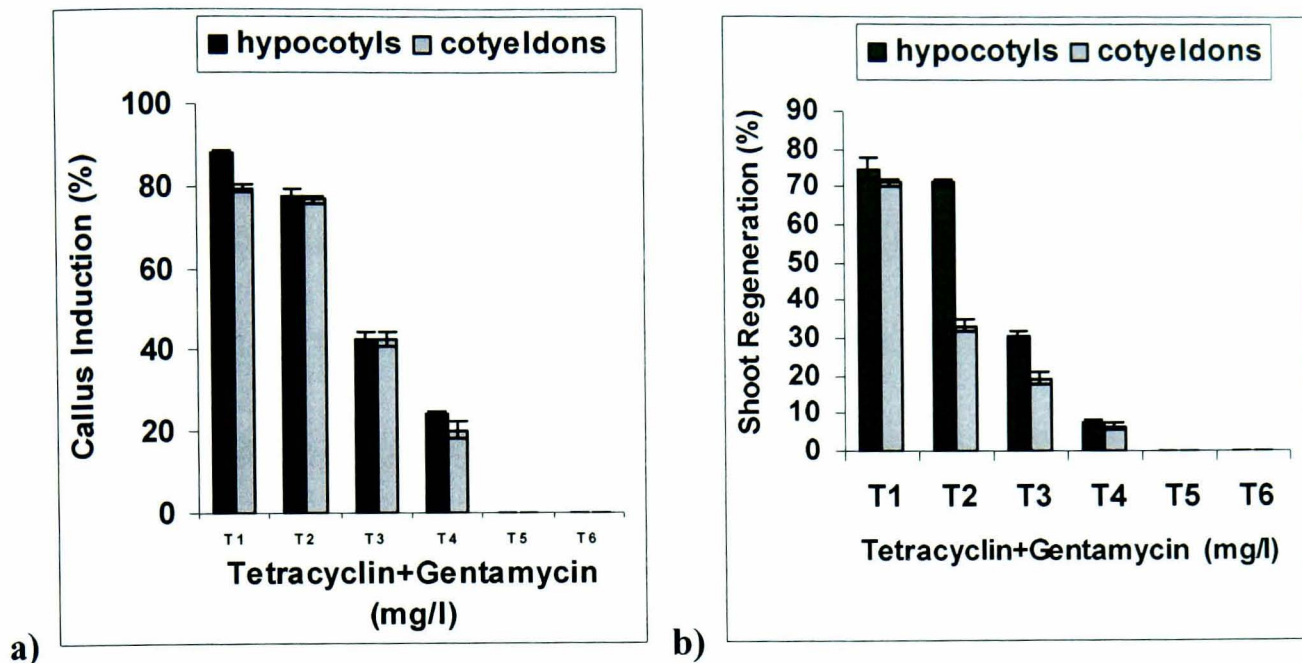
Figure 38 . Effect of combination of tetracyclin and gentamycin on a) callus induction and b) shoot regeneration. I bar=SE & n=27.

Plate 17. Effect of different concentration of gentamycin and tetracycline on shoot regeneration. {T1=0.0 Tet. + 0.0 Gent.; T2= 1.0 Tet. + 1.0 Gent.; T3=3.0 Tet. + 2.0 Gent.; T4=6.0 Tet. + 4 Gent.; T5= 9.0 Tet. + 6 Gent.; T6= 12.0 Tet. + 8 Gent. (mg l^{-1})}

3.4.7 Optimization of parameters enhancing *Agrobacterium* mediated transformation of cauliflower hypocotyls and cotyledons

In the present experiments the focus was placed on the optimization and evaluation of some transformation parameters with the aim of developing the efficient transformation of hypocotyls and cotyledons of cauliflower. Such parameters include: density of Agrobacterium culture (OD 600 0.5, 1;10 and 1:20); seedling age (4, 8 and 12 days); pre-culture period (0, 2 & 4 days); Co-cultivation (1, 2, 3 & 4 days); acetosyringone concentration (0.0, 35 , 70 &105 μM). Transformation rate was recorded as the number of shoots regenerated and surviving under selective medium supplemented with different concentrations of antibiotics.

3.4.7.1 Effect of *Agrobacterium* density

The influence of *Agrobacterium* density (OD_{600} 0.5) without dilution and with dilution (1:10 and 1:20) on the transformation rate % was evaluated and is presented in Figure 39. Explants treated with *Agrobacterium* strains APX, SA and TA without dilution failed to produce any transformation plantlets whilst those treated with 1:10 dilution showed the highest transformation rate and lower at 1:20 dilution. This shows that the number of *Agrobacterium* cells that were available to infect the plant tissues was important in affecting transformation efficency. Transformation rate was higher for hypocotyl explants compared to cotyledon explants under all the treatments except for

1:10 dilution of strain TA where cotyledon explants recorded a higher transformation rate (10%) compared with hypocotyls explant (8.3%).

In conclusion OD_{600} volume 0.5 at dilution 1:10 was selected for subsequent transformation experiments as the suitable *Agrobacterium* density for transformation.

3.4.7.2 Effect of seedling age

In the present study three types of seedling age 4, 8 and 12 days were compared. Results in Figure 40 showed that transformation rate was highest with the use of 8 days seedlings. Eight days hypocotyl explants treated with TA strain recorded the highest transformation rate (11.6 %). Explants from 12 day old seedling showed a lower rate of transformation than 8 days. Necrotic reaction on explants from 4 day old seedling was so high that none survived the treatment demonstrating the highly sensitive nature of young tissues. Hypocotyl explants showed the highest value of transformation compared with cotyledons under most of the treatment except that treated with SA strain where transformation percentage was equal for both hypocotyls and cotyledons from 8 and 12 day old seedling. Eight day old seedlings were selected to apply in all subsequent transformation experiments.
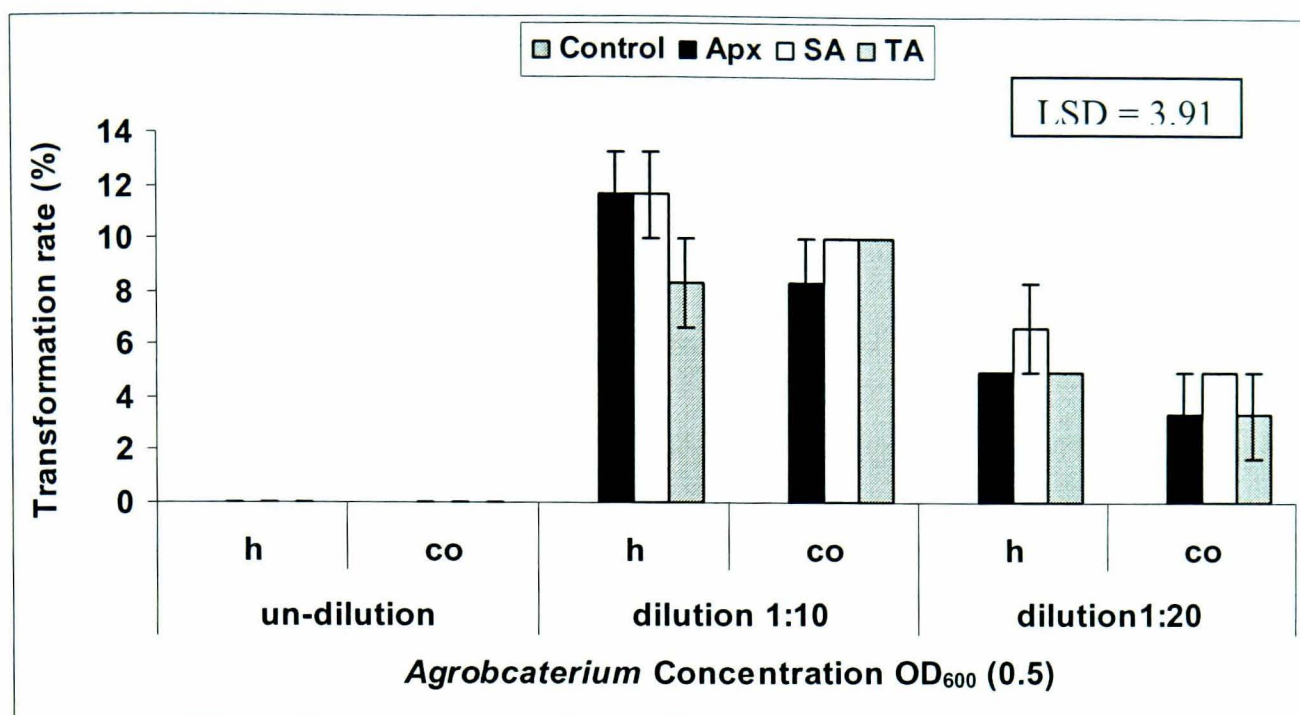
Figure 39 . Effect of *Agrobacterium* Density on Transformation Percentage. h= hypocotyls and co=cotyledons. I bar=SE & n=30.
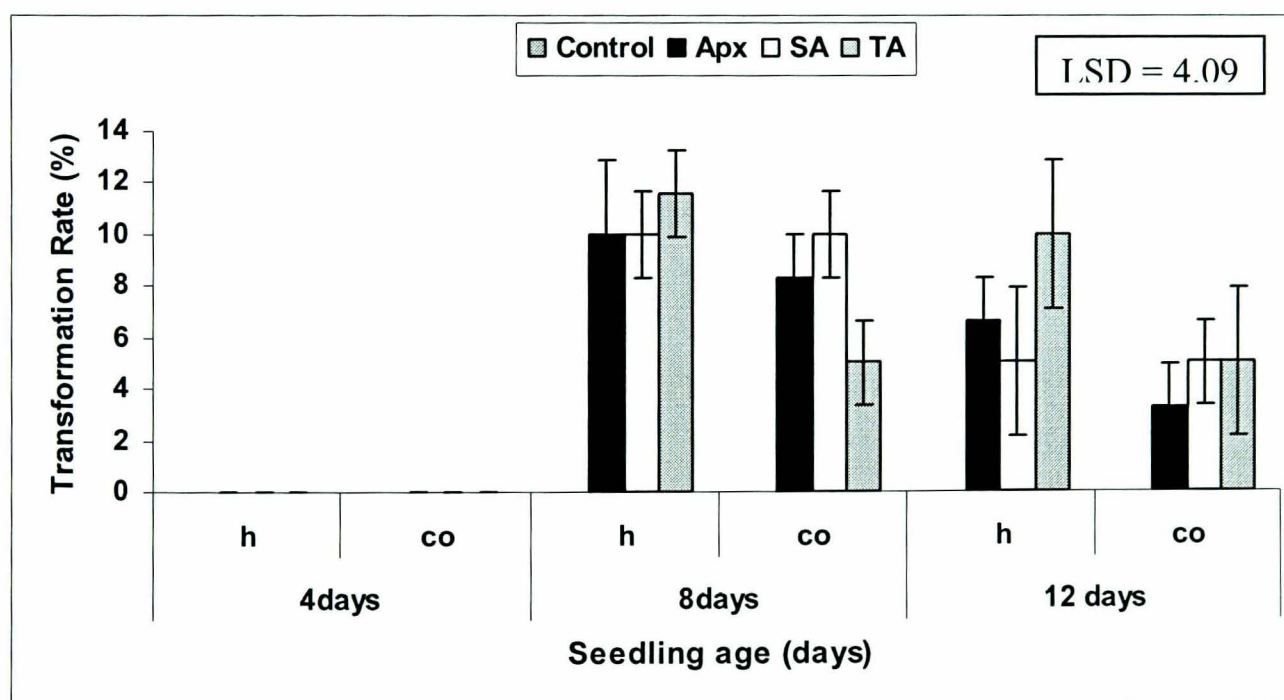
Figure 40 . Effect of Seedling age on Transformation Percentage. h= hypocotyls and co=cotyledons. I bar=SE & n=30.

3.4.7.3 Effect of pre-culture period

Prior to inoculation with *Agrobacterium*, explants were pre-cultured on S23 medium for 0, 2 and 4 days and the results are presented in Figure 41. No pre-culture period inhibited transformation but 4 days raised the transformation percentage and this was better than 1 day pre-culture. There was no significant difference between the two explant types. Hypocotyls and cotyledons with no pre-incubation, necrosis was evident showing a hypersensitive response.

