GENETIC TRANSFORMATION OF *Cicer arietinum*, L FOR INSECT RESISTANCE

Thesis submitted to
SRI VENKATESWARA UNIVERSITY
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IN
BOTANY

By

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JANUARY, 2003

Dedicated to my
Beloved Father

CERTIFICATE

Certified that the entire work embodied in this thesis entitled **"Genetic Transformation of *Cicer arietinum* L. for Insect Resistance"** has been carried out by **B. Jayanand** under my guidance in the Genetic Transformation Laboratory, International Crops Research Institute for the Semi Arid Tropics (ICRISAT), Patancheru, Hyderabad, India and that no part of it has been submitted elsewhere for any degree or diploma.

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Certified that the entire work embodied in this thesis entitled **"Genetic Transformation of *Cicer arietinum*, L. for Insect Resistance"** *has been carried out by* **B. Jayanand** *under my guidance in the Department of Botany, Sri Venkateswara University, Tirupati, Andhra Pradesh, India and that no part of it has been submitted elsewhere for any degree or diploma.*

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DECLARATION

I hereby declare that the disseration entitled "GENETIC TRANSFORMATION OF *Cicer arietinum*, L. FOR INSECT RESISTANCE" is an original and independent record of research work undertaken by me during the period of my study at Sri Venkateswara University, Tirupati, under the supervision of Dr. G. SUDARSANAM, Department of Botany, S.B.E.S., S.V. University, Tirupati, and that it has not previously been submitted for the award of any other degree or diploma of any University.

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"I'm convinced biotechnology is going to help us. There's fear, but biotechnology has been going on since the beginning of time. Mother Nature was crossing plant genes long before scientific man and agricultural man began doing it. If you like to eat spaghetti, you are eating a GMO that Mother Nature made."

Norman Borlaug

Father of Green Revolution

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ABBREVIATIONS

2-iP 2-isopentenyl adenine
2,4-D – 2,4-Dichloroacetic acid
2,4,5-T – 2,4,5-Trichloroacetic acid
AB *Ascochyta* Blight
ABA – Abscissic acid
BAP – Benzyl Amino Purine
BGM – *Botrytis* Grey Mold
Bt – *Bacillus thuringiensis*
Bti – *Bacillus thuringiensis-israeliensis*
CaMV 35S – Califlower Mosaic Virus 35S promoter
CAT – Chloramphinical Acetyl Transferase
DNA – Deoxyribonucleic acid
FAO – Food and Agriculture Organization
GA – Gibberellic acid
IAA – Indole-3-acetic acid
IBA – Indole-3-butyric acid
ICRISAT – International Crop Research Institute for Semi-Arid Tropics
ICARDA – International Center for Agricultural Research in the Dry Areas
IPM – Integrated Pest Management
min – minutes
MS medium – Murashige and Skoog medium
NAA – Naphthalene Acetic Acid
*npt*II – Neomycin Phospho Transferase II
PCR – Polymerase Chain Reaction
PIs – Protease Inhibitors
QTL – Quantitative Trait Loci
RNA – Ribonucleic acid
SBTI – Soybean Trypsin Inhibitor
SCRI – Scottish Crop Research Institute
sec – seconds
T-DNA – Transfer DNA
TDZ – Thidiazuron
Ti plasmid – Tumor inducing plasmid
*uid*A - β-Glucuronidase

SUMMARY

Agriculture contributes major part of world's food especially in the developing world. Over 200 plant species are cultivable out of which rice, wheat and maize make 70% of the total output. Legumes have very important attributes such as protein and lipid rich seeds and symbiotic nitrogen fixation, which qualifies them as best alternative food crops. Legumes are broadly divided into food and forage legumes out of which forage legumes form major part. Chickpea is one of the most important leguminous, cool season, alternative food crop cultivated prevalently in Asia Pacific region. Even though it has convincing nutritional importance, its area of cultivation has been low and improvement virtually stagnant. Conventional breeding has not been an effective crop improvement strategy for chickpea and recent advances in biotechnology such as plant tissue culture and genetic transformation, paved the way for alternative crop improvement methodologies. This work was carried out with principal objectives of optimization of tissue culture variables and transformation of selected explants by using genes from *Bacillus thuringiensis BtCry1Ab* and soybean trypsin inhibitor (*SBTI*) genes to confer resistance to *Helicoverpa armigera* or the legume pod borer. The work can be broadly divided into three parts, 1. Somatic embryogenesis, 2. Organogenesis and 3. Genetic transformation.

Somatic embryogenesis: Different explants like mature embryo axis, parts of mature embryo axis, leaflets, stem segments and root segments were derived from in vitro grown seedlings of different ages. Somatic embryos were induced by using 2,4,5-T and 2,4-D as principal growth regulators in combination with hormones like kinetin, zeatin, TDZ and BAP. Efficient induction of somatic embryos was observed and best

frequency was observed with 2,4,5-T in combination with kinetin. However, efforts for maturation and regeneration of embryos induced were unsuccessful though good number of media combinations involving ABA, zeatin, BAP and TDZ were tested. Hence further work on the somatic embryogenic pathway of regeneration was not carried forward.

Organogenesis: Various explants such as mature embryo axis, shoot tip, leaflets, leaf base, stem segments, hypocotyl, epicotyl, root segments, root tip, axillary bud, cotyledonary node and axillary meristem explants (AM1, AM2, AM3 and AM4) were prepared from in vitro grown seedlings and their multiple shoot regenerating efficiency was tested via direct and indirect organogenic pathways. Multiple shoot induction frequencies were tested with shoot induction medium (SIM) that consisted of 4 µM TDZ, 10 µM 2-iP and 2 µM kinetin. Explants that do not contain any traces of pre-existing meristems, such as hypocotyl, epicotyl, leaflets were also used so as to achieve regeneration via callus phase. It was observed that indirect organogenesis via callus phase cannot be achieved from the above-mentioned explants with any of the tested media combinations. It was also observed that asynchronous multiple shoot regeneration could be achieved from pre-existing meristems of explants like shoot tip, axillary bud. Very low frequency of shoot induction was observed with hypocotyl and epicotyl and it was found that few shoots that originated were from traces of meristems associated with some of the explants. Based on these results it was apparent that adventitious regeneration is very difficult with most of the explants and modification of the tested explants. Four axillary meristem explants namely AM1, AM2, AM3 and AM4 were prepared so as to achieve adventitious regeneration and three of them AM1, AM2 and AM4 were found to be better candidates. Among these explants AM4 were more responsive and provided

adventitious shoot buds. These seemed more applicable to genetic transformation experiments where negation of the apical dominance of axillary bud and shoot tip was the salient feature of this explant. Removal of axillary bud followed by regeneration of multiple shoot buds gave better adventitious regeneration and multiple wounding sites. Multiple shoot induction was found to be better on medium containing TDZ, when compared to the medium containing BAP. However, it was observed that prolonged culture of the explants on TDZ-containing medium negatively interfered with further elongation. Hence, the inclusion of TDZ was restricted to germination and first phase of induction. Several other factors played an important role in shoot bud induction. For example, acidic pH showed efficient induction of multiple shoots. Age of the seedling was optimized for all axillary meristem explants where AM4 preparation required 5 to 6 days for the removal of axillary bud and another week for removal of multiple shoot buds. Inclusion of cotyledon with AM4 explant was found to enhance multiple shoot regeneration frequency. Two step elongation using shoot elongation medium (SEM1; MS with 5 μM 2-iP and 2 μM kinetin) in the first step followed by 2 to 3 passages of sub-cultures on SEM2 (MS with 2 μM GA_3) resulted a better elongation frequency. A novel rooting system was developed by employing filter paper bridge technique and the liquid root induction medium (RIM) consisted of MS with 5 μM IBA. This method resulted in high rooting as well as transplantation frequencies. An efficient hardening and transplantation method was standardized by optimizing variables such as potting medium, temperature, humidity, irrigation and photoperiod. Conviron™ growth chamber was found to be better facility for hardening and initial phase of transplantation while pod maturation and harvesting was done in specially designed P2 facility. Best hardening of

in vitro grown plants was achieved with static hydroponics system containing Arnon's nutrient solution.