Pre-culture of explants for 2 days was used in all subsequent transformation protocols.
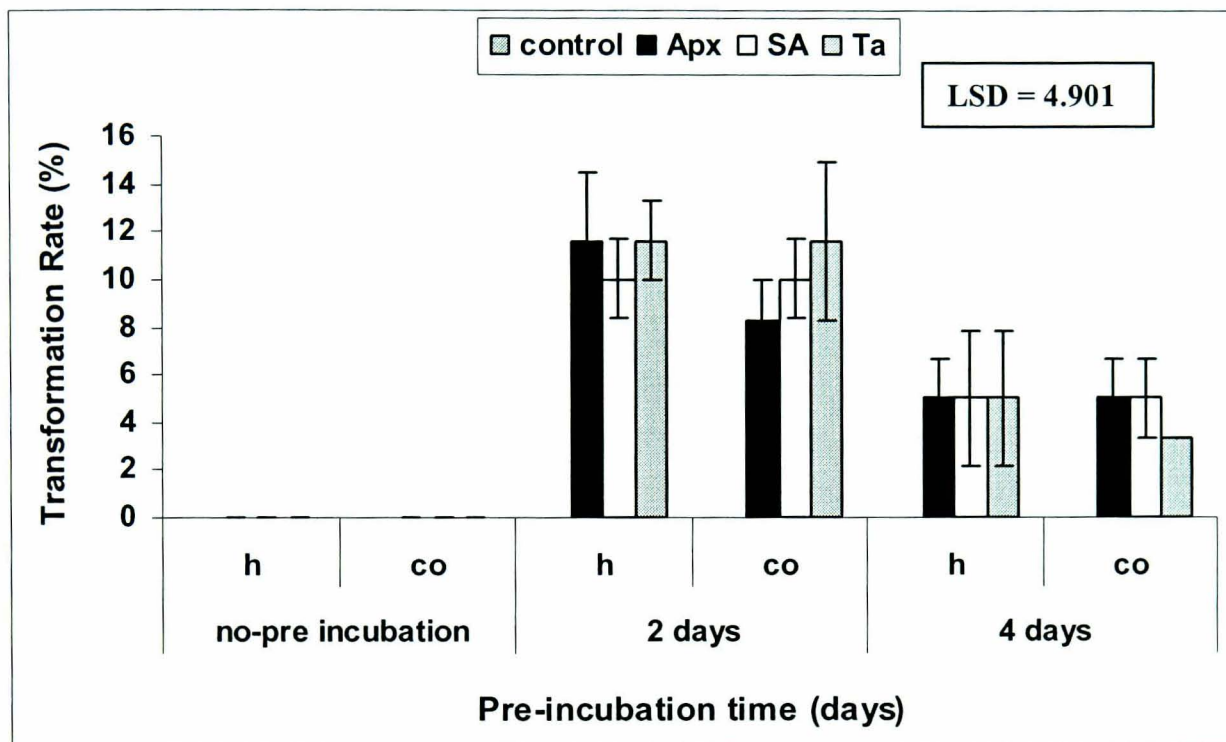
Figure 41. Effect of pre-incubation Periods on Transformation percentage. h= hypocotyls and co=cotyledons. I bar=SE & n=30

3.4.7.4 Effect of co-cultivation periods

It known that when *Agrobacterium* are mixed with plant cells, the *Agrobacterium* tends to aggregate, due to the presence of soluble plant exo-cellular polysaccharides, rather than binding to the plant cells. For this reason it is necessary to co-cultivate the bacteria and explants for a period of time. Hypocotyl and cotyledon explants after infection with different strains of *Agrobacterium* were incubated for 1, 2, 3 or 4 days in order to select which should apply in transformation experiments.

The results showed that co-cultivation for 1, 2, 3 days was suitable and significantly increased the percentage of transformation (13.3%). After 4 days co-cultivation transformation was significantly reduced and the explants failed to be survived due to *Agrobacterium* overgrowth in the cultures (Figure 42).
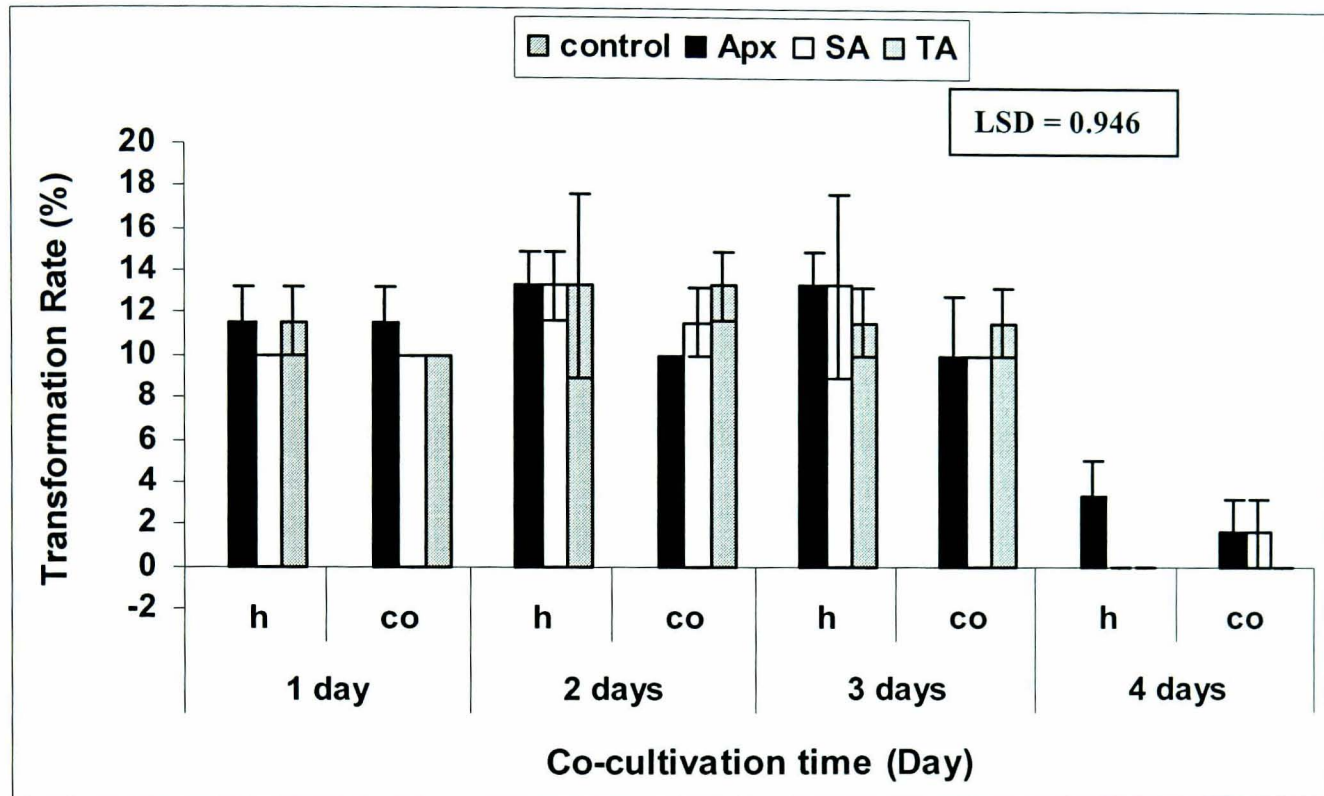
Figure 42 . Effect of co-cultivation periods on transformation percentage. h= hypostyles and co=cotyledons. I bar=SE & n=30

3.4.7.5 Effect of Acetosyringone

One virulence inducer acetosyringone was tested in an attempt to increase the infection probability of *Agrobacterium tumefaciens*. The results showed that the presence of acetosyringone significantly increased the percentage of transformation and the optimum concentration was 70 µM (Figure 43).

The addition of 105 µM acetosyringone was found to be unsuitable due to high degree of tissue browning at cut ends. Accordingly, acetosyringone at a concentration of 70 µM was added to the *Agrobacterium* suspension during its virulence induction period in all consequent experiments.
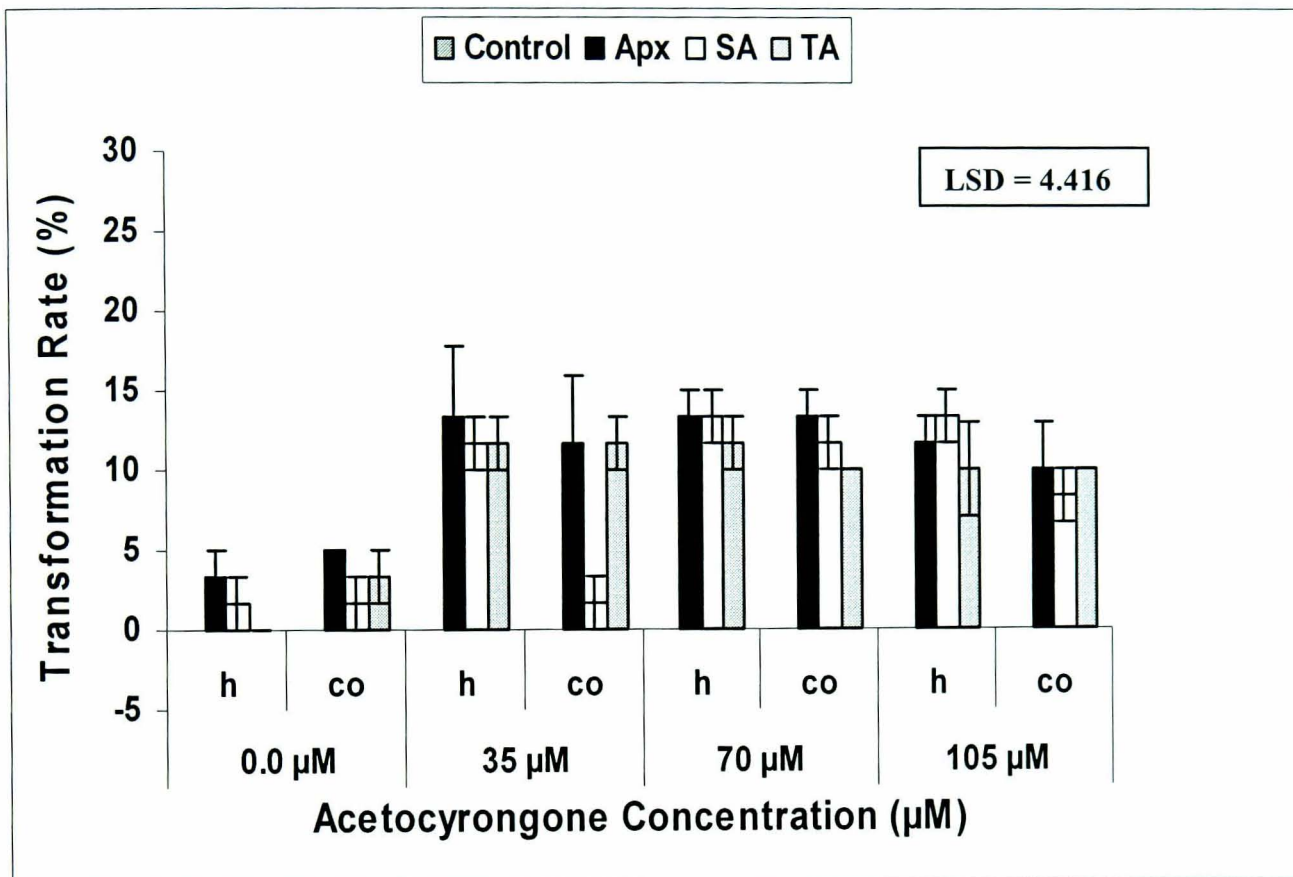
Figure 43. Effect of different concentration of Acetosyringone on Transformation Percentages. h= hypostyles and co=cotyledons. I bar=SE & n=30.

3.4.8 Selection of transgenic plants

After co-cultivation the infected explants were washed for 30 seconds with 250 mg/l cefotaxime to inhibit *Agrobacterium* growth, followed by three washes with sterilized distilled water and transferred to S23b (S23a medium with 250 mgl^{-1} cefotaxime medium) plus (25 mg l^{-1} kanamycin + 6 mg l^{-1} gentamycin) or (5 mg l^{-1} kanamycin + 6 mg l^{-1} tetracyclin) or (6 mg l^{-1} gentamycin + 9 mg l^{-1} tetracyclin). Within 2-4 weeks most of the untransformed shoots turned either pink or white and no further growth of these shoot was observed, while transformed shoots remained green on this medium and continued to grow. Transfer of regenerated green shoots to transformant selection medium was effective in avoiding false positives (Plate 18 & 19).
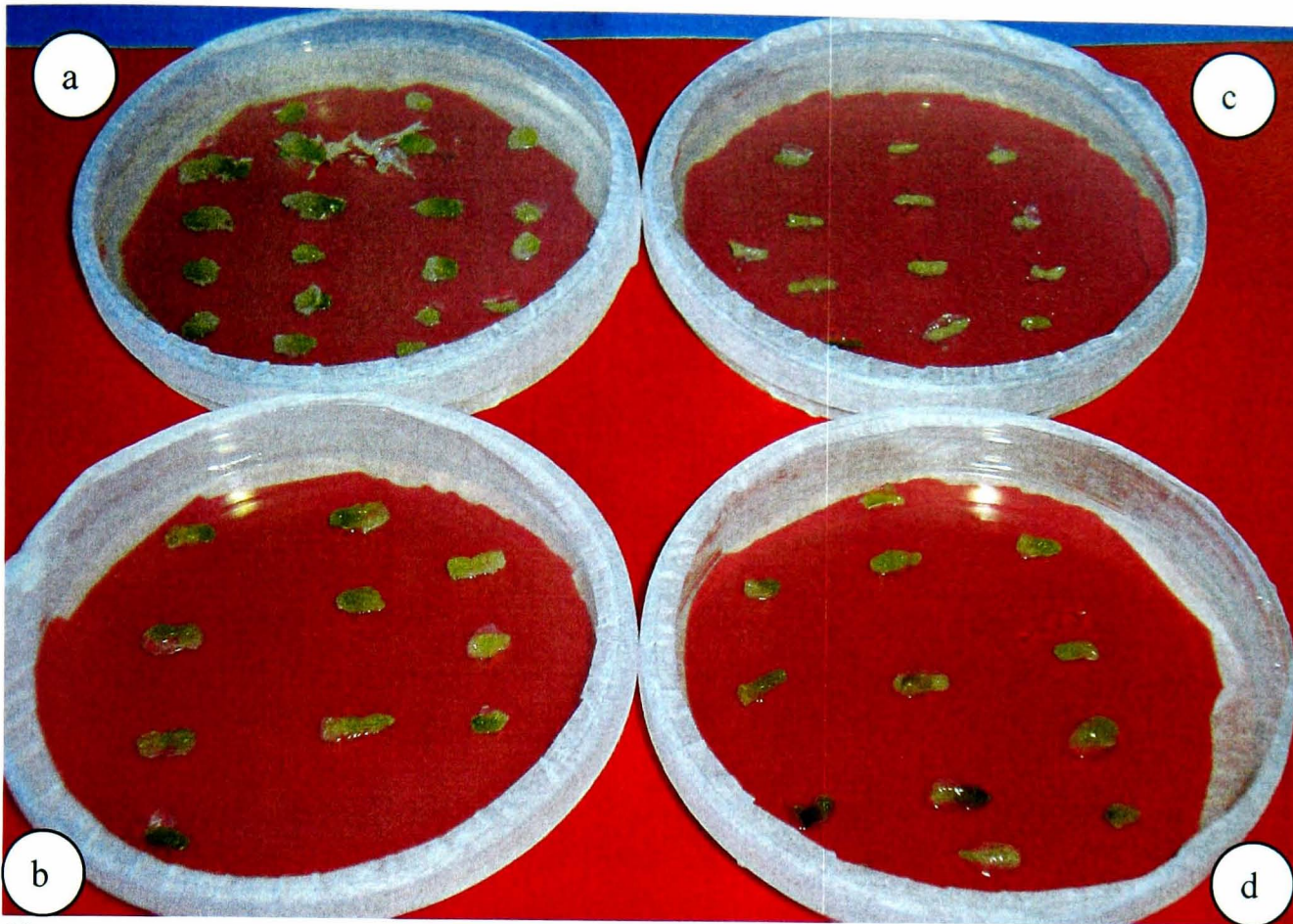
Plate 18. Selection of transgenic explants of 1) hypocotyls and 2) cotyledons for a)non-transgenic plant and b) transgenic plant culture on non-selective medium and c) non-transgenic plant and b) transgenic plant culture cultured on selective medium.

1)

2)

Plate 19. Selection of transformed plants infected with 1) APX strain and 2) SA strain. a) transgenic plant   b)non-transgenic plants.

3.4.9 Molecular analysis of *Agrobacterium* plasmid using PCR

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.

Three constructs were tested in this study. One contained APX inserted into $P^{CGN1578}$ as a vector, and the others contained SOD in *Agrobacterium* one using $P^{BIN\ +\ ARS}$ and other using $P^{CGN1578}$. The APX or SOD encoding sequences were detected at 478 bp after PCR amplification from the appropriate colonies (Plate 20).
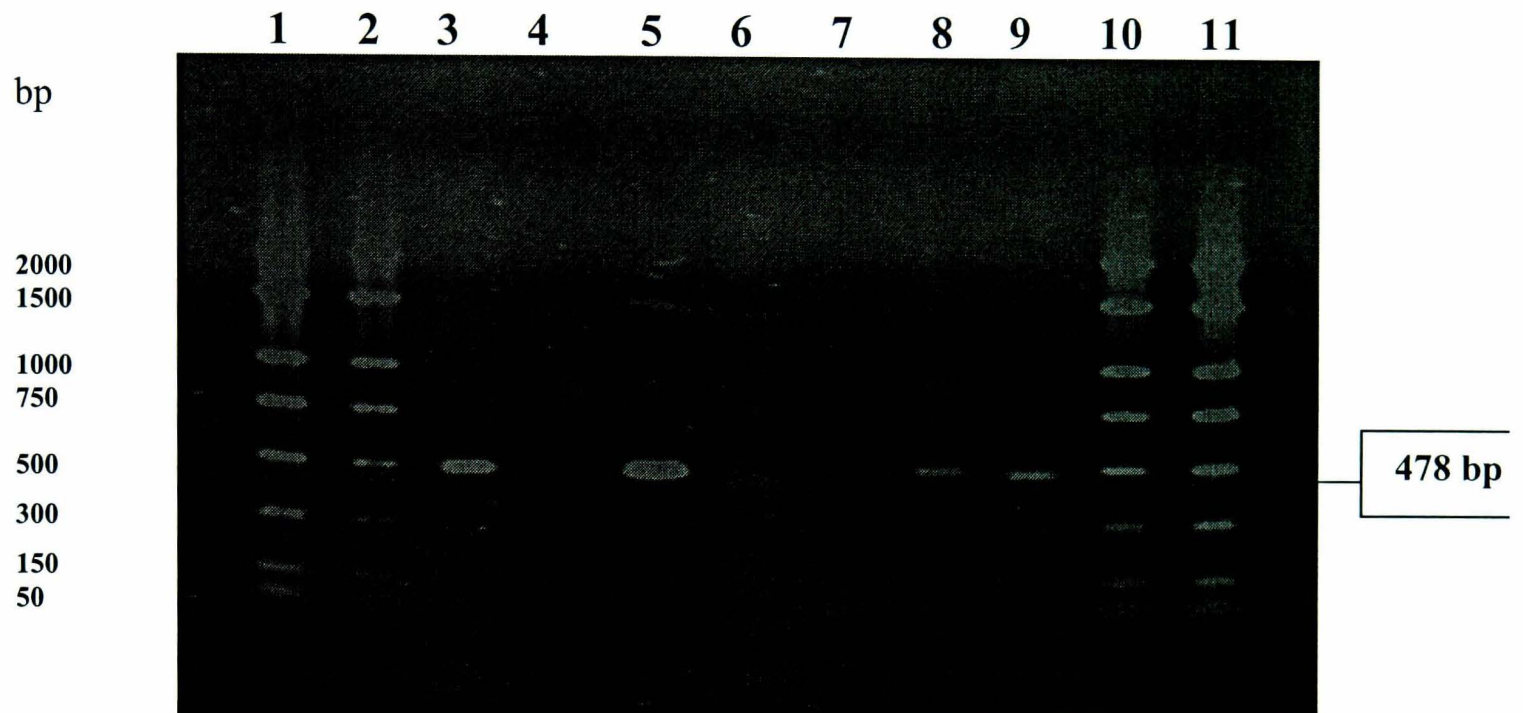
Plate 20. PCR detection of insert DNA in recombinant plasmids harboured in *Agrobacterium*. A. PCR product from *Agrobacterium* colonies contained recombinant plasmid with APX or SOD insert. Lanes 1,2, 10 and 11 are DNA markers; lanes 3 and 5 are from colonies harbouring PBIN + ARS (SOD insert) and PCGN1578 (SOD insert); lanes 8 and 9 are from colonies harbouring PCGN1578 (APX insert), consequently ;lanes 4 and 7 are the result of PCR without bacterial colony; lane 6 is a colony harbouring vector without insert.

3.4.10 Confirmation of transformtion

3.4.10.1 Leaf disc assay

APX and SOD gene expression were assayed on small pieces of leaf placed on S23e (S23 plus selective antibiotic) medium without sucrose. Untransformed leaf pieces did not swell on this medium and turned white within 5-6 days and lost the ability to form callus; transformed leaf pieces expanded in size and remained green with callus formation (Plate 21)

3.4.10.2 Histochemical and fluorescence GUS assay

Enzymatic analysis revealed GUS activity in hypocotyls and cotyledons used in this experiment. Samples taken from both explants showed GUS activities which indicated the presence of functional GUS enzymes in regenerated plantlets (Plate 22 & 23).

3.4.10.3 DNA analysis (RAPD-PCR)

Polymerase chain reaction was carried out (Plate 24) to provide further evidence for the presence of the APX and SOD gene in the genomes of transformed plantlets. DNA from leaf tissues of putative transgenic plants was analysed. In addition, a DNA extract from the APX and SA strains was prepared (as in section 3.3.7.3.c) and used as a positive control. DNA from non-transformed cauliflower leaves was used as a negative control. Specific

forward and reverse primers were used (as described in 3.3.7.3.c) to produce a

DNA fragment of about 478 base pairs if the transformed gene is present.

PCR results confirmed that a fragment of about 478 bp appeared in transgenic

plants whilst it was not present in non-transformed plants.
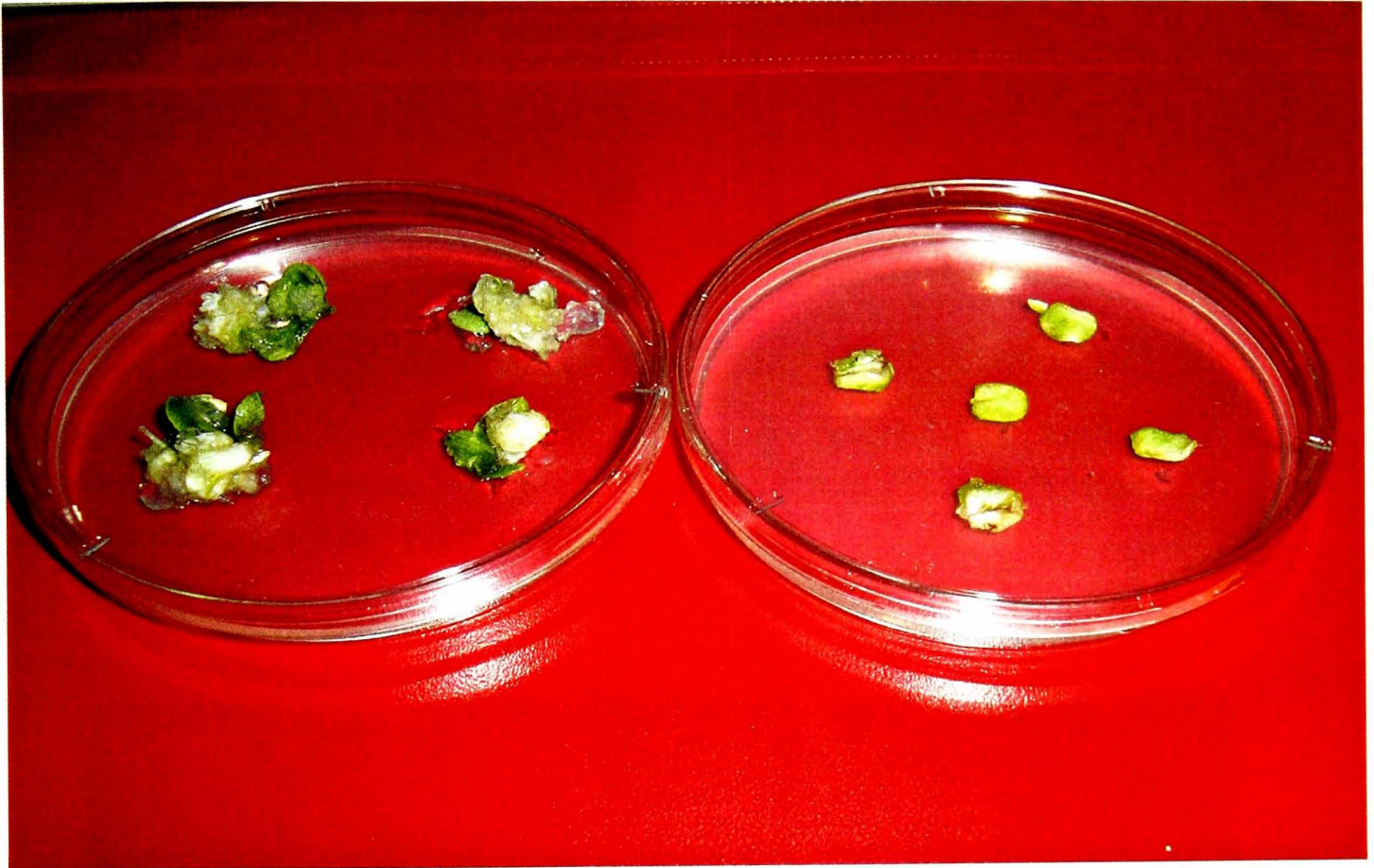
Plate 21. Leaf disc assay. Left, leaf pieces from transformed plants remained green and produce callus. Right, leaf pieces from untransformed control turned white without callus formation.
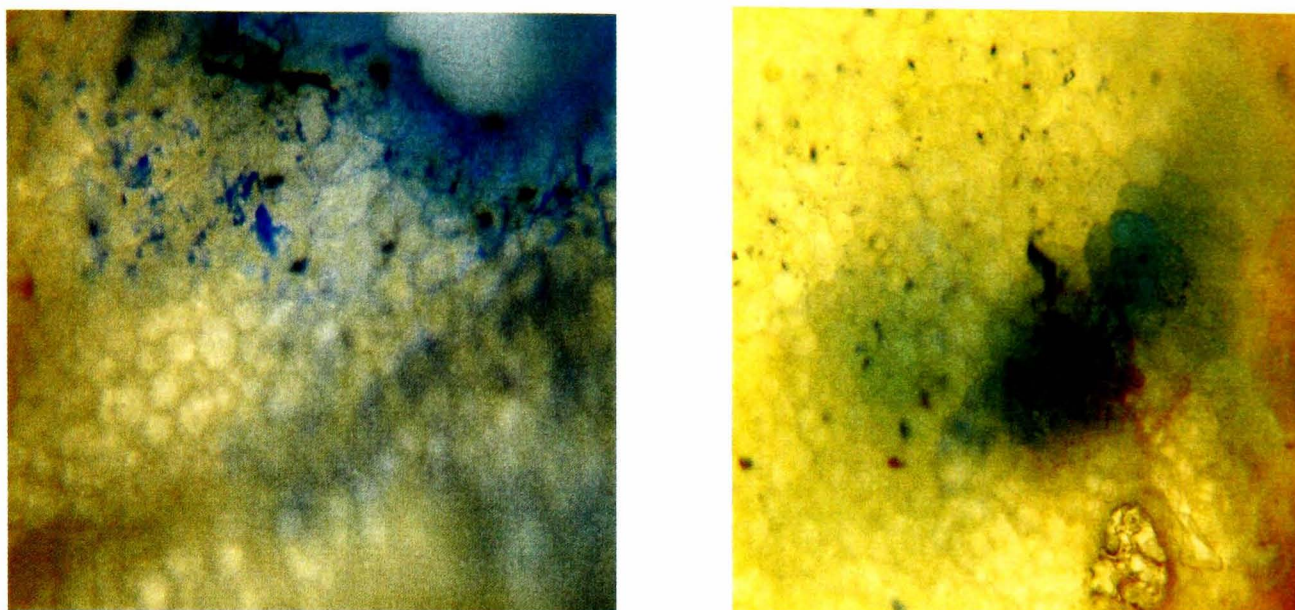
Plate 22. Transformation evidence in the peripheral region of an a) cotyledon and b)hypocotyl after co-cultivation with *A. tumefaciens* for 3 days and assayed for GUS activity.