✓*Genetic transformation:* Genetic transformation of chickpea was achieved by using AM4 explants as the starting plant material and both biolistic and *Agrobacterium*-mediated methods were employed. A vector, pRT99:GUS-Int maintained in *E. coli* was used in biolistic process. This vector had *npt*II gene as the selectable marker and *uid*A gene as the reporter. Preparation of the explants, microcarrier preparation and bombardment of the explants was carried out by standard procedures. Large size of the AM4 explant was a limitation in this process as only few explants could be grouped at the center of the petri plate. Transformation by this method did not result in creation of any transgenics as some of the selected shoots died at the stringent selection step.

✓*Agrobacterium*-mediated transformation resulted in relatively high frequency of transgenics as far as the T_0 results were considered. Two binary vectors namely pHS 723:Bt and pHS 737:SBTI based in strain C 58, were used for co-cultivations. The former has *BtCryIAb* as agronomically important gene, *npt*II as the selection marker and *uid*A gene as reporter where as the later binary vector had soybeen trypsin inhibitor (*SBTI*) as the gene of agronomic interest, *npt*II as the selectable marker and *uid*A gene as reporter. In both the vectors *npt*II and *uid*A genes were fused into a single unit though their products exhibit independent activities. AM4 explants were co-cultivated with *Agrobacterium* strain of interest following the standard procedures. Cefotaxime was used to terminate the growth of *Agrobacterium* and kanamycin was used for selection of the putative transformants. Standardized protocol of regeneration was used for regeneration and recovery of the transgenic plants. Hardened and transplanted transgenics were

initially maintained in Conviron™ growth chamber and later in the P2 facility especially designed for growing transgenics. A total of 11 plants with *BtCrylAb* and 9 plants with *SBTI* genes were obtained. Molecular analysis of these putative transformants was done initially by GUS histochemical assay followed by confirmation by PCR and Southern blot analysis. Southern blotting was done by non-radioactive method by using the commercially available non-radioactive AlkPhos direct labeling kit from Amersham (USA). PCR amplification of *npt*II, *uid*A, *BtCrylAb* and *SBTI* genes was carried out for preliminary screening of the putative transformants. About 60% of the putative transformants showed positive reaction for PCR for *nptII*, 70% for *uid*A genes, 30% for *BtCrylAb* and 10% for *SBTI* gene. Factors affecting restriction of genomic DNA such as enzyme concentration, water and BSA were optimized. Southern blot analysis of *BtCrylAb* plants showed 70% of plants with *npt*II and *BtCrylAb* gene integrations.

In conclusion the regeneration protocol developed during the course of this study, was found to be very efficient since culture conditions for all the stages of regeneration and recovery of in vitro grown plants that included seed germination, multiple shoot induction, elongation, rooting hardening and transplantation were optimized. This protocol was effectively used for successful genetic transformation of chickpea with insecticidal genes like *BtCrylAb* and *SBTI* genes. Following this protocol, 40% of the selected putative transgenic plants can be obtained in period of 90 to 100 days. In the present study, over 30transgenic plants carrying *BtCrylAb* were obtained. Eight of these plants are being maintained for experimental work on insect bioassays and field trials.

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Lane 1: Unrestricted genomic DNA (5 µg. was added to the well)

Lane 2: 1 µg. genomic DNA digested with 10 units of enzyme

Lane 3: 5 µg. genomic DNA digested with 10 units of enzyme

Lane 4: 5 µg. genomic DNA digested with 15 units of enzyme and double the quantity of water

Lane 5: 5 µg. genomic DNA digested with 20 units of enzyme with double the quantity of water

Lane 6: 5 µg. genomic DNA digested with 10 units of enzyme and 0.1% BSA

Lane 7: 5 µg. genomic DNA digested with 10 units of enzyme, 0.1% of BSA and double the quantity of water

Lane 8: 5 µg. genomic DNA digested with 10 units of enzyme, 0.3% of BSA

Lane 9: 5 µg. genomic DNA digested with 10 units of enzyme, 0.3% of BSA and double the quantity of water

Lane 10: 5 µg. genomic DNA digested with 10 units of enzyme, 0.5% of BSA

Lane 11: 5 µg. genomic DNA digested with 10 units of enzyme, 0.5% of BSA and double the quantity of water

Lane 12: 5 µg. genomic DNA digested with 10 units of enzyme, 1.0% of BSA

Lane 13: 5 µg. genomic DNA digested with 20 units of enzyme

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Southern analysis of theT_0 generation of *BtCryIAb* plants. Genomic DNA was restricted with *Eco*RI enzyme that has two restriction sites within the *BtCryIAb* gene. This double cut releases the 3 kb fragment of *BtCryIAb* gene. Analysis of the transgenics for the *BtCryIAb* gene. Lanes 1 to 11 were added with transformant samples, Lane 1: CB1, Lane 2: CB2, Lane 3: CB3, Lane 4: CB4, Lane 5: CB6, Lane 6: CB5, Lane 7: CB7, Lane 8: CB8, Lane 9: CB9, Lane 10: CB10 and Lane 11: CB11. Lane 12 has the negative control and lane 13 has the positive control Restricted plasmid pHS723:Bt with *Eco*RI enzyme).

1.0 INTRODUCTION

Life is the term used to explain the co-ordinate integrity of defined matter energy transactions manifested in the living organisms. These transactions have been made possible with the availability of fixed energy. Early life derived energy from anaerobic environment and evolution of autotrophic organisms diversified course of evolution. Plants have been the largest source of fixed energy, as they transform the portion of solar energy into chemical energy, which makes them eligible for the title of 'the Producers'. This fixed energy is being used at various levels of food chain in various forms. In plants, this energy has been used for various metabolic activities and stored in material forms of macro molecules such as carbohydrates, protein and lipids, collectively structured into organs like seeds, grains, fruits, vegetables etc., to be used at the time of need. The concept of agriculture was born when man realized the nutritional significance of these plant parts. Agriculture brought about revolutionary social changes by transforming nomadic tribes into more advanced static civilizations, and methods of agricultural practices have been the principal measure to assess the advancement of civilizations.

Abundantly water supplying regions and sustainable rainfed areas have become the havens for settling and advancement of static civilizations. Agriculture gained significance principally by nourishing the human world and their domesticated animals. Consistent discovery of plants with diversified nourishing values expanded the span of agriculture while one faculty of agriculture concentrated on discovery and cultivation of plants with added benefits such as medicinal importance, timber, ornamentals etc., that have varied degrees of economic importance. Variety of plant organs such as seeds, fruits, leaves etc. were identified to be nutritionally very significant. A nutritious food is identified as a balanced diet that provides optimal energy and various organic and inorganic molecules of

structural and functional importance. Carbohydrates and lipids are the principal sources of energy while lipids and some proteins are structurally significant. Another class of proteins, vitamins, carbohydrates and various small organic and inorganic molecules are functionally very important. Most of these organic macromolecules in combination with several inorganic molecules must be supplied externally in the form of diet though there is synthesizing machinery within the body for some of the molecules. Most of the countries depend upon meat, poultry, eggs etc. for these dietary supplements. Developed world derives much of its protein (46%) and carbohydrates (20%) from animal products like meat, poultry, eggs and fish and other seafoods, while developing countries derive the protein (64%) and energy source (65%) primarily from agri-based foods. Henceforth, economies of the later are directly dependent on agriculture.

Agricultural practices and crop selection dates back to over ten thousand years and ancient agriculture was mostly characterized by few crop varieties that nourished relatively few people and their domesticated animals. The early moderate populations demanded equally moderate agricultural outputs. However, static civilizations stimulated wide array of social and cultural transformations that steadily increased the population as well, which in turn necessitated better yield from the selected crops. It was only since 19^{th} century that dramatic advances took place in agriculture in terms of quality and quantity which was made possible by well-concerted efforts of scientists from all over the world committed for a common cause of food-for-all.