a   b   c
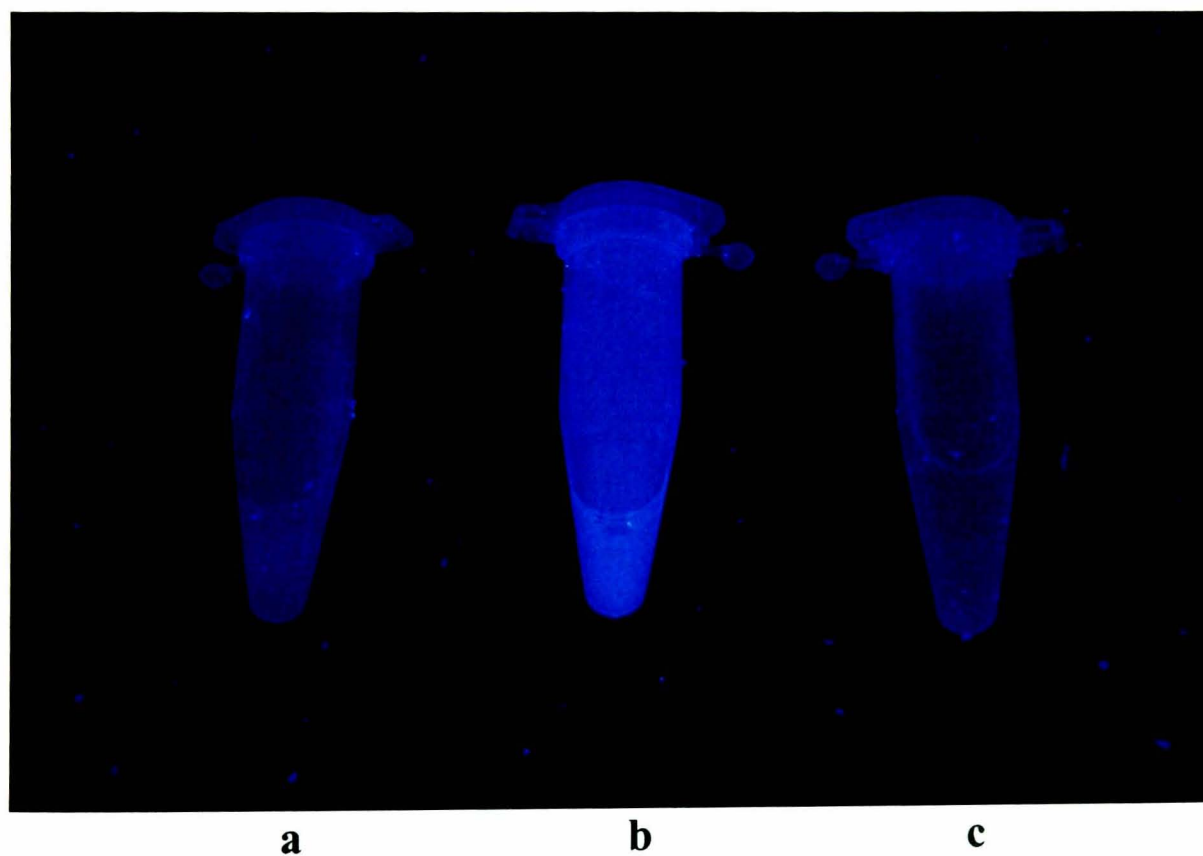
Plate 23. GUS assay showing strong fluorescence a) from *A. tumefaciens* culture b) from transformed leaf tissues of cauliflower plantlets. Non transformtion was detected from leaves of non-transformed plantlets (c).
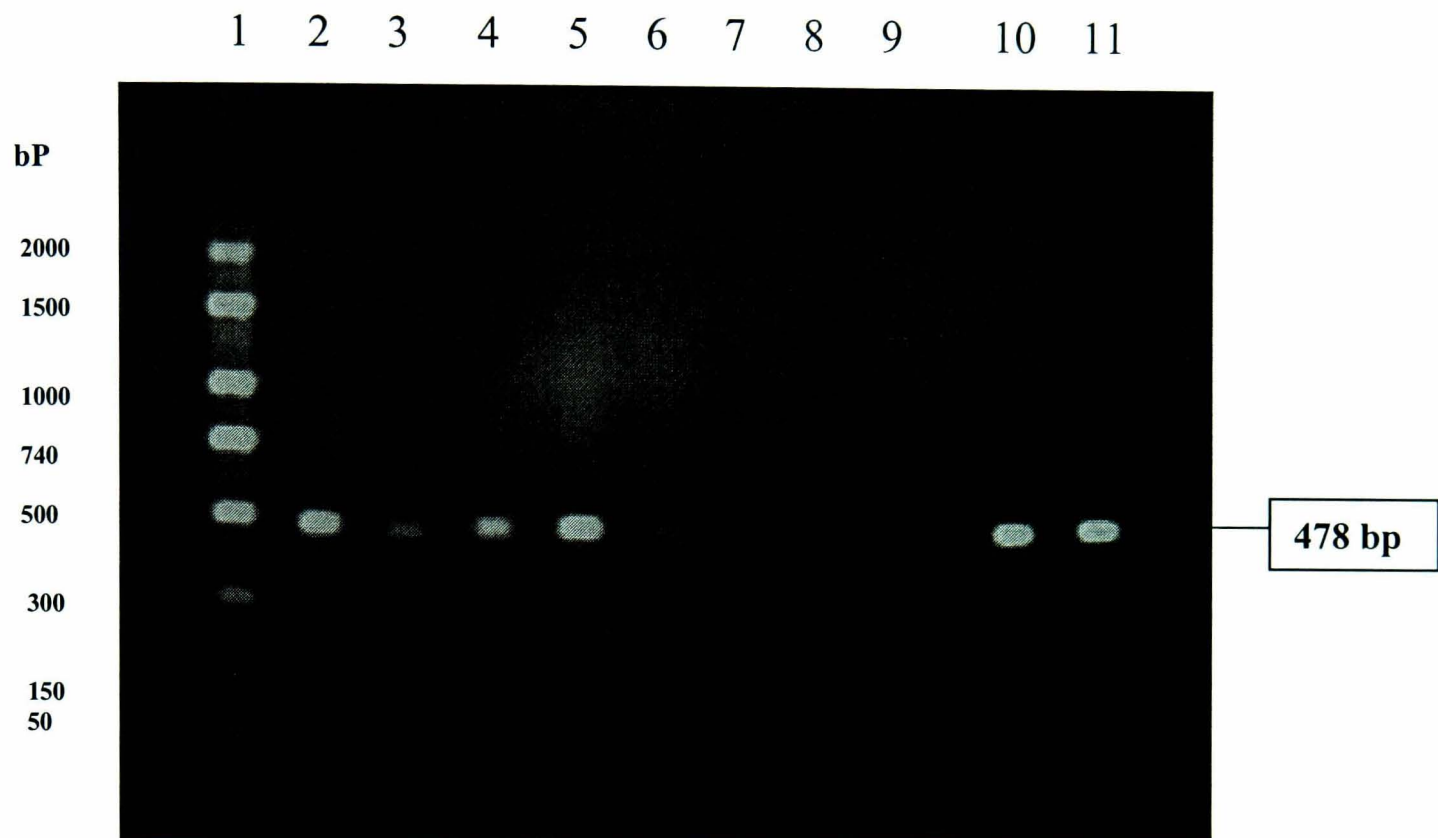
Plate 24. PCR analysis of the presence of the APX and SOD gene in putative transgenic plant. DNA molecular size marker (lane 1), negative control (non-transformed cauliflower leaves) (lane 7 & 9), transformed plants carrying SOD gene (lane 2, 4 & 5), positive control (*Agrobacterium* DNA of SA or TA Strain) (lane3&6), transformed plant carrying APX gene (lane 10), positive control (*Agrobacterium* DNA of APX strains) (lane 11) and water (lane 8).

3.4.11 Transformation experiment by using curd explants

No data was recorded because all the explants failed to survive the infection with *Agrobacterium*.

3.4.12 Expression of transgenes (APX and SOD) in the putative transgenic plant under salt stress

Transgenic plants were evaluated after 28 days of growth on solid S23 medium supplemented with 300 mM NaCl. Morphological and biochemical parameters were used to test the transgenic plant under salinity treatment as follows:

3.4.12.1 Effect of salt tolerance on shoot fresh weight of transgenic plants

In control plants, shoot fresh weight was adversely affected by salinity treatment (Figure 44; Plate 25 & 26), whilst in transgenic plants carrying APX and SOD this depression in growth was significantly less.
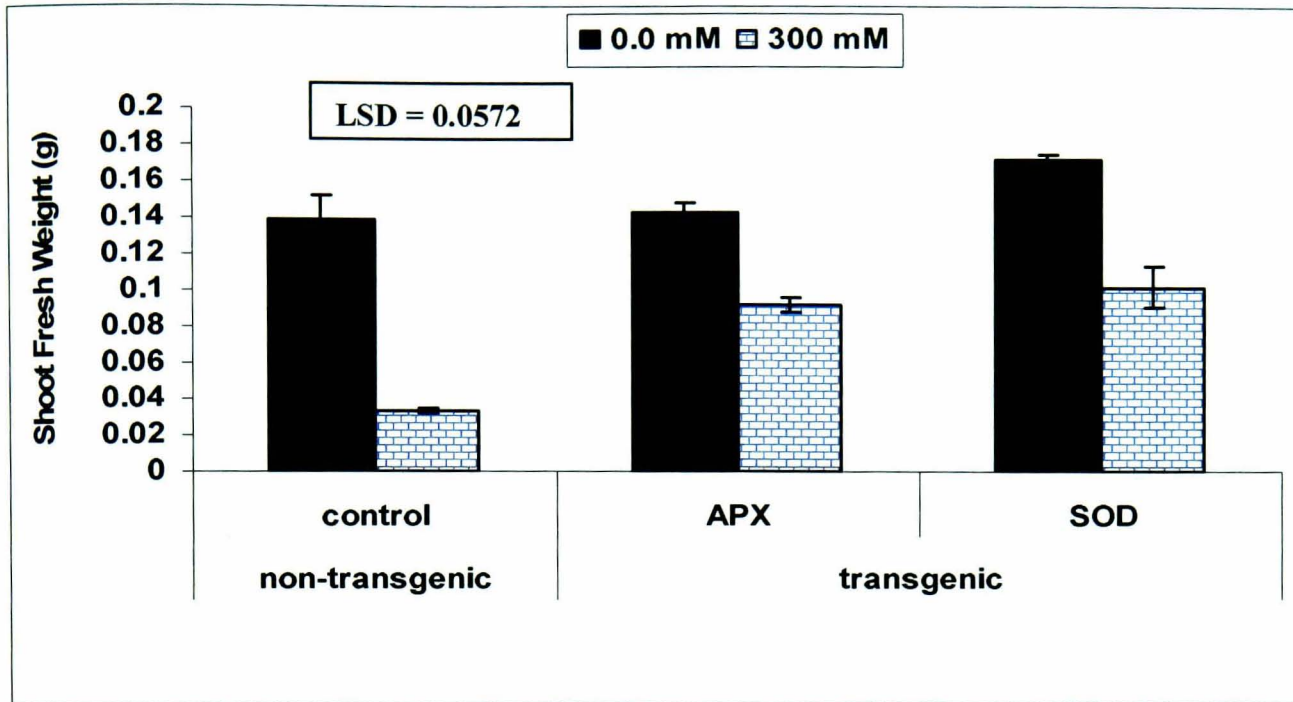
Figure 44. Effect of salt treatment on fresh weight in transgenic and non-transgenic plants. I bar = SE and n=15

Plate 25. Effect of salt treatment on shoot growth in control plants.

1)
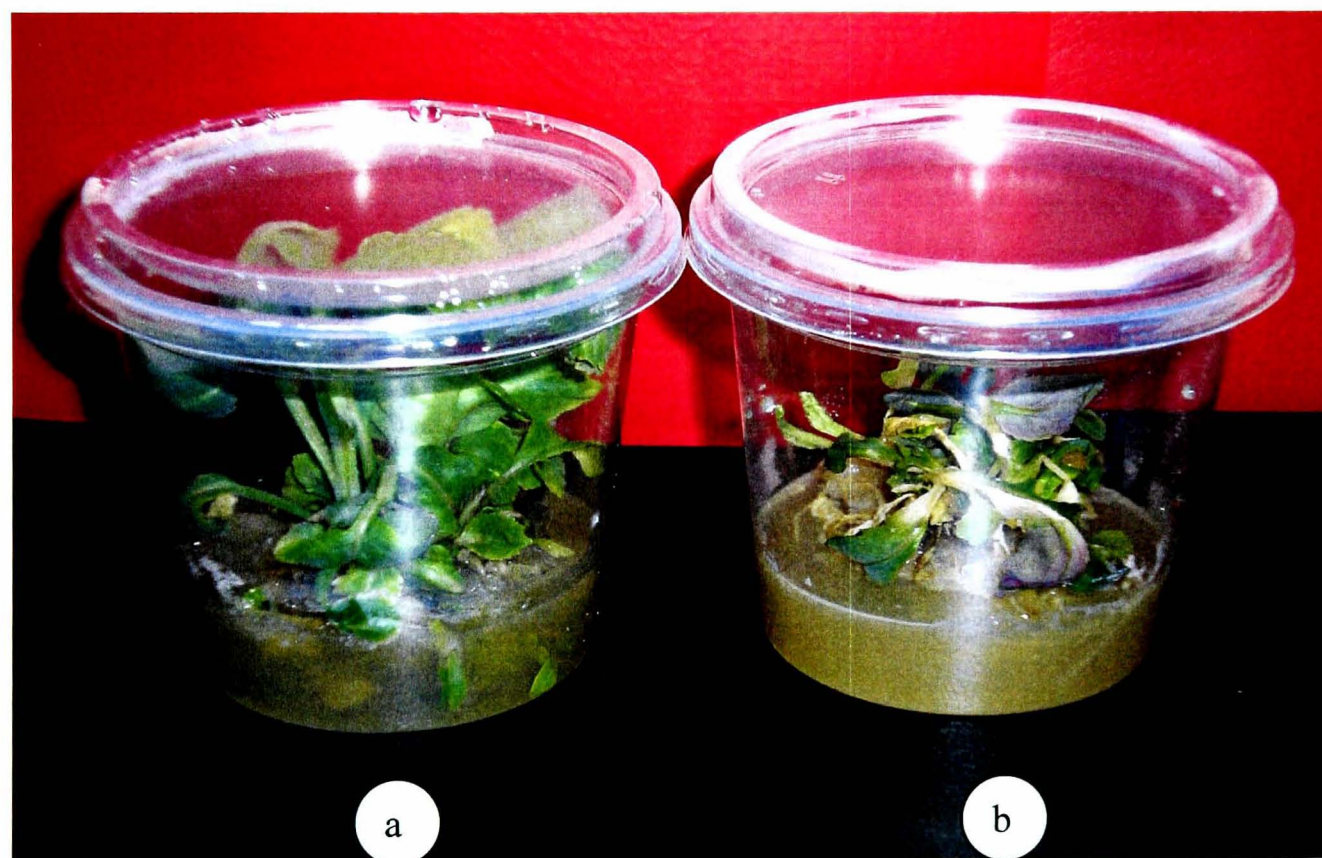
2)

Plate 26. Effect of salt treatment on shoot growth in transgenic plant 1) carrying APX gene   2) carrying SOD gene. a) 0.0 NaCl and b) 300 mM NaCl

3.4.12.2 Effect of salt treatment on proline content of transgenic plants

The level of proline in transformed and non-tranformed plants grown under zero and 300 mM NaCl. The results showed that for the control plants the level of proline was low and stayed low when challenged with NaCl. These levels were comparable to those seen with control plants in mutation experiments in chapter 2. The transgenic plants however showed a significant rise in proline when challenged with NaCl of the order of 5 to 6 times control levels (Figure 45).
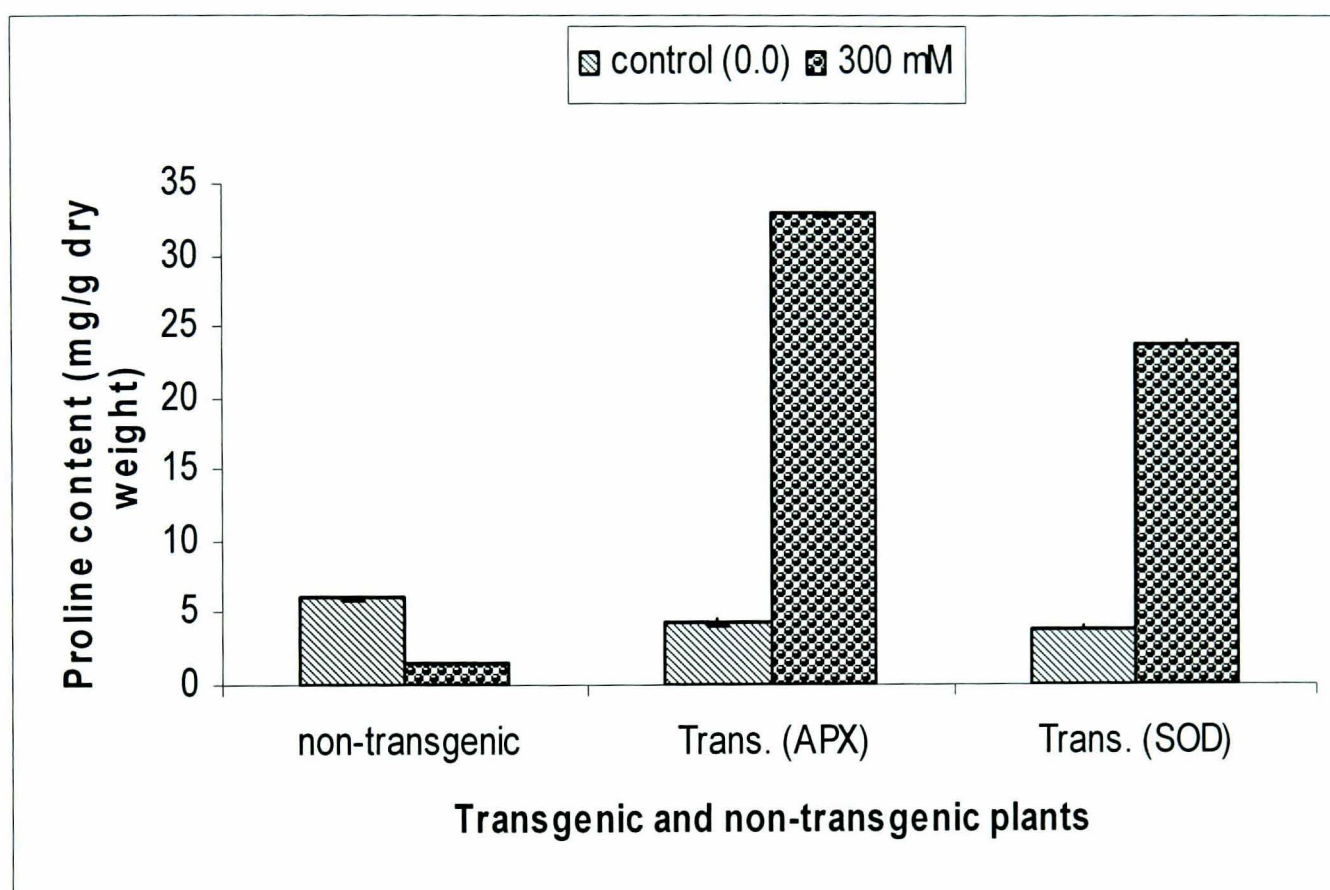
Figure 45. Effect of NaCl on proline content in transgenic and non-transgenic plants. LSD was equal = 1.11. I bar=SE & n=3

3.4.12.3 Effect of Salt tolerance on Na, K, Ca and Mg content of transgenic plants

Leaf tissue Na, K, Ca and Mg content were expressed as mg/g dry weight. The results showed that Mg, Ca and K concentration tended to be reduced under salinity treatment compared with the control (Figure 46). The Mg, Ca and K content as lower in non-transgenic plants than transgenic plants.

Sodium concentration showed the opposite trend with Na level in transgenic plants higher under salinity treatment compared with control treatment. Highest Na concentration (13.13 mg/g) was recorded for transgenic plants infected with SA strain under salinity treatment, while lowest Na concentration was recorded for non-transgenic plants under the same level of salinity. Analysis of variance for experiments indicated Na, K, Ca and Mg content significant differences between the treatment and the type of plants (transgenic or non-transgenic) except for Na, K, and Mg and Ca (untreated).

Significant interaction between treatments and type of plants was found for Ca and K concentration of the plant tissue. There were no significant effects of interaction between the salinity and type of plants for Mg and Na concentration.
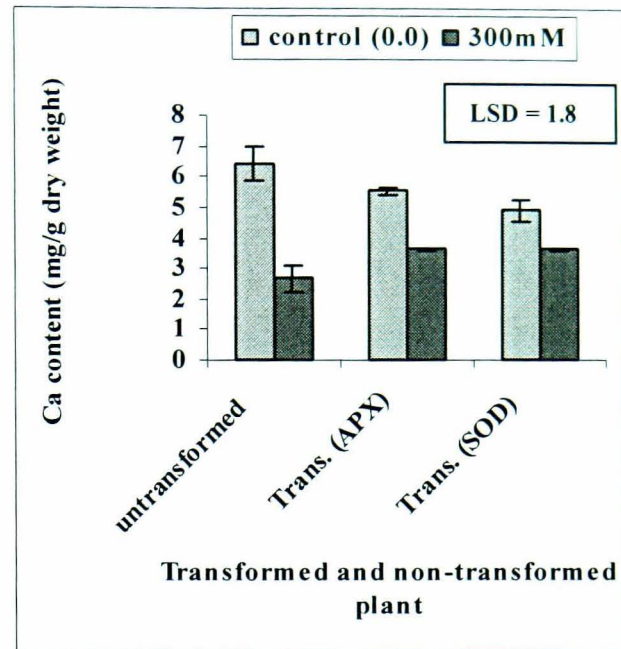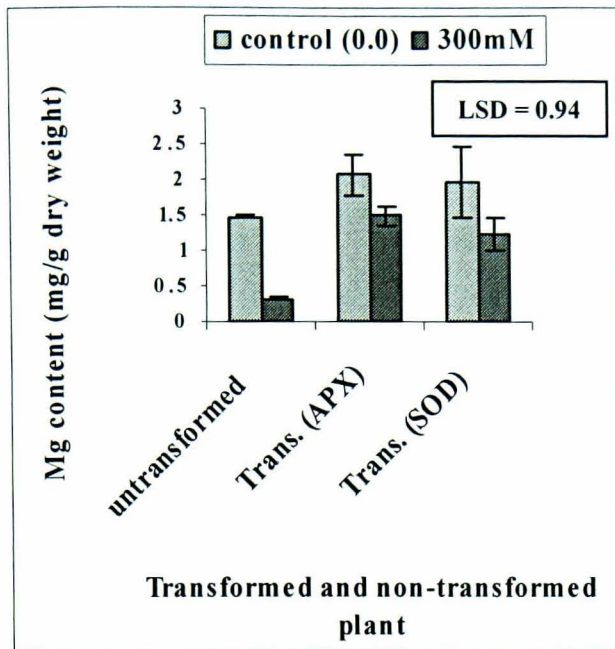
a)

b)
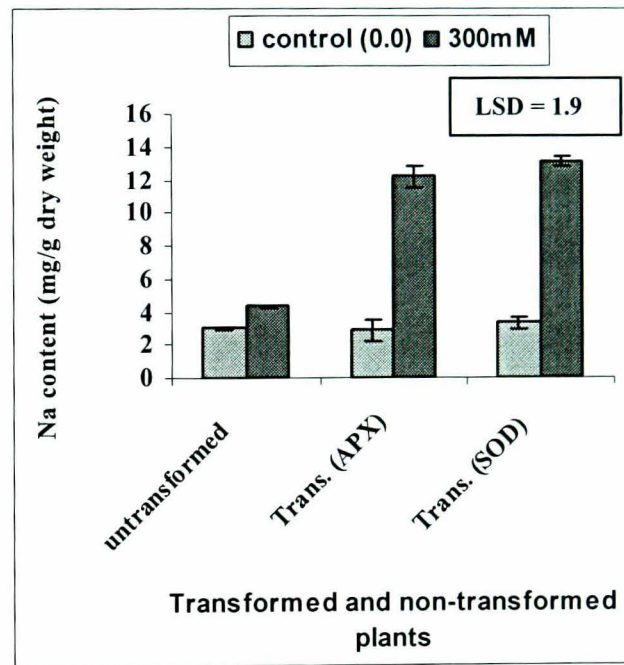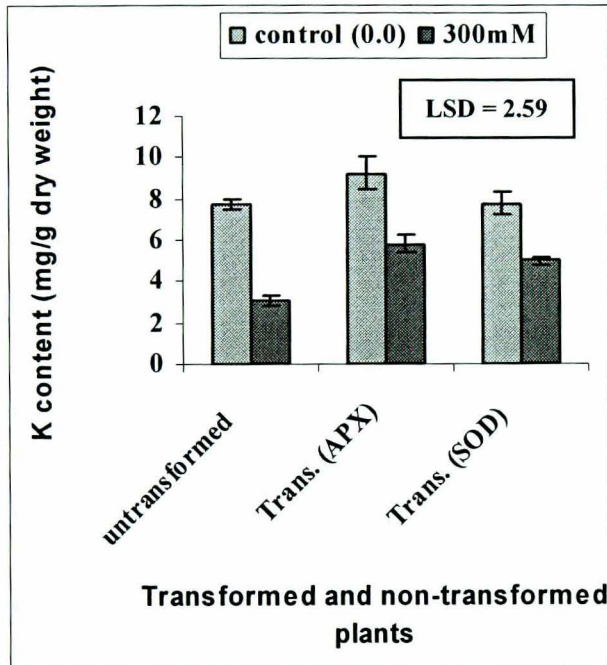
c)

d)