Presently around 200 plant species with thousands of cultivars address the issue of human diet. Among these only 15 species contribute 90% of diet where rice, wheat and corn contribute up to 70%. Though these three crops are known as staple food crops of the world, some of the third world countries are not in a position to cultivate and maintain

them. About 10% of the Earth's land surface is arable that is fast approaching saturation, with only 1% of water available for cultivation and with inevitable population outburst there is an increasing concern of how to feed people as cautioned by eminent economist of yesteryears, Malthus. When demand was more than the supply, 1960s witnessed major changes by revolutionary agricultural outputs, all put together under the inspiring phrase 'Green Revolution' that was carefully crafted by eminent breeder Norman Borlaug, who was rightly awarded the Noble Prize for peace. Incepted with wheat and extended to almost all the crops, green revolution paved the way to light up smiles on the faces of the hungry though it shot down the world food prices. This led to a more sustainable agriculture, land use efficiency and opened the doors to many hopeful avenues, which could address various other imminent problems. These results came when demographic realities were sending ripples through the scientific community about the ways and means to manage the problems of starvation and malnutrition. With over 6 billion people on board, an estimated increase of 1.7 billions more by 2020 and with around 800 million people still starving, there are more questions than answers. Nevertheless, all the efforts put together will record an estimated increase in the annual growth rate of 2.6% food grain production while population increase rate is 1.4%. Unfortunately even with these results the hunger still remains since the outputs are not reaching the needy because of the low purchasing power and standard of living. These concerns led to cultivation of alternative food supplements such as legumes other than the popular cereals.

Pulses are dry seed legumes that have relatively lesser nutritional popularity. Nutritionally important pods bearing seeds and herbaceous nature characterize these crops. More than 50% of the world pulse yield is being contributed by Asia-Pacific region. The protein and oil rich seeds of these plants have an indispensable consideration in the human diet and animal feed as food additives. Fermented or processed seeds make preferred

dishes for people of the developing countries as accessory foods. Most important feature of these plants is that they fix atmospheric nitrogen in symbiosis with *Rhizobium,* which qualifies them to be used in inter cropping and rotation cropping practices in combination with the cereals to enhance the productivity of the later. However, despite their protein rich and nitrogen fixing attributes, the production of pulses has increased at much slower pace. This pace could have been the result of factors like rice-wheat rotation, inter cropping with few other cereals and lesser availability of nutritionally dependable legumes in hot season. Most of the important legumes are cool season crops where the cereals relatively dominate the cool season. These facts pushed the areas of legume cultivation to marginal regions that are mostly rainfed. Significant crop losses are observed due to various biotic and abiotic stresses, as farmers of the developing countries are not inclined to use expensive remedial inputs such as fertilizers and pesticides. This has resulted in a wide gap between the yield ratios of pulses to cereals at 1:32 in 1990 (Paroda, 1995). However, various scientific, social and nutrition concerns indicate that the legumes must be given due importance in the regular agricultural practices which would be more beneficial to the third world countries. In accordance with this pointer, various governmental and non-governmental research institutes including ICRISAT, India; ICARDA, Syria; IATA, Nigeria etc., embarked onto understanding the metabolic and agronomic intricacies of various food and pasture legumes and organize maneuvers for improvement and preservation of the elite germplasm of peas, beans, lentils, grain legumes and various other pulses.

During early agriculture, selection of cultivable varieties was principally based upon natural selection. However, it was in later part of 19th century that actual plant breeding programmes started. Varieties with elite phenotypic traits were selected and crossed for combination of necessary characters in the offspring. Mendel's famous pea experiments and observations provided a more logical base for the breeding trials and

observations. GH Shull's experiments in 1907 gave an impetus for plant breeding that resulted in an exponential increase in breeding programs, which was in turn expanded to countless members of cultivable varieties. Socio-economic importance of these programs gave birth to thousands of varieties initially in cereals and later in other domesticated crops. However, breeding is a highly time consuming process and labor intensive as the crossing means pollinating every plant manually. Crops with long duration life cycles pose variety of problems in conventional breeding. Another limitation of the conventional breeding lies in the sexual incompatibility of the cultivated varieties with their wild relatives, where the ancestral wild varieties were proven to be the reservoirs of several agronomically important traits. Tools of modern biotechnology have come to the rescue for some of the difficult constraints to crop improvement by understanding their molecular basis and providing remedies at the molecular level itself. Identification of physical basis of life and molecular characterization of inheritance patterns made breeding a more systematic and meaningful science of crop improvement. Statistical appropriation of polymorphisms inherited with the help of techniques like Southern blotting and polymerase chain reaction (PCR) gave birth to a new faculty of modem agriculture that is, marker assisted selection (MAS). This was not only useful in identification of quantitative trait loci (QTLs) but also useful in isolation of genes for a specific trait. Identification and isolation of agronomically important genes from different prokaryotes and eukaryotes ignited a desperate wish in scientists to introduce them into plants and observe their effect in the new environment. The existing bacterial transformation systems (Mandel and Higa, 1970) gave some logistic support to this idea of plant transgenesis by recombination events in the genetic material. Discovery of the ability of a crown gall inducing soil bacterium, *Agrobacterium tumefaciens* to introduce the genetic material into the plants (Drummond, 1979), in late 70s revolutionized the genetic transformation research. *Agrobacterium*

tumefaciens, popularly known as a 'natural genetic engineer' transfers genetic material with the help of the tumor inducing (Ti) plasmid (Chilton, 1983). The native genes of Ti plasmid perform the function of cancerous establishment of the *Agrobacterium* in the infected plant cells. Among various attributes of this plasmid, the most important one is that it transfers virtually any genetic sequence present between the left and right borders of the famous T-DNA. Though this organism's infectivity is restricted to dicots, technological advancements made this organism useful to transfer genetic material to monocots (Siemens and Schieder, 1996) and even to animal and human cell cultures as well according to recent reports (Kunik et al., 2001). Various other techniques evolved with the inspiration from this organism, but they are host and technique specific monocots (Siemens and Schieder, 1996). Biolistics or particle bombardment technique is one potential technique (Sanford, 1993) that could solve the problem of host incompatibility.

Chickpea (2n=16) is one of the important food legumes prevalently cultivated in Asia-Pacific region where large portion of the yield is contributed by the Indian sub-continent. Broadly there are two varieties of chickpea, Desi and Kabuli. It is a cool season pod bearing crop, seeds of which are excellent source of proteins rich with nitrogenous amino acids especially lysine and arginine making the products of this crop, very good food additives. Besides, it is also known to improve soil fertility with the help of symbiotic nitrogen fixation. It contributes 15% of the world's pulse harvest of about 58 million tons, annually. Despite significant gains in world pulse production during the last two decades with an annual growth rate of 1.9% chickpea production growth has been slow. Chickpea yields worldwide have risen by 0.6% annually which amounts to 800 kg/ha, and the area of cultivation has remained virtually stagnant. This slower pace has been the result of various refractory biotic and abiotic constraints such as *Ascochyta* blight (AB), *Botrytis* Grey Mold (BGM), dry root rot, collar rot, *Fusarium* wilt, pod borer, drought and low temperature

(Nene and Haware, 1980). The legume pod borer has been the worst of all accounting for over 20% of the total crop loss (Vyas et al, 1983). The enhancement of insect and disease resistance in chickpea could increase its yield potential by as much as three times. The available chickpea germplasm also lacks effective resistance sources for use in developing pest resistant genotypes. An attractive option is to introduce genes for insect resistance from other sources to chickpea by the use of transgenic technology that has shown a great promise (Sharma and Ortiz, 2000).

Attempts to create transgenic chickpea to combat the above constraints have been the short cut strategy adopted by various groups working for creation of elite germplasm of chickpea. However, reliable regeneration and transformation protocols have been evading such efforts due to the perceived recalcitrant nature of chickpea towards tissue culture. Several regeneration protocols involving somatic embryogenesis and organogenesis have been published during past one and half decade only to show the difficulty in regenerating chickpea in the in vitro environment. Micropropagation has not been a serious problem and it can be achieved using explant containing pre-existing meristems such as shoot tip and cotyledonary nodes (Rao and Chopra, 1987; Riazuddin et al., 1988; Rao and Chopra, 1989). Considerable work has been done for regenerating whole plants via somatic embryogenesis from mature leaflets (Rao and Chopra, 1989) and immature leaflets (Barna and Wakhulu, 1993), mature embryo axis (Suhasini et al., 1994) and immature embryo axis (Sagare et al., 1993) or cell suspension cultures (Prakash et al., 1994). However, the success rates on the maturation of induced embryos into fully differentiated plants have been very low (<2%) making it an inefficient system for genetic transformation studies. Regeneration of shoot buds from various explants have also been reported to produce shoots either directly (Shri and Davis, 1992; Kar et al., 1996; 1997; Subhadra et al., 1998) or indirectly through callus phase (Khan and Ghosh, 1984; Prakash et al., 1992; Barna and

Wakhulu, 1994). However, regeneration of quiet a few shoots from traces of pre-existing meristem with that of the explants such as hypocotyl and epicotyl were mistaken for indirect regeneration from callus phase. To date, effective chickpea regeneration has been possible only through the use of explants based on cotyledonary nodes or shoot apices derived from seedling explants. In most of the cases the shoot buds originated asynchronously making these systems inefficient for genetic transformation studies. However, rooting and transplantation of the in vitro recovered plants has remained a major bottleneck in the meaningful application of this technology for serious crop improvement programmes. Such systems have been used to genetically transform chickpea and the transformation frequencies reported were very low (Fontana et al., 1993; Kar et al., 1997; Krishnamurthy et al., 2000).