Figure 46. Effect of salt treatment on a) Magnesium b) Calcium c) Potassium d) Sodium content in transgenic and non-transgenic plants. I bar=SE & n=3

3.5 Discussion

One of the main objectives of the work described in this thesis was to develop an efficient protocol for genetic modification of cauliflower from hypocotyls or cotyledons. Development of an efficient gene transfer system largely depends on a rapid and reliable *in-vitro* regeneration system for the desired plant species (Christy and Sinclair 1992). Hence a further objective was to establish an efficient regeneration system via organogenesis that may result 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 the regeneration and transformation experiments.

Optimization protocols for hypocotyls and cotyledons regeneration

Tissue culture of cauliflower has been carried out for long time with plant regenerated from tissue such as curd (Kieffer *et al.,* 2001), hypocotyls (Lv Lingling *et al.,* 2005; Chakrabarty *et al.,* 2002), cotyledons (Prem and Nicole 1998), root (Grout, 1980), and stem discs (Eimert and Siegemund 1992; Suman *et al.,* 1985). Shoot regeneration from these different tissues are affected by several variable factors. These variables include the type and character of explants and the medium composition (Gregco *et al.,* 1984; Finer, 1987, Burrus *et al.,* 1991; Sun *et al.,* 1998; Muller *et al.,* 2001; Dhaka and Kothari, 2002; Lv Lingling *et al.,* 2005; Flavio and Beatriz 2005).

Effect of growth regulator on callus induction, shoot regeneration and root formation

For these reasons two types of cauliflower explant were cultured on media containing 20 combinations of plant growth regulators NAA, kinetin and BAP. Depending on the explant type and concentration of the growth regulators in culture media, callus formation, shoot regeneration and root formation was induced in both types (hypocotyls and cotyledons). In general terms, a high cytokinin ratio to auxin was found to stimulate shoot production.

In this study the highest regeneration response (79.6 and 76.8 %) was observed on S23 media with a high concentration of cytokinin (3mg l^{-1} BAP) and auxin (0.2 mg l^{-1} NAA). Similar results were obtained by Omer and Nurey (2000), who found 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) similarly found that all callus and cell suspension failed to regenerate shoots on MS media containing kinetin and IAA in *Brassica* whereas Murata and Ortan (1987) also used MS media supplemented with kinetin and IAA and obtained regeneration from callus in *Brassica* species.

Limited shoot regeneration was also obtained on medium without growth regulators. Similar results were obtained in *Vigna radiata* (Gulati and Jaiwal 1990) and in linden (Omer and Nurey 2000). This response could be related to the endogenous hormones balance in the explants.

Analysis of the response of different explant types to different shoot induction media showed that the interaction highly significant. Similarly, Punia and Bohorova (1992) reported that regenerating intact 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 0.2 NAA mg l^{-1}. This auxin (NAA) has been reported to stimulate a high frequency of root formation on regenerated shoots in many plant species such as Arabidopsis (Jocelyn and Katherine 2001); Orchidaceae (Kerbauy, 1984); Tobacco (Prisca and Peter 2005); *Posidonia oceanica* (Balestri and Lardicci 2006). This probably indicates that cells in the highly regenerative area of hypocotyls and cotyledons have the potential to develop into roots, depending on the level of auxin as suggested by Mante *et al.*, (1989) and Wayne (1996).

Previous work with *Brassica napus, Brassica oleracea* (Biddington and Robinson 1991); *Brassica rapa ssp.* Oleiffere (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. In this study, shoot regeneration and elongation of *Brassica oleracea's* substantially increased in the presence of $AgNO_3$ (3.5 mg l^{-1}). This indicated that $AgNO_3$ acts with explants and exogenous growth regulators to improve shoot regeneration; similar responses have been recorded by Witrzens *et al.,* (1988); Malone-Schoneberg *et al.,* (1994) ; Flavio and Beatriz (2005); and Kuvshinov *et al.,*(1999). The addition of more than 5-10 mg l^{-1} of $AgNO_3$ may have a toxic effect on shoot cultures of cauliflower (Chakrabarty *et al.,* 2002).

Elongation of plantlets was performed also in the presence of GA_3 (0.01 mg l^{-1}). From several reports it is known that GA_3 promotes shoot elongation (Power 1987; Malone-Schanoberg *et al.,* 1994). Recently, it has been demonstrated that gibberellins control meristem identity in *Arapidopsis* and *Sinapsis* flower mutants, with flower bud induction by activation of MADS-box gene (Lie *et al.,* 2002). By increasing shoot elongation the competition between leaves to catch the light will be less and this may lead to increased leaf area and also increased the photosynthesis.

Antibiotic effects

Effect of kanamycin, gentamycin and tetracyclin on callus induction and shoot regeneration

The use of antibiotics in plant tissue culture is not generally recommended because plant cells are known to be sensitive to antibiotics such as kan. ,tet. and gent. (Dix *et al.,* 1977) and a selectable marker for identification of the rare transformants is required. One of the objectives in this work was to obtain plant transformation using kan., tet. and gent. resistance genes as selectable markers. It was thought that the selective agents might also influence callus induction and shoot regeneration. Generally, the regeneration frequency of either hypocotyls or cotyledons decreased as the concentration of kan. or tet. or gent. increased and, at high concentrations, the regenerated shoots were weak, had white leaves and died after a short time. The same observation was reported in *H. annus L.* by Pugliiesi *et al.,* (1993), rootstocks by Yepes and Aldwinckle, (1994b) and plum plants by Padilla *et al.,* (2003).

The latter authors reported that once the explants had been exposed to kanamycin, the initiated shoots began a slow bleaching process which progressed over several subcultures. This phenomenon was also observed in both transformed and non-transformed shoots in the preliminary transformation experiments reported here. Moreover, starting from 6 mg l^{-1} tet. + 4 mg l^{-1} gent. or 5 mg l^{-1} kan. + 3 mg l^{-1} tet. or 15 mg l^{-1} kan. + 4 mg l^{-1}

gent. the necrosis appeared on the regenerated shoot while 9 mg l^{-1} tet. + 6 mg l^{-1} gent. or 15 mg l^{-1} kan. + 6 mg l^{-1} tet. or 25 mg l^{-1} kan. + 6 mg l^{-1} gent. totally prevented shoot and callus induction. This indicated that both antibiotics have detrimental effects on the callus and shoot regeneration. There are many reports referring to the interactions between selective agents and subsequent regeneration ability (Schopke *et al.,* 1996). From the results it was concluded that 9 mg l^{-1} tet. + 6 mg l^{-1} gent. or 15 mg l^{-1} kan. + 6 mg l^{-1} tet. or 25 mg l^{-1} kan. + 6 mg l^{-1} gent. are effective for an irreversible suppression of growth for non-transformed cauliflower, these concentrations are, therefore appropriate for use in experiments involving selection of kan. or tet. or gent. resistant transformed cultures.

Effect of carbenicillin and cefotaxime on *Agrobacterium* elimination and shoot regeneration

For effective *Agrobacterium* mediated transformation, the antibiotic regime should control *Agrobacterium* overgrowth without inhibiting the regeneration capacity of the plant cells. For this purpose different concentrations of carbenicillin and cefotaxime were evaluated to study the effect on shoot regeneration and *Agrobacterium* elimination and to determine which antibiotic will be more effective and at what concentration.

The presence of carbenicillin and cefotaxime in the medium had significant effects on regeneration but the effect of cefotaxime was less compared to carbincillin. Increasing the concentration dramatically inhibited the shoot regeneration put at the same time led to elimination of the *Agrobacterium*. These results were also reported by Mathias and Mukasa (1987); Lv Lingling *et al.,* (2005). The results also demonstrate the different sensitivity of *Brassica oleracea* hypocotyl and cotyledon tissues to the two commonly used antibiotics for *Agrobacterium* elimination. The results suggested that cefotaxime at the concentration 250 mg l^{-1} was best concentration so as to be non toxic for cauliflower and to eliminate *Agrobacterium* after co-cultivation explants. These results confirmed Borrelli *et al.,* (1992) results since they found that cefotaxime strongly promoted plant regeneration in two durum wheat cultivars. Furthermore, Mihaljevic *et al.,* (2001) has compared the effect of carbenicillin and cefotaxime at 500 mg l^{-1} on the embryonic tissue growth and revealed that cefotaxime was less toxic the embryonic tissue growth and more suitable for *Agrobacterium* elimination. Chakrabarty *et al.,* (2002) indicated 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 investigations

prefer to use cefotaxime (Lv Lingling *et al.,* 2005; Eimert and Siegemundand 1992) others used carbincillin (Chakrabarty *et al.,* 2002; Prem and Nicole 1998) to eliminate *Agrobacterium*. These could be results from the interaction between the genotypes and antibiotics or the mechanisms of action of these antibiotics which is intimately connected to the physiological reactions of the cultured tissues (Escandon and Hane 1991). In our experiment 250 mg l^{-1} cefotaxime were added to cauliflower cultures to enhance the regeneration frequency and efficiency and at the same time suppress the *Agrobacterium* growth.

Factors affecting *Agrobacterium* mediated transformation of cauliflower

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

One of these parameters *is Agrobacterium* density which plays an important role in the transformation process. Our results showed that there were negative effects between *Agrobacterium* density and transformation rate. The reduction in transformation rate caused by inoculation with high

concentration of *Agrobacterium* (OD_{600} = 0.5) related to an apparent hypersensitivity response of explants to the *Agrobacterium*. The same affect of high bacterial concentration was also reported by Orlikowska *et al.,* 1995; Changhe *et al.,* (2002); and Ismail *et al.,* (2004). Diluted concentration (1:10 and 1:20 dilution) reduced necrosis to a great extent. The maximum transformation rate (10 %) was obtained with 1:10 dilution.

In general the results confirmed that 1:10 (OD_{600}=0.5) bacteria density helped to improve transformation rate. The reduction of transformation rate caused by inoculation with 1:20 dilution (OD_{600} 0.5) related to a hypersensitivity response of explants to the *Agrobacterium* or low *Agrobacterium* density, receptivity. These results agree with Srivestava *et al.* (1988); and Henzi *et al.,* (2000). Whereas Chakrabarty *et al.,* (2002) obtained that maximum transformation efficiency with 1:20 dilution.

In the present study explants from 8 days old seedlings were better for transformation than 4 and 12 day old seedlings as very few green shoots were recovered when older seedlings were used and these was poor survival of the explants during co-cultivation when 4 days old seedlings were used. In contrast Ponnti-Kaerlas *et al.,* (1990); Chakrabarty *et al.,* (2002); and Lv

Lingling *et al.,* (2005) produced 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 0, 2 and 4 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; Caradoza and Stewart 2003). This means that explants were hypersensitive to the *Agrobacterium* culture without any pre-culture. While pre-culturing treatment of the explants before co-cultivation supported explants to overcome the stress resulting from co-cultivation with *Agrobacterium* and subsequently improving gene transfer. These results were in agreement with Molinier *et al.,* (2002).

After pre-culture, the explants were transformed by co-cultivation with *Agrobacterium* for 1, 2, 3 or 4 days. The results showed 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 explanation 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 showed that 2 days co-cultivation period significantly increased cauliflower transformation (Chakrabarty *et al.,* 2002; Lv Lingling *et al.,* 2005). Also the results are consistent with those of Fillati *et al.,* (1987) where about 605 tomato cotyledons co-cultivated with *Agrobacterium* for 48 hrs produced Kanamycin resistant shoots. In most transformation protocols, 2 days of co-incubation is commonly used. For example, Horsch *et al.,* (1986) reported high frequencies of transformation in tomato and tobacco when they used 2 days of co-incubation.

Two possible explanations could be used to explain the reduction in transformation after 4 days of co-incubation. First, the introduction the of too many bacteria may prevent tumor formation perhaps by an excessive synthesis of T-DNA gene product, resulting in a hypersensitive response. Data in support of this possibility have been obtained 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 4 days 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 findings of Matthysse *et al.*, (1978). In their experiment, dead *Agrobacterium tumefaciens* blocked tumorogenesis when co-incubated with live *Agrobacterium tumefaciens* and tobacco tissue culture. The phenolic compound acetosyringone has been known to induce the vir gene (Shimada *et al.*, 1990) and the highest transformation rate (13%) was recorded when 70 μM acetosyringone was added to culture medium. Similar results were obtained by Sheikaoleslam *et al.*, (1987); Boloton *et al.*, (1986); Ismail *et al.*, 2004 and Heniz *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 vir gene is mediated by acetosyringone which is normally released by the wounded plant cell.

Confirmation methods of interrogation APX and SOD genes into cauliflower plants

In this study, the most reliable results were obtained if small quantities of cells and fresh bacterial colonies (one to two weeks after streaking) were analyzed using PCR method. 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. SOD and

APX genes were detected after PCR amplification from appropriate colonies at 478 bp. This method can be used routinely to evaluate insert sequence constructed into vectors harboured in individual *Agrobacterium* colonies, substantially reduces time and effort required to evaluate the authenticity of inserts in *Agrobcaterium* binary vectors (Ping *et al.,* 1997; Jones *et al.,* 1987).

Although Perm and Nicole (1998) indicated that PCR is a fast and sensitive method, it is expensive and susceptible to cross-contamination. On the other hand, the leaf disc assay 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 necessary 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 β glucuronidase. In this study, *Agrobacterium* containing some of the GUS plasmids showed significant GUS activity. The substrate used in this study, X-Gluc, works very well but the quality of the histochemical localization are effect by numerous variables such as tissue preparation and fixation. The results of transformation with Bt-

gene construct indicated that the transient GUS assay approach is an easy reliable method of establishing optimal conditions of transformation, (Chakrabarty *et al.,* 2002). All the positive plants in histochemical assay showed bands in PCR analysis.

Effect of salinity treatment on transgenic cauliflower plants

Generally, Data for shoot fresh weight, proline content and Na, Ca, Mg and K content of non-transgenic and transgenic plants showed that shoot fresh weight, K, Mg and Ca under 300mM NaCl was lower compared to the control treatment while the proline content and sodium concentration was higher. The reduction in shoot fresh weight was very low in the non-transgenic plants than transgenic plants under salinity treatments. Proline content and sodium concentration in transgenic plants under salinity treatment recorded highest value (28.4 and 12.66 mg/g dry weight), respectively.

From these results it is clear that transgenic plant (carrying APX and SOD genes) have a high level of superoxidase dismutase and ascorbate peroxidase isozymes which are essential effectively to maintain the antioxidant system that protect plants from oxidative damage due to abiotic stresses. This explanation agrees with that of Shigeru *et al.,* (2002).

Also Hernandez *et al.,* (2000) reported NaCl induced activity of SOD and APX enzymes in tolerant pea cv. Granada while in salinity sensitive cv.Chillis no significant changes in activity of the above enzymes were observed. This confirmed that APX and SOD successfully integrated into the plant and it is expressed under salinity treatment as superoxidase dismutase and ascorbate peroxidase isozymes and transgenic plant become more tolerant to salinity treatment than non-transgenic plants.

Chapter 4

General Discussion

4 General Discussion

4.1 Using mutations and DNA transfer to improve abiotic stress in cauliflower

There is an extremely large body of literature concerning plant abiotic stress resistance and many ways to improve resistance have been put forward but to date only a limited number of techniques are showing success. Traditional plant breeding approaches are very limited (Richards 1996), mutagenic approaches have had some success in cereals and other crop species (Gengenbach, 1984; Gottschalk 1981; Maliga, 1984) and there are only a limited number of successes via genetic modification (Cardoz and Stewart 2003; Cao and Earle 2003 and Cheng *et al.,* 2003). Furthermore there are only a limited number of researchers working in this field working in crop species, most work is being carried out in the model species *Arabidopsis* (XW *et al.,* 1991; Maria *et al.,* 2003).

The demonstration of the success of mutagenesis in the first phase of this project confirmed that this is one method of producing lines with improved stress resistance. The disadvantage of this technique is that it can be considered as a "blind" technique where specific genes are not targeted.

The second phase of the project investigated an alternative approach, that of targeted gene supplementation (*Agrobacterium* mediated transformation). This technique succeeded to achieve the same goal to produce lines of GM cauliflower tolerant to abiotic stress.

The mutation approach has an advantage compared with the *Agrobacterium* mediated transformation as it is rapid, simple, requires minimal use of chemicals and plant tissues, low cost, retains abiotic stress resistance over 2-3 year periods and caused no contamination or damage to the explant.

In this investigation tissue culture techniques were used for screening among the mutant lines and also employed for the transfer of APX and SOD genes from the binary vectors to the plant. The use of tissue culture techniques can overcome some of the limitations in the application of mutation and DNA transfer techniques. *In-vitro* culture in combination with induced mutation can speed up breeding programmes from the generation of variability, through selection, to the multiplication of the desired genotypes. The work presented here clearly demonstrated that a short selection process with relatively high selection pressure can produce useful stable mutants very quickly. This confirms that this kind of selection is useful in finding genotypes resistant to abiotic stress (Ashraf and Harris 2004).

4.2 Screening of mutant lines under abiotic stress

The results of screening cauliflower lines under elevated NaCl and hydroxyproline concentrations using leaf strip and leaf disc resistance assays under *in-vitro* conditions provided several advantages over the study of intact plants. The reaction took place over a short time, under well-controlled experimental conditions and provided excellent repeatability. Baraka *et al.*, (1997) and Vijayana *et al.*, (2003) reported similar techniques as powerful tools for studying plant responses to different abiotic and biotic stress.

Mutant lines always contained more proline than controls possibly resulting from increasing activity of the enzymes involved in the synthesis, or inhibition of enzymes involved in the degradation, of proline, an explanation supported by Aspinall and Paleg (1981). The results presented in this thesis support the hypothesis that proline acts as a protective compound during NaCl and hydroxyproline treatment. Similar observations in other species led to the suggestion that proline can be used as a metabolic marker for more specific screening or selection *in-vitro* (Bhaskaran *et al.*, 1985; Martinez *et al.*, 1996). Many studies have shown that free proline accumulating cell lines, resistant to proline, show increased resistance to stress such as salt and freezing (Reccardi *et al.*, 1983; Ashraf and Harris 2004; Juan *et al.*, 2005).

The evidence from this investigation suggests that whilst mutant lines of cauliflower can be created which demonstrate correlated resistance to more than one abiotic stress, there are equally lines which are clearly only resistant to a single stress. This together with the proline data suggests that many of the mutations that have been induced are likely to be in "downstream" operational genes rather than transcription factors, since some mutant lines were resistant to salt and/or frost but had low proline. Zhu (2000) indicated that many genes that are important for salt tolerance may not actually be induced by salt stress. The current findings also provide physiological evidence to support the molecular evidence that many genes are involved in the up-regulation of resistance to abiotic stress (Zhu *et al.*, 1997; Pearce, 1999; Guy 2003; Vinocur and Altmann 2005). These gene products are involved not only in the protection of cells against stress, but also in the regulation of gene expression and signal transduction pathway in abiotic stress response. These gene products are classified into either functional or regulatory groups.

The metabolism of stress resistance in plants and the exact role of increased compatible solutes remains a major gap in our knowledge (Vinocur and Altmann 2005) and research efforts are being coordinated to investigate this in model species. The results of such efforts will then need to be corroborated in crop species and the existence of the range of mutants described in this

research work will greatly assist future corroborative studies. In the meantime, agronomic evaluation of resistant mutants described here needs to be carried out to determine the stability of the mutant traits in the field.

Although, nearly all of the mutant lines which demonstrated good resistance to stress had high proline contents there were exceptions – lines S42, S65 and S81 were all resistant to salt and/or frost but had relatively low proline. What is also clear from the results it that there is no absolute correlation between proline level and stress resistance. The range of mutants however provides abundant material to explore this question more thoroughly in the future. Many previous studies have shown that free proline accumulating cell lines show increased resistance to abiotic stress such as salt and freezing (Riccardi *et al.* 1983; Ashraf *et al.,* 1986a; Ashraf and McNeilly 2004; Kueh & Bright 1981; Dix *et al.,* 1984; Tantau & Dorffling 1991; Dorffling *et al.,* 1993). Bhaskaran *et al.* (1985) and Martinez *et al.,* (1996) indicated that proline can be used as a metabolic marker for more specific screening or selection *in-vitro* and the data presented here do not contradict these results but illustrate that resistance level is not necessarily proportional to the free proline content. It is concluded that elevated proline is not essential for improved resistance to abiotic stress in cauliflower, but where it does occur it does improve resistance

4.3 Transformation protocol for cauliflower

Another way of improving the plants ability to tolerate abiotic stress was by using *Agrobacterium* mediated transformation. This method was employed as a second stage of this research and as another method to improve abiotic stress resistance in cauliflower by inserting APX and SOD genes into the plant. Development of an efficient gene transfer system largely depends on a rapid and reliable *in-vitro* regeneration system for the desired plant species. Even though in our lab a simple methodology allowing the production of ten of thousands of shoots, from the curd meristem has been developed which would allow the necessary level of replication, this technique was rejected for transformation because of bacterial overgrowth. All the meristem cultures were contaminated and no meristem was able to survive the infection with *Agrobacterium*. As a result of this and because of the success of many previously published reports using hypocotyls and cotyledons (Chakrabarty *et al.*, 2002; prem and Nicole 1998), these were used in this investigation. The transformation of meristem could also lead to the production of chimeric plants which would be undesirable.

Adaxially oriented cotyledons (cotyledon explants placed upright with the distal cut-end placed onto the media) responded better than explants in the abaxial orientation for plant regeneration. In peanut (Ozias-Akins, 1989), chickpea (Shi and Davis, 1992) and cauliflower (Prem and Nicole, 1998) the

same observation has been shown. The highest shoot regeneration rates were obtained from explants placed with the distal end down and this could be related to the fact that there is no direct contact between the medium and the regenerative proximal end of the explants thus allowing gradients of hormones to build up. But it may also be because of limited movement from the medium to the regenerative tissue which could be partly responsible for setting up local hormone concentration gradients that might stimulate the high frequency of shoot regeneration.

Many factors limit plant morphogenesis however the presence of plant regulators in the medium was the main factor to optimise shoot regeneration from hypocotyls and cotyledons explants in cauliflower. A common feature of the protocols used to achieve adventitious shoot regeneration in many plant species has been the use of high cytokinin and low auxin concentration in the media. Plant tissue culture has generally shown that 2,4-D was the best hormone to use for callus initiation and maintenance. Franco *et al.,* (1990) suggested that 2,4-D was required for inducing cell division and formation *in-vitro* and has been commonly used for many species (Murata and Orton 1987; Fuller and Fuller 1995). Murata and Orton (1987) and McCoy (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 subsequent regeneration. The regeneration of

shoots from callus tissues has been improved by the addition of $AgNO_3$ to the medium in order to inhibit ethylene action and these results were confirmed by Purnhauser *et al.,* (1987); Chi *et al.,* (1990); and Roustan *et al.,* (1990). It has been suggested that the effects of silver nitrate are mediated by an inhibition of ethylene action; accumulation of this gas in tissues is correlated with a reduced frequency of adventitious shoot regeneration from explants (Jackson *et al.,* 1987).

Several factors are critical for successful production of transgenic cauliflower. One of these factors is the control of *Agrobacterium* overgrowth or *Agrobacterium* elimination by using carbenicillin or cefotaxime. Both carbenicillin and cefotaxime prevented the *Agrobacterium* overgrowth but cefotaxime had less effect on plant regeneration compared to Carbenicillin. The suggested explanation for the different effects on plant regeneration of the two antibiotics rests in their modes of action. Cefotaxime binds with the penicillin binding proteins of the inner membrane of the bacterial envelope that are essential for cell division (Mathis and Boyed 1986). Cefotaxime however is metabolised to an unknown compound with growth regulator activity (Borreli *et al.,* 1992). While one possible reason for the carbenicillin results is that the breakdown products of the antibiotic may act as growth regulators, thus modifying defined tissue culture conditions (Lin *et al.,* 1995), another possibility is that antibiotics cause DNA hypermethylation. This

process is known as an epigenetic process that acts as an alternative to mutation to distrupt tumor-suppressor gene function and can predispose to genetic alternations through inactivating DNA repair genes that could act by slowing down the cell cycle to prolong the S phase. This would provide a wider window for the DNA methyltransferase to act on the DNA, therefore affects gene expression and plant development (Schimth *et al.,* 1997).