In the ongoing efforts at ICRISAT to develop suitable tissue culture and transformation protocols for chickpea, the present work was aimed at the improvement of existing protocols for all the stages of regeneration and transformation. The work was carried out with the following objectives.

1. Development of efficient protocols for plant regeneration in tissue cultures of chickpea.
2. Optimization of various factors affecting regeneration via organogenic pathway.
3. Optimization of factors affecting hardening and transplantation of in vitro regenerated plantlets.
4. Genetic transformation and successful recovery of chickpea transgenic plants by using agronomically important *BtCryIAb* and *SBTI* genes.
5. Molecular characterization of putative transgenic plants of chickpea for future use.

2.0 REVIEW OF LITERATURE

Large part of the world's food is being contributed by agriculture though most of the developed countries derive their food from animal source. World food grain production touched 2 billion ton mark at the turn of the last millennium. The economic, commercial and social realities resulted in an uneven distribution of the food grains amply available that left 800 million people still undernourished (FAO, 2001). The surpluses are used for exports and the costs were dearer for the developing countries that kept the number of undernourished people still alarming. Alternative food supplements from pulse crops gained an increasing significance in the recent times that prompted developed country like United States to include the lentils, broad beans and chickpeas in their farm bill 2002 so as to encourage the exports in this area (Food Outlook, FAO, 2002). Pulses are the edible dry seeds of leguminous plants. They are of special nutritional and economic importance due to their contribution to the diets of millions of people worldwide. The main importance of pulses lies primarily in their high protein content (two to three times higher than most cereals) as well as in being a valuable source of energy. In addition, pulses contain good amounts of nutritionally essential minerals such as calcium and iron. The use of pulses as food is concentrated in developing countries, accounting for about 90% of global human pulse consumption. In most low-income countries, pulses contribute about 10% of the daily protein and about 5% of energy intakes in the diets of people (Paroda, 1995). During the initial years of legume cultivation breeding was an important means of crop improvement. These efforts met with considerable success that resulted in improved yield of biomass in terms of whole plant dry matter or the seed. However, breeding for traits

against various biotic and abiotic constraints met with low success rates where biotechnology may prove to be an effective alternative.

Evolution is a process of natural creation of variations in the genetic make-up of organisms so that it fits into the variable conditions of the changing environment. This process takes millions of years and it goes on. As the requirements doubled and tripled, there is a necessity to induce variations deliberately to meet the need. This fast emerging field of plant science gains its significance and advantage over the limitations of the genetic recombination by means of conventional breeding. Though this technique is being applied to many organisms, including microbes and animals, the scope of this thesis is restricted to deal with plants only. Moreover, most of the published and on going work is on plants when compared to the animals. Microbe transformation is rather a procedure for basic research and only a part of preparative technique for the higher organism transformation.

2.1 Genetic transformation

Genetic transformation, in principle is integration of alien genes into the foreign organism. Stable integration and inheritance of useful genes is the main objective of gene transfer experiments. The concept of genetic transformation started with Avery et al. (1944). In angiosperms, gene transfer, however, is a regular process where paternal chromosomes from the exploding sperm are transferred to the egg cell of the female gametophyte (Frankel and Galun, 1977). So, the problem is to transfer the genes deliberately. Advent of plant tissue culture especially the protoplast isolation (Cocking, 1960) and cybrid formation gave an encouraging impetus to the concept of gene transfer. Protoplast isolation resulted in cell-wall-stripped cells and regeneration from the whole

plants from them enthused scientists for adding new genetic elements and earliest reports were of Hess (1969; 1970) when no tools for transfer and characterization of transformed plants were available. Discovery of ability of the soil microorganism *Agrobacterium tumefaciens* to transfer its T-DNA to the plant genome revolutionized this area and created altogether a different faculty of science.

2.1.1 Various methods of genetic transformation in plants

Various methods of gene transfer into plants have been designed with *Agrobacterium*-mediated transfer as the principal method. Its miraculous ability to transfer part of its genetic material, T-DNA into the plant genome for its own benefit was one of the significant discoveries of 20th century. Vast literature accumulated in describing the whole mechanism and apparatus used by the microbe for gene transfer. Hence, this method will be dealt in detail in the following section. Methods other than those relying on the *Agrobacterium* such as biolistics, microinjection etc. will be described in this section.

2.1.1.1 Genetic transformation by the biolistic process:

"Biolistic" is a short term for biological ballistics; the process is one by which biological molecules, such as DNA and RNA, are accelerated (usually on microcarriers, termed microprojectiles) by gunpowder, compressed gas or other means. The biological molecules are driven at high velocity into the target, in this context, the plant cells. A team of nanofabrication facility of Cornell University developed this technique, and Sanford (1988) gave early descriptions. This is basically simple device where the genetic material is coated on to the tungsten or gold particles and accelerated with high pressures into the plant cells. During subsequent years, this device took series of changes to fit commercial

requirements (Sanford, 1993). The original driving power – the real gunpowder was replaced by safer compressed helium system. A different acceleration system was also developed based on the spark discharge chamber in which a water droplet was placed between two electrodes and a high voltage capacitor caused an instant vaporization of the water, creating a shock wave. This shock wave accelerates DNA coated particles into the target plant cells (Christou et al., 1990). Several labs tried their own homemade particle guns (Perl et al., 1992). Various devices and intricacies of biolistic processes were reviewed by Potrykus and Spangenberg (1995). The first application of the biolistic process was made by its inventors using chloramphenicol acetyl transferase (CAT), a marker gene (Klein et al., 1987). Later years saw the two important publications of successful transformation of chloroplasts in *Chlamydomonas* and mitochondria in yeast (Johnston et al., 1988).

This protocol becomes significant when the host cells are not complacent with the methods like *Agrobacterium*-mediated and direct protoplast transformation. Especially *Agrobacterium*-mediated transformation is, to some extent restricted to dicots and it also requires wounding. Hence, several reports appeared using method to obtain transformed plants in rice (Datta et al., 1990), soybean (Christou et al., 1990), maize (Fromm et al., 1990; Koziel et al., 1993) and barley (Wan and Lemaux, 1994).

2.1.1.2 Alternative methods of genetic transformation into plant cells:

Alternative methods can be divided into two types: direct physical introduction of DNA and transmission of genetic material by modified plant viruses. Viral gene transfer can also involve physical transmission to the plant (e.g., rub inoculation). Most important method of direct introduction of DNA is the protoplast transformation. Initially direct

introduction into protoplasts using ply-L-ornithine (Davey et al., 1980) and this compound was later replaced with calcium phosphate/polyethylene glycol (PEG) (Krens et al., 1982). However, the success of protoplast transformation lies in the successful regeneration of the whole plants from them. Most of the direct DNA transformation involves usage of *E.coli* plasmids such as pBR 322 and pUC derivatives. Both plant DNA and RNA viruses offer possibilities as plant transformation vectors.

2.1.2 Miracle microorganism: *Agrobacterium*

Early in twentieth century, Smith and Townsend (1907) studied these crown gall tumors of cultivated Paris daisy and for the first time established that this "plant tumor" is of bacterial origin. The ineffective bacteria isolated from these samples produced tumors on the stems of other crops. This bacterium changed its name many times from *Bacterium tumefaciens* through *Phytomonas tumefaciens*, *Bacillus tumefaciens* and finally settled at *Agrobacterium tumefaciens*. Riker (1923) and Pinckard (1935) studied many intricacies of the plant-microbe interactions that resulted in various cell stimulations in plants. The opines were detected in the tumors (Petit, 1970) and the vital role of these opines in the establishment of "genetic colonization" was revealed by Shell (1979). Initially it was perceived that the genes for opine synthesis is plant borne and only after rigorous studies the source of opines was confirmed to be from the infecting bacterium (Montoya et al., 1977). Much earlier to that Kerr (1971) found that the virulence could be transferred from *Agrobacterium* to saprophytic bacteria through DNA transformation. Persistence of bacterial DNA in bacterial free tumor cells was observed by Johnson et al., (1974) and DrLica and Kado (1974) with the less efficient techniques of their times. A large plasmid with a size of around 200 kb. was found to be necessary for the virulence of the

Agrobacterium (Zaenen et al., 1974). Eventually, Chilton et al., (1977) found that the genes of Ti plasmid were responsible for the synthesis of opines which were necessary for the progression of tumor without the addition of growth hormones. They also found that only 5% of the plasmid DNA was responsible for virulence (Chilton, 1978). Since then many groups embarked upon exploring the intricacies of the *Agrobacterium* and its ability to transform the host cells.

2.1.2.1 Ti plasmid and its characteristics

Many scientific groups in early 70s found that the Ti plasmid, precisely part of it's DNA is responsible for the tumorigenic nature of the infecting bacterium. The process of identification of causal sequence for the tumorigenic activity was many folds expedited by the discovery of Southern blot (Southern, 1975) and DNA sequencing methods (Maxam and Gilbert, 1977). Hooykaas, Schilperoot and their associates found additional evidence for the role of Ti plasmid in tumor induction (Hooykaas et al., 1977). A detailed description of early events in crown gall research was provided by Shell et al., (1979). It also became evident from Chilton's experiments that part of Ti DNA (termed as T-DNA) was transferred to the plant cell. Transcription of the T-DNA was confirmed by northern blot experiments (Drummond et al., 1977). All the T-DNAs were found to be similar and has a length of 23 kb flanked by almost identical borders (Zambryski et al., 1980). Spontaneous deletions studies revealed the genetic components of the T-DNA and their oncogenic nature was confirmed (Gelvin et al., 1981). Schilperoot and colleagues revealed many aspects of the T-DNA by inducing mutations and tracking them down in the host plant cells by transforming the tobacco protoplasts (Hockema et al., 1984). By this time it was also evident that a set of *vir* genes was involved in the transfer of T-DNA. It became

evident by 1983 that the *Agrobacterium* is a sure candidate for genetic transformation (Chilton, 1983; Herrera-Estrella et al., 1983). Where T-DNA of the Ti plasmid can be transferred to the plant cells and that can serve as an excellent tool for the genetic transformation of plants by disarming the T-DNA and introducing the genes of interest into that area.

2.1.2.2 Molecular mechanism of T-DNA transfer into plants

The details of these mechanisms can be obtained from several reviews (Hooykaas and Schilperoot, 1992; Zambryski, 1992; Greene and Zambryski, 1993; Zupan and Zambryski, 1995). Winans (1992) provided a critical review of chemical signaling between *Agrobacterium* and plants cells.

2.1.2.3 Tools for genetic transformation

Understanding the plant gene structure and its essential components is a prerequisite for designing the tools for genetic transformation. By the time the conceptual basis for the genetic transformation was ready using *Agrobacterium*, the components of a complete plant gene to be effectively expressed was also ready. The amalgamation of various molecular biology techniques led to the designing of vectors for transforming the plant cells.

Transformation vectors: Most widely used vectors are binary vectors (Bevan, 1984) and cointegrate type vectors (Draper et al., 1988; Rogers et al., 1987; Deblaere et al., 1987). The cointegrate type vectors have become less popular since they are more difficult to engineer than binary type ones and are less efficient.

Binary vectors: A binary vector should contain essentially at least one of the borders, it should have the ability to replicate in *E.coli* and it should contain a selectable marker (Armitage et al., 1988; Hood et al., 1993). In principle, a binary vector consists of two plasmid; a plasmid that is transferred and a helper plasmid. In the initial years it was pBI101, and later many versions were constructed by Becker et al., (1992). Additional information on binary vectors was provided by several authors (Jones et al., 1992; Futterer, 1995). Recently it has been suggested to use binary vectors that contain two separate T-DNAs (Crameri et al., 1996). The logic of the authors is that the antibiotic resistance marker will be lost during subsequent generations. A helper plasmid contains the *vir* gene complements that are essential for transfer of T-DNA.

Promoters: Futterer (1995) reviewed the subject of promoters for genetic transformation of plants. In the early years of genetic transformation of plants, investigators were merely interested in showing that integration and expression of transgenes is a reality in plants. So, initially promoters endogenous to the T-DNA were used. Soon it was observed that the promoters for opine synthesis were weak. Chua and his collaborators (Odell et al., 1985) isolated the CaMV 35S promoter from turnip leaves infected with the Cauliflower mosaic virus (CaMV). This promoter was found to be many folds stronger and resulted in constitutive expression of the introduced genes. However, sub-domains of this promoter were found to be exerting tissue specific expression (Benfey and Chua, 1989). Since then this promoter became an attractive candidate for plant molecular biology research. Its fusion with part of mannopine synthase (MAS) promoter increased the potency of this promoter (Kay et al., 1987). Valuable information can be found in the reviews by Benfey and Chua (1989), Wang and Cutler (1995).

The above promoters were found to be more efficient in dicots and there was a distinguished interest for finding out the promoters for monocots. In the early studies with rice (Shimamoto et al., 1989) the CaMV 35S promoter was used to activate the selective and reporter genes. However, it was found that this promoter was more efficient in dicots. Combination of this promoter with other promoter segments and introns were even tried. This concept was followed by the usage of cereal alcohol dehydrogenase 1 (*Adh1*) gene (Callis et al., 1987; Kyozuka et al., 1990). A similar approach to integrate the first intron of the *Shrunken 1* gene of maize was also followed in cereal transformation (Mass et al., 1991), but it became less popular in the subsequent years. Rice actin gene promoter (*Act1*) was found to be even more potent than the above two (Zhang et al., 1991). This promoter showed more or less similar potency as that of *Emu* promoter that is a recombinant promoter containing a truncated *Adh1* promoter with other elements (Last et al., 1991). The current most effective promoter is the *Ubiquitin 1* (*Ubi1*) of maize (Christensen et al., 1992). This promoter was used successfully to transform wheat (Weeds et al., 1993), barley (Wan and Lemaux, 1994) and rice (Toki et al., 1992). Another promoter of the rice *Aldolase P* (*AldP*) gene was found to be one of the better alternative (Kagaya et al., 1995).

Terminators: Knowledge of the elements for gene expression is as important as the promoters. It is considered that fundamentally mRNA is stable unless destabilizing motifs are involved. Specific examples of the studies that handled the polyadenylation signals in plants are investigations of Mogen et al. (1990). Rothnie et al. (1994) studied the essence of the terminator regions and impact of 3'-end regions on the level of gene expression of octopine synthase gene and other gene constructs was studied by Ingelbrecht et al. (1989). Hence, the usage of terminator region at the 3' end of the transgene was found to be

essential. In practice, terminator of nopaline synthase gene or of the CaMV was fused into the respective chimeric gene.

Selectable and reporting markers: These are essential components of the total cassette that is to be transferred to the plant cells. These genes select the transformed cells from that of the untransformed. Some of the popular selectable markers are antibiotic markers such as kanamycin, hygromycin, streptomycin etc. Other groups of selectable markers are the ones that confer resistance to herbicides such as phophinohtricin, biolophos, glyphosate, dalaphon etc. As noted above this group of selectable markers can serve a dual purpose: to select transformants and to render crops resistant to respective herbicides (D'Halluin et al., 1992). The third group is diverse, including genes that cause resistance to high nitrate, high amino acid levels (lysine or threonine) or amino acid analogues (Schrott, 1995). Most commonly used selectable gene is the kanamycin resistance gene neomycin phosphotransferase (*nptII*). This gene product detoxifies aminoglycoside antibiotics such as kanamycin, neomycin, geneticin and paromomycin (Vardi et al., 1990). The gene *hpt* was isolated from *E.coli*. It codes for hygromycin phosphotransferase that detoxifies antibiotic hygromycin.

Reporter genes are coding sequences that, upon expression in the transgenic plant, provide a clear indication that genetic transformation did take place. They are useful also for transient expression experiments, in which the transgene is not necessarily integrated into the host genome. Schrott (1995) reviewed a review of genes used and their assay methods. Most commonly used genes used, as reporters are the ones that code for CAT, GUS, Luciferase and Green Fluorescent Protein (GFP). The assay for the *uidA* that codes for GUS was developed by Jefferson and his associates (Jefferson, 1987). This gene has

gained an instant popularity owing to its efficiency and localization of the expression without extracting the tissue. The luciferase reporter gene was developed by de Wet and associates (De Wet et al., 1985) and was reviewed by Luehrsen et al. (1992). The *gfp* gene that codes for green fluorescent protein (GFP) was the recent one isolated from jellyfish by Chalfie et al. (1994). Many reports appeared in support and against the usage of GFP (Haseloff and Amos, 1995).

2.2 Legume tissue culture and transformation

Leguminosae is a very important family of angiosperms comprising of many species in relation to human nutrition, pasture and fodder needs. Important protein rich seed bearing plants, mostly herbaceous, such as peas, lentils, beans collectively known as pulses are members of this family. They rank next to cereals in terms of human nutrition. In quantitative significance they are far behind the cereals, however, gaining some due importance as food additives in the recent years. Domination of the cereals in the food sector allowed only marginal increases in the overall yield of pulses. Recent concerns over the importance of these crops led to augmented efforts to improve the quality and quantity. Classical and modern breeding technologies proved result in limited success in interchange of the desirable characters in these crops. Biotechnological improvement has emerged as a potential supplement to these efforts. Advances in plant tissue culture, genetic transformation methods and simultaneous improvements in molecular biology techniques and gene isolations gave an impetus to these efforts. Amalgamation of all these strategies laid foundation for many potential strategies for crop improvement.

2.2.1 Food legumes

There are several species and subspecies classified as food legumes. But, only few (15 to 20) genera are very important. Hundreds of cultivars within these genera are included in the agricultural practices, each having some selected attributes. Most important of these species are *Glycine max, Arachis hypogaea, Cicer arietinum, Lens culinaris, Pisum sativum, Lathyrus sativus, Cajanus cajan, Vigna radiata, Vigna mungo, Vigna aconitifolia, Vigna umbellata, Vigna unguiculata, Phaseolus vulgaris, Macrotyloma nuflorum* etc. These species constitute over 80% of total food legume output. Initially many of these species were thought to be recalcitrant in tissue culture and later advancements of biotechnological techniques gradually eased the technical difficulties. Micropropagation was relatively easier when compared to adventitious shoot regeneration, where the shoots originate from pre-existing meristems. Adventitious regeneration is a pre-requisite for a successful genetic transformation. Each species responded differently in tissue culture and some of the protocols were successfully used for genetic transformation.

2.2.1.1 Organogenesis

Organogenesis is a widely used tissue culture strategy for regeneration of whole plants via direct and indirect induction of various plant organs such as shoots and roots. Generally shoots are induced initially from selected explants followed by roots. Multiple shoots are induced either directly or through callus phase where the techniques are termed as direct and indirect organogenesis respectively.

Direct organogenesis: Following are some selected reports where organogenesis without any intermediary callus phase has been reported in various economically important

legume crop species. Cotyledon explants for the indirect regeneration shoots was developed for soybean (*Glycine max*) using 2,4,5-T for embryogenic callus induction. Somatic embryogenesis was best on SE (soyabeen embryo) medium supplemented with BAP and best regeneration of shoots was found on hormone free medium or on medium with IBA (Cho et al., 1992). Multiple shoot regeneration was obtained from leaf and hypocotyls explants of *Glycine wigtii*. 3-4 day old seedlings cultured on NAA and IBA containing medium gave rise to multiple shoot buds (Pandey and Bansal, 1992). A wild relative of soybean, *Glycine clandestina*, was induced with brown, compact and nodular callus and plants were regenerated from it (Sharma and Kothari, 1993). Culturing of zygotic embryos and multiple shoot regeneration was studied envisaging their use for micro projectile bombardment in *Arachis hypogaea* (Schnall and Weissinger, 1993). Effects of auxins (IAA, NAA, IBA and 2,4-D) and cytokinins (kinetin and BAP) were studied for multiple shoot regeneration from cotyledons and cotyledonary node explants of *Arachis hypogaea* (Venkatachalam and Jayabalan, 1997). Various concentrations of BAP, 2-iP, chloropyridylphenylurea (4-PU), TDZ and zeatin in combination with NAA were used to improvise the regeneration from cultured leaf segments in *Arachis hypagaea* (Akasaka et al., 2000). Explants such as petioles, epicotyl sections and other seedling explants were used for regeneration of *Arachis hypogaea* (Cheng et al., 1992). Regeneration via caulogenesis (shoot organogenesis) was achieved in *Arachis hypogaea*, from plumular explants. The shoot buds regenerated on medium containing brassin, BAP and β-naphthoxy acetic acid (Ponsamuel et al., 1998). In vitro regeneration of *Arachis hypogaea* was achieved via organogenesis by employing BAP as the principal multiple shoot inducer. Optimal temperature for culturing of leaf explants was standardized, and

effect of silver nitrate was studied (Pestana et al., 1999). Wild species are generally less responsive in tissue culture. However, differentiation in tissue culture using mature leaf explants was first reported in *Arachis villosulicarpa* (Johnson and Pittman, 1986). A protocol for tissue culture based regeneration of *Arachis pintoi*, a wild perennial peanut, was developed. Day length and media effects were directly correlated with variations in regeneration (Ngo and Quesenberry, 2000). Effect of aluminium on the tissue cultures of *Phaseolus vulgaris* was studied (Espino et al., 1994). Direct plant regeneration and multiplication was obtained from the embryo and cotyledons of common bean *Phaseolus vlugaris* that were imbibed for 3 days and cultured on a soil medium for 7 days (Mohamed, 1990). A small portion of split embryonic axis showed a genotype dependent multiple shoot regeneration in *Phaseolus vulgaris* and *P. coccineus* (Santalla et al., 1998). Various factors such as light intensity and duration plant growth regulators etc. were studied while regenerating multiple shoots from mature embryonal axes of *Cajanus Cajan* (Franklin et al., 2000). Epicotyl, hypocotyl, leaf, and cotyledonary nodal explants were shown to regenerate a high frequency of multiple shoots with high BAP and kinetin of pigeon pea (*Cajanus cajan*) (Geetha et al., 1998). Multiple shoots were regenerated from distal ends of cotyledonary segments of *Cajanus cajan*. This was achieved using combinations of BAP, kinetin with adenine sulfate (Mohan and Krishnamurthy, 1998). Cotyledonary node culture using BAP and IAA was reported in pigeonpea, *Cajanus cajan*. A mass of multiple shoot-initials formed at the axillary bud region of the cotyledonary node of the seedlings within two weeks. The cotyledonary node along with the mass of shoot-initials excised from the seedling, continued to form new shoot-initials on MS medium containing 6-benzylaminopurine and supplemented topically with indole-3-acetic acid. (Prakash et al.,

1994). Clonal propagation of F1 interspecific hybrids of *Vigna radiata* and *V. mungo* was done. Multiple shoots were induced from the cotyledonary node explants of F1 hybrids (Avenido et al., 1991). Effect of culture medium on plant regeneration from cotyledons of *Vigna radiata* was studied. Genotype size, orientation and age of the explant showed very significant effects on plant regeneration (Gulati and Jaiwal, 1990). Differences in shoot regeneration from cotyledonary node explants in Asiatic *Vigna* species were used for genomic grouping within subgenus *Cerototrophis* (Avenido and Hattori, 1999). Shoot tip cultures were established for plant regeneration of mungbean, *Vigna radiata.* Complete plants were regenerated directly without an intervening callus phase from shoot tips on basal medium (MS salts + B5 vitamins). Regeneration frequency varied with cultivars, explant size and growth regulator combinations in the medium. Addition of cytokinins induced a variable amount of callus at the base of the shoot tip, followed by multiple shoot formation. BAP, kinetin and zeatin each induced multiple shoots in 100% of the explants but the highest number of regenerants per explants (9) was produced with BAP. (Gulati and Jaiwal, 1992). Regeneration was achieved using cotyledonary nodes giving rise to axillary shoots of *Vigna mungo* (black gram). Regeneration has been achieved through organogenesis using explants from axillary shoots originating from the nodes of seedlings germinated in cytokinin containing medium. Seeds germinated in thidiazuron (TDZ) supplemented MS produced 11 axillary shoots/cotyledonary node. Stem and petiole explants derived from these axillary-shoots produced callus along with shoot-buds after 2 weeks of culture on half strength MS supplemented with NAA. Shoot-buds were also produced from various sites of injury caused by incisions on the stem explants. Full strength MS salts inhibited bud formation (Das et al., 1998). Cotyledon explants derived

from germinated seeds of a multipurpose leguminous tree, *Sesbania grandifolia*, showed a high percentage (96%) of explants producing at least 30 shoot bud per explant (Detrez et al., 1994).

Indirect organogenesis: Regeneration and analysis of callus from flow-sorted heterokaryons of soybean (*Glycine max*) and *G. canescens* was done (Hammatt et al., 1988). Pieces of leaves from seedlings of *Arachis pintoi* were regenerated via organogenesis and somatic embryogenesis pathways. Plant regeneration was obtained via two developmental pathways: organogenesis and somatic embryogenesis. Organogenic callus cultures were initiated from pieces of leaf on MS medium supplemented with NAA or 2,4-D in combination with BAP, kinetin or 2-iP. The most suitable combination for plant regeneration through organogenesis was an initial medium composed of NAA and BAP followed by transfer of the callus to a shoot induction medium (MS+ BAP). Rooting of regenerated shoots was readily achieved by culture on MS with NAA. Embryogenic callus cultures were initiated from pieces of leaf on MS medium supplemented with picloram in combination with kinetin, zeatin, BAP or 2-iP, and the most suitable combinations were picloram, BAP or 2-iP. (Rey et al., 2000). A comparative study of callus formation and plant regeneration was done using different explants of *Phaseolus vulgaris* and *P. coccineus* (Ruiz et al., 1986). A different synthetic auxin 2,3,5-triiodobenzoic acid was shown to induce callus and roots on stem cuttings of mungbean, *Phaseolus aureus* (Ali and Jarvis, 1988). A novel method of culturing leaf disc explants of pigeon pea (*Cajanus cajan*) on multiple shoot induction medium with IAA and BAP where the shoots originated via callus phase (Rathore et al., 2000). Plants were regenerated from leaflet-derived callus of *Aeschynomene sensitiva*, *A. americana* and *A. villosa*. Explants

were induced to form callus when aseptically cultured on Murashige and Skoog medium solidified with 0.8% agar and containing NAA and benzyladenine. Shoot regeneration was readily achieved and roots were induced when shoots were transferred to medium devoid of growth regulators or with NAA. Callus from *A. falcata* failed to regenerate shoots. Explants from leaflets of *A. fluminensis* did not produce callus when cultured in vitro. (Rey and Mroginski, 1996). Pre-soaked seeds of *Vigna radiata* showed variable callus growth when exposed to various intensities of light where the shoot regeneration was also variable under different intensities of light. Hormonal supplements of the culture medium had some promotive effect on regeneration under various light intensities (Narciso et al., 1997). Analysis of tissue culture borne genetic variations (sòmaclonal variations) was done in *Pisum sativum* (Griga et al., 1995). One hundred and forty six somaclones were generated that were resistant to the purified toxin of *Cercospora canescens* (Kaushal et al., 1997).

2.2.1.2 Somatic embryogenesis

Somatic embryogenesis is another efficient strategy where regenerating tissue initially attains some defined globular, elliptical etc. shapes and those units gradually regenerate into whole plants. Usually the globular proembryos split into torpedo shaped ones and shoot primordium regenerate from the axillary portion. Relatively regeneration via somatic embryogenesis pathway consumes more time than organogenic pathway. A fast and efficient regeneration system via somatic embryogenesis was developed using BAP and NAA on cotyledon explants of *Glycine max*. Plants were ready within six weeks from explant stage (Fu et al., 1995). An efficient regeneration system was developed for *Glycine tomentelta* via somatic embryogenesis pathway. Effect of plant growth regulators and pH was studied and conditions were standardized (Lee, 1992). Promotive role of

thidiazuron (TDZ) was studied to induce direct somatic embryogenesis and regeneration from seedling explants of *Arachis hypogaea* (Gill and Saxena, 1992). Direct somatic embryogenesis from zygotic embryos derived from 40-day-old immature pods of *Arachis hypogaea* using 2,4-D were studied where various factors like growth regulators, sucrose, genotypes and length of embryonic axis influenced frequency (Reddy and Reddy, 1993). 2,4-D-induced somatic embryogenesis was obtained from embryogenic calluses derived from hypocotyls explants of *Arachis hypogaea*. High concentration of 2,4-D decreased the frequency of somatic embryogenesis (Venkatachalam et al., 1997). Synthetic seeds of *Arachis hypogaea* were obtained by encapsulating 5 to 30-day-old somatic embryos and germinated by on medium with various concentrations of sucrose, maltose, BAP and NAA. 25% of these embryos were finally converted into plantlets (Padmaja et al., 1995). A refinement of embryo rescue technique to improve plant recovery from early heart shaped embryos of interspecific hybrids of *Phaseolus polyanthus* and *P. vulgaris* was reported (Geerts et al., 1999). Rates of ethylene production were determined in highly embryogenic and virtually non-embryogenic tissue cultures of *Medicago sativa* ssp. *falcata* during a 10-day induction period on medium containing 2,4-D and kinetin, and during the first 10 d of somatic embryo formation on growth regulator-free medium. It was concluded from these experiments that the high rates of ethylene production during embryo induction are not essential for subsequent embryo differentiation (Meijer, 1989). Efficient plant regeneration via somatic embryogenesis has been developed in pigeonpea (*Cajanus cajan*). Cotyledon and leaf explants from 10-day-old seedlings produced embryogenic callus and somatic embryos when cultured on MS supplemented with 10 μM thidiazuron (TDZ). Subsequent withdrawal of TDZ from the induction medium resulted in the maturation and growth of

the embryos into plantlets on MS basal medium. (Sreenivasu et al., 1998). Distal ends of cotyledons were used to induce somatic embryogenesis in *Cajanus cajan* by applying BAP, kinetin and adenine sulfate and whole plants were regenerated (Patel et al., 1994). Direct somatic embryogenesis was induced from excised seedling leaf segments of vegetable legume, *Psophocarpus tetragonolobus* by using NAA and BAP and the conversion frequency of cotyledonary embryos was 53.3% upon culture on MS medium containing ABA for 7 days followed by transfer to MS medium supplemented with IBA and BAP. (Dutta Gupta et al., 1997).

2.2.1.3 Other methods

Besides organogenesis and somatic embryogenesis the techniques such as regeneration of plants from isolated protoplasts, microspore, anther and ovule cultures etc. are less frequently applied in the tissue culture studies. Excellent yields and quality were achieved for soyabean (*Glycine max*) protoplasts and plants regenerated from agitation-derived protoplast preparations had a higher chance of being derived from intact cells. (Zaghmout et al., 1990). Protoplast isolation in *Arachis hypogaea* is relatively rare phenomenon and there is one report for isolation and regeneration of plantlets through this method and this method was effectively used for electroporation mediated transformation (Li et al., 1995). A different method using thin cell layer technique and transverse thin cell layer (fTCL), where the fTCLs were cultured on TDZ for *Phaseolus vulgaris* was employed. Shoot multiplication was enhanced using BAP with silver nitrate (Carvalho et al., 2000). A reproducible protocol for plant regeneration from seedling hypocotyls protoplasts using various growth regulators such as zeatin riboside, GA_3 and IBA was reported in *Vigna sublobata* (Bhadra et al., 1994). Callus regeneration was achieved from

protoplasts isolated from mesophyll tissue of sweet pea *Lathyrus odoratus* (Razdan et al., 1980). Protoplasts were isolated from leaf tissue of *Lens culinaris* by using cellulase, macerozyme dissolved in 0.5 M mannitol with pental salts. However, the callus cultures could not be regenerated into plantlets (Stiff et al., 1991). Genetically variable plants were obtained from anther derived callus cultures obtained from microspore cultures of *Cajanus cajan* (Kaur and Bhalla, 1998). Cryopreservation is an excellent method for germplasm conservation provided an efficient method is available for regeneration. Methods for pollen embryo cryopreservation and conservation of germplasm of *Arachis, Brassica* and *Triticum* sp. were explicitly reviewed (Bajaj, 1983).

2.2.1.4 Genetic transformation

Agrobacterium-mediated transformation: Direct crown galls were induced by infecting stem explants of Lentil (*Lens culinaris*) with four strains of *Agrobacterium tumefaciens*. Opines were detected in the crown gall and Southern analysis showed that T-DNA was transferred (Warkentin and McHughen, 1991). Inclusion of potato suspension culture in the culture medium enhanced the transformation frequency of the callus obtained from in vitro grown seedlings of the *Glycine max* (Chang and Chan, 1991). An efficient protocol for *Agrobacterium*-mediated transformation of cotyledon explants from in vitro grown seedlings of *Arachis hypogaea* that resulted in a very high frequency of transformation (55%) was reported. The explants were transformed with binary vectors pBI121 and pROKII:IPCVcp that consisted of *npt*II as selectable marker gene and Indian Peanut Clump Virus coat protein gene as the agronomically important gene (Sharma and Anjiah, 2000). Co-cultivation of cotyledonary node explants of *Arachis hypogaea* was done with *A. tumefaciens* strain harboring binary vector containing *uid*A gene as reporter

and *npt*II for selection. PCR and Southern analyses confirmed the integration of transgene (Venkatachalam et al., 1998). Transformation of *A. hypogaea* was also done using the somatic embryogenesis pathway. The cotyledons were co-cultivated with *A. tumefaciens* strain LBA4404 containing *uid*A and *npt*II genes. Somatic embryos were induced with NAA and BAP and later regenerated into whole plants with a transformation frequency of 47% (Venkatachalam et al., 2000). Embryo axis explants were used for the *Agrobacterium*-mediated transformation in *Arachis hypogaea* (McKently et al., 1995). In vitro grown seedlings of *Cajanus cajan* were inoculated with three types of wild strains of *Agrobacterium* A281, A6 and T37 and cultivar-*Agrobacterium* strain specific induction tumors was found (Rathore and Chand, 1997). Leaf disks of pigeon pea cv. ICPI5164 were transformed by *A. tumefaciens* strain LBA4404 plasmid pBAL2 carrying kanamycin resistance and GUS reporter genes under the control of the 35S promoter. The optimum period of cocultivation was 4 days, giving 47.8% transformed calluses (Arundhati, 1999). Transformation of pigeonpea was achieved using *A. tumefaciens* strain GV2260, containing the construct of isolated cowpea proteinase inhibitor gene, *pCPI*. The gene was driven by CaMV 35S promoter containing kanamycin resistance as plant selection marker. Molecular analysis of the putative transformants was done by Northern blotting technique (Lawrence and Koundal, 2001). *Agrobacterium*-mediated transformation of *Vigna sesquipedalis* was achieved using cotyledonary node explants where 2% of the shoots showed integration of *npt*II, phosphinothricin acetyl transferase (*pat*) and *uid*A genes. Integration was confirmed using GUS histo-chemical assay and Southern blot analysis (Ignasimuthu, 2000). Hypocotyl and primary leaves excised from 2-day-old in vitro grown mungbean (*Vigna radiata*) were used for the transformation studies. This particular grain

legume was considered to be highly recalcitrant. A convincing transformation frequency was obtained and the frequency was confirmed using GUS histochemical assay and Southern blot analysis (Jaiwal et al., 2001). Culture and co-cultivation of primary leaves of *Vigna mungo* resulted in transformed calluses that did not regenerate into whole plants although the selected calluses exhibited positive NPTII assay (Karthikeyan et al., 1996). A genomic fragment encoding *Phaseolus vulgaris* arcelin-5a protein that confers resistance to an insect pest *Zabrotes subfasciatus*, along with *npt*II and *uid*A genes were constructed into binary vector which was used to transform *Phaseolus acutifolius* where bud explants were used from genotypes (Dillen et al., 1997a). Callus of *Phaseolus acutifolius* var. *acutifolius*, the tepary bean, was co-cultivated with Agrobacterium tumefaciens strain C58C1Rif. Due to the high regeneration competence of *P. acutifolius*, transformed plants could be raised and transformed seed was obtained. It is suggested that by interspecific hybridization of transformed *P. acutifolius* with the regeneration recalcitrant *P. vulgaris*, introgression of desirable genes into *P. vulgaris* could be achieved. The relevance of this approach with reference to alternative techniques aimed at reducing or omitting the need for in vitro regeneration (e.g. pollen transformation, meristem transformation) is assessed (Dillen et al., 1997b). Epicotyls and nodal explants of *Pisum sativum* were transformed using binary and co-integrate vectors. The transformation frequency was found to be the function of explant source, *A. tumefaciens* strain, pea genotype and duration of co-cultivation (Kathen et al. 1990). Some commercial breeding lines of *Medicago sativa* (alfa alfa) were transformed by *Agrobacterium* method. Stable transgenic material was screened with *npt*II specific PCR amplification and Southern hybridization (Desgagnes et al., 1995). Shoot and leaf explants of non-regenerable *Medicago* sp. were infected with

Agrobacterium and in other experiment transgenic plants were obtained by eloctroporating protoplasts (Kuchuk et al., 1990). *A. tumefaciens* mediated transformation was performed with some members of a population of *Medicago sativa* into which a trait of somatic embryogenesis was incorporated via breeding and the transgenic plants were analyzed by PCR (Du et al., 1994). *A. tumefaciens* mediated transformation of *Lupinus mutabilis* was done using shoot apical explants. A first report in this particular plant, transformants were confirmed with non-radioactive Southern hybridization (Babaoglu et al., 2000). An elite accession, CIAT 184, of an important pasture legume, *Stylosanthes guianesis*, was transformed by *Agrobacterium*-mediated method with binary vector harboring *npt*II and *uid*A genes (Sarria et al., 1994). A rapid and reproducible protocol for *Torfolium subterraneum*, a subterranean clover, was standardized using *npt*II, *uid*A and an alpha-amylase inhibitor gene. The protocol shows that glucose and acetosyringone was required in the co-cultivation medium. Four commercial cultivars were successfully transformed (Khan et al., 1994). Genetic transformation of the broad bean, *Vicia faba*, was done using *A. tumefaciens* and *A. rhizogenes*. Three cultivars and mutants were used for the transformation studies (Jelenic et al., 2000).

Biolistics: Biolistic transformation of *Glycine max* was done using a bovine milk protein, β-casein cloned under seed specific lectin promoter (Maughan et al., 1999). The method of electroporation was used to transform the protoplasts of *Glycine argyrea*. Protoplast colonies developed into callus and 78% develop into transformed shoots that showed *npt*II activity (Jones and Davey, 1991). Embryogenic callus tissue from various cultivars of *Arachis hypogaea* was used for biolistic-mediated transformation. Callus from mature seeds, escape free selection on hygromycin, brief osmotic desiccation followed by

sequential subculture on cytokinin medium are the salient features of this protocol (Livingstone and Birch, 1999). A novel method for transformation of *Cajanus cajan* via biolistic bombardment was developed using a vector containing heterologous oat arginine decarboxylase cDNA that is an important gene in polyamine metabolism. An increase of putrescine levels was found in the transgenic lines (Sivamani et al., 2001). Genetic transformation of *Phaseolus coccineus* and *P. vulgaris* was done using novel nylon micro projectiles. Ethanol co-precipitation method for DNA/particle preparation was superior to that of Ca^{2+}/spermidine (Genga et al., 1992). A novel method of electric discharge particle acceleration was used to transform seed meristem explants of *Phaseolus vlgaris*. 0.03% of confirmed transformed plants were recovered (Russell et al., 1993).

Other methods: Electroporation, microinjection etc. are some methods of genetic transformation of plants that were used less often. Mesophyll protoplasts of *Cajanus cajan* were elctroporated with plasmid construct containing *npt*II gene as selectable marker and the transgenic callus was produced. The transformation frequency was observed to be 30% (Sarangi et al., 1991).

2.2.2 Forage and pasture legumes

Leguminoceae is family of over 18,000 species, which has a distinct economic importance by virtue of processing protein rich seeds and ability to fix atmospheric nitrogen through symbiotic nitrogen fixation. Over 70% of the legumes are forage and pasture legumes (NAS, 1979). Many domesticated and wild animals feed on these forage legumes deriving most of their nitrogen requirements from this class of pasture legumes. Owing to the magnitude of their importance several groups carried out studies on

biotechnological improvement of the same so as to provide better food for the domesticated animals.

2.2.2.1 Organogenesis

Direct organogenesis: A complete in vitro