Identification and optimization of the parameters affecting T-DNA delivery in a recalcitrant crop such as cauliflower were required in this research to establish an efficient *Agrobacterium* mediated transformation protocol. A reduction in transformation rate was caused by inoculation with a high concentration of *Agrobacterium* OD_{600} = 0.5 related to a hypersensitivity response of the explants. This is in contrast to many other transformation protocols. Reduced bacterial concentrations (1:10 and 1:20 dilution) reduced explant necrosis by a great extent. Explant age was also found to be very important with an explant age of 8 days recommended for transformation experiments. Younger (4 days) or older explants (12 days) were much less receptive as very few green shoots were recovered with older seedlings and with the younger seedlings the necrotic reaction was so high that none could survive the treatment. A short pre-incubation period (2 days) was required to recover transgenic plants from hypocotyl and cotyledon explants in preference to a long pre-incubation period (4 days). A short pre-incubation

period increased the transformation rate because pre-culturing of the explant before co-cultivation supported explants to overcome the stress resulting from the co-cultivation with *Agrobacterium* and subsequently improved gene transfer. Lengthening the *Agrobacterium* co-incubation time of explants to 2 days was very effective in cauliflower transformation whilst with 4 days co-cultivation transformation rate was reduced. There are two possible reasons to explain the reduction in transformation after 4 days of co-incubation. First, the introduction of too many bacteria may prevent tumor formation perhaps by an excessive synthesis of T-DNA gene product, resulting in the hypersensitive response. Data in support of this possibility have been obtained by studying the crown gall-grape system (Yanofsky *et al.*, 1985). 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 8 days of co- incubation, dead bacteria would accumulate. Lysis of bacterial cell walls would result in the release of the lipopolysaccharide components of the cell wall that block tumorogenesis (Whatley *et al.*, 1976). These results are consistent with the findings of Matthysse *et al.*, (1978), in their experiment, dead *Agrobacterium tumefaciens* blocked tumorogenesis when co-incubated with live *Agrobacterium tumefaciens* and tobacco tissue culture. One of the important processes in gene transfer is the induction of bacterial virulence mediated by the virulence (*vir*) gene coded by the Ti plasmid and organized in six operons (Hooykass

and Beijersbergen 1994). 70 μM acetosyringone was most efficient to improve transformation frequency in this research and it has been found that the *vir* genes are inducible by acetosyringone which act synergistically with other synthesised phenolics and lead to improved transformation frequency (Shimoda *et al.,* 1990).

Histochemical, fluorometric and leaf disc assay as well as molecular analysis confirmed that the DNA was transferred and integrated into the cauliflower genome. Enzymatic assays are however time-consuming, laborious and expensive procedures. Although PCR is a fast and sensitive method, it is also expensive and susceptible to cross-contamination 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 inserts in *Agrobacterium* binary vectors. It may possible to improve the sensitivity of the PCR by increasing the annealing temperature or by reducing PCR cycles if non-specific amplification is a problem. On the other hand, both of leaf disc and GUS assay used in this study have advantages as they are rapid, simple, require minimal use of chemicals and plant tissues, and do not cause permanent damage to the plant.

The present study provides a reliable transformation system for integration of some genes into the cauliflower genome. This provides an opportunity to

introduce genes of agronomic interest such as abiotic resistance (salt, drought and frost). In this investigation, putative transgenic cauliflower plants and non-transformed plants were tested under NaCl stress. Measurement of shoot fresh weight, proline content and Na, K, Mg and Ca indicated that transformed plants were more tolerant to salt stress compared with non-transformed plants under salt stress treatment. The results indicated strongly that the stress resistance in transgenic plants was caused by SOD and APX gene expression. Elevated level of SOD and APX enzymes have been correlated with increased levels of oxidative stress resistance in several cases (Jansen *et al.*, 1989; Jahnke *et al.*, 1991). Over expression of antioxidant genes in plants has been previously shown to provide enhanced resistance to oxidative stress in several crop species (Allen 1997; Gueta-Dahan *et al.*, 1997; Payton *et al.*, 2001; Roxas *et al.*, 2000). The results support previous reports and demonstrate that ectopic expression of the APX and SOD gene in cauliflower confers increased resistance to oxidative damage caused by exposure to salt. The data, and the cited literature, also suggest, however, that antioxidant enzymes, such as APX and SOD may serve as good physiological and molecular markers in marker assisted breeding programmes aimed at increasing resistance to environmental stress.

Finally, most breeding programmes looking for improvied abiotic stress resistance are using genetic engineering as a rapid method to create plants

tolerant to abiotic stress, nevertheless this study confirmed that the mutation approach is more efficient, gene stable, low cost, gives no contamination with no damage to explants and a low risk compared to gene transfer. This result suggests that a mutation approach should be recommended to improve abiotic stress in cauliflower.

4.4 Conclusion

Mutagenesis and direct gene transfer are both promising approaches to improve abiotic stress resistance in cauliflower compared with traditional plant breeding approaches. In this study both mutagenic and gene transfer approaches were successful of producing plants with improved stress resistance.

The mutation approach has an advantage compared with the *Agrobacterium* mediated transformation as it is rapid, simple, requires minimal use of chemicals and plant tissues, low cost, retained abiotic stress resistance over 2-3 years periods and caused no contamination or damage to the explants.

Experimental work in the first phase of research showed no significant differences between the performances of selected lines from mutagenesis under *in-vitro* and *in-vivo* conditions. This means that *in-vitro* screening of cauliflower, using leaf strips and leaf discs, is an easy and efficient method to identify salt and hydroxyproline adapted genotypes within a limited space and time period.

The level of resistance however was not necessarily correlated with the level of proline and some lines showed resistance without elevated proline. It is concluded that elevated proline is not essential for improved resistance to

abiotic stress in cauliflower, but where it does occur it does improve resistance.

Mutant lines also demonstrated improved frost stress under non-acclimated conditions and this difference increased when plants were acclimated. Some of the mutant lines proved to be resistant to salt, hydroxyproline and frost and this cross-resistance suggests a common resistance mechanism, which requires further investigation. Other mutant lines proved to be double resistance and the rest single resistance.

In the present study it was shown that the resistance to salt and hydroxyproline is stable in mutant lines during many *in-vitro* subcultures. Furthermore, parallel work has demonstrated that these differences are heritable and expressed in the F1 population of a mass pollinated cross of selected lines (Efekodo, 2004).

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

Leaf disc assay as a method to distinguish between transgenic and non-transgenic plants in this study showed the advantage as being rapid, simple and requiring minimal use of chemicals and plant tissues that causes no permanent damage to the plant. The results of transformation with APX and SOD gene constructs indicated that the transient GUS assay approach is easy reliable method of establishing optimal conditions of transformation.

The main question posed was: is it possible to transform hypocotyls, cotyledons and curd explant via *Agrobcterium tumefaciens* ? It is shown here that it is possible to transform hypocotyls and cotyledons of cauliflower while it is not possible to transform curd explants with *Agrobacteriumm tumefaciens* strains harbouring the co-integrated Ti-based vectors $P^{CGN1578}$ and $P^{BIN+ARS}$

Optimal conditions for transformation of hypocotyls and cotyledons explants were: density of bacteria culture 1:10 dilution (OD600 = 0.5); seedling age (8 days); 2 days of pre-culture; 2 days co-cultivation; 70 μM acetosyringone. The frequency of transformation was still low (10 to 13 %) but the protocol was successful to mobilize APX and SOD stress gene into cauliflower.

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

It is speculated that the transgenic plants (carrying APX and SOD) had a high level of superoxidase dismutase and ascorbate peroxidase isozymes although due to logistical restraints these were not measured. It is hypothesised that the increased activity of the antioxidants system helped protect the plants from oxidative damage due to abiotic stress. It is recommended that these plants are assessed by measurement ascorbate peroxidise (APX) and Superoxidae dismutase (SOD) activity using spectrophotometry method (Nakano and Asda, 1981) or determine activity by an in gel assay method according to Chen and pan (1996) .

4.5 Overview of the research

For a 3 year project the outputs of the research project were successful in confirming both mutagenesis and transformation as technologies for improving stress resistance in cauliflower. It did not aim to assess the relative merits of different mutagenesis or other transformation construct and so the techniques used cannot be the most efficient or effective. It could be that other mutagens or other constructs prove to be more effective and/or reliable in

future studies. This thesis illustrates more that theses technique can be effective. Thus the thesis does not claim to be exhaustive study but in more a proof of concept.

4.6 Future work

The mechanism 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.

This study was carried out on a number of mutant selected plants and more genetic and molecular genetic investigations are needed to determine the aspecific gene(s) responsible for the resistance and the inheritance of the stress resistance genes in cauliflower.

The further Development of transformation protocols for hypocotyls and cotyledons are required in the future to increase the transformation percentage. Also more investigations are necessary to optimize transformation protocols for curd explant.

It would be important to Transform the high performing lines from mutagenesis and assess their performance and determine whether there are any effective "gene stacking" effects.

Due to the limitation of time, funds and facility (GM greenhouse) the transgenic plants were not cultured under *in-vivo* conditions, it is necessary

transfer the transgenic plants to *in-vivo* conditions to evaluate their agronomic characters under abiotic stress, assess their gene stability and to ensure no gene escape possibilities.

Appendices

Appendix 1

1a- NaOH 1 M:
4 g NaOH + 100 ml water

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

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

Appendix 2

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
Ammonium Nitrate	1650
Boric Acid	6.2
Calcium Chloride Anhydrous	332.2
Cobalt Chloride Hexahydrate	0.025
Cupric Sulphate Pentahydrate	0.025
Disodium EDTA Dihydrate	37.26
Ferrous Sulphate Heptahydrate	27.8
Glycine (Free Base)	2.0
Magnesium Sulphate Heptahydrate	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 Heptahydrate	8.6

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

Appendix 3

S23 medium (Callus induction medium)

Content	g/l
M&S	4.4
Adenine	0.080
Thiamine	0.0004
Sodium Phosphate	0.170
Sucrose	30
Agar	7
2,4-D (2,4-Dichlorophenoxyacetic acid)	0.002

Appendix 4
Growth regulator stock Solution

4a- 2,4 D (2.4-Dichlorophenoxyacetic acid) 2 mg/l
10 mg/l 2, 4 D was dissolved in NaOH (1M)then volume was made up to 5 ml by adding distilled water. 1ml was taken from the stock solution and added to 1L medium.

4b- NAA (Naphthalene acetic acid)
10 mg/l NAA was dissolved in NaOH(1M) then volume was made up to 50ml by adding distilled water.
To get 0.2 mg/l NAA: 1ml was taken from stock solution and added to 1 L of medium.
To get 0.1 mg/l NAA: 0.5 ml was taken from stock solution and added to 1L medium.
To get 0.05 mg/l NAA: 0.25 ml was taken from stock solution and added to 1 L medium.

4c- Kinetin or BAP (6-benzylamino purine)
150 mg/l K or BAP was dissolved in HCL (1M) then volume was made up to 50 ml by adding distilled water.
To get 1 mg/l K or BAP: 0.33 ml was taken from stock solution and added to 1 L of medium.
To get 3 mg/l K or BAP: 1 ml was taken from stock solution and added to 1 L of medium.

Appendix 5

S23a medium (Shoot regeneration medium)

Content	g/l
M&S	4.4
Adenine	0.080
Thiamine	0.0004
Sodium Phosphate	0.170
Sucrose	30
Agar	7
NAA (Naphthalene acetic acid)	0.00005 or 0.0001 or 0.0002
Kinetin or BAP (6-benzyl aminopurine)	0.001 or 0.003

Appendix 6

S23b medium (Shoot regeneration medium)

Content	g/l
M&S	4.4
Adenine	0.080
Thiamine	0.0004
Sodium Phosphate	0.170
Sucrose	30
Agar	7
$AgNO_3$	0.0005 or 0.035
GA_3 (Gibberellic acid)	0.00001
NAA(Naphthalene acetic acid)	0.0002
BAP (6-Benzyl aminopurine)	0.003

Appendix 7

Luria-Broth medium (LB)

Bacto-tryptone   10 g/l
Bacto-yeast extract   5   g/l
NaCl   10 g/l
PH   7.2 adjust with 1 m NaOH or HCl
Agar (Solid only)   15 g/l
Complete volume to 1000 ml (distilled water)

YE Broth medium (YEB)

Beef Extract   5 g/l
Yeast Extract   1 g/l
Peptone   5 g/l
Sucrose   1 g/l
$MgSO_4$ 2 m/l of a 1 M solution
PH   7.2 adjust with 1 m NaOH or HCl
Agar (Solid only)   15 g/l
Complete volume to 1000 ml (distilled water)

Appendix 8

S23d medium (Shoot regeneration medium)

Content	g/l
M&S	4.4
Adenine	0.080
Thiamine	0.0004
Sodium Phosphate	0.170
Sucrose	30
Agar	7
$AgNO_3$	0.035
GA_3 (Gibberellic acid)	0.0001
NAA(Naphthalene acetic acid)	0.0002
BAP (6-Benzyl aminopurine)	0.003

Appendix 9

Antibiotic stock solution:

Kanamycin: 50 mg/l
0.5 g Kanamycin was dissolved in 10 ml distilled water.

Gentamycin: 10 mg/l
0.1 g was dissolved in 10 ml distilled water

Tetracyclin: 12 g/l
0.120 g tetracyclin was dissolved by adding some drops from 50 % ethanol and the volume was made up to 10 by distilled water.

Carbenicillin or Cefotaxime 500 mg/l
5 g carbenicillin or cefotaxime was dissolved in 10 ml distilled water

Appendix 10

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 mM 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
1mM EDTA
This solution is 50 x TAE.

For Agarose gel 1 x TAE is needed.

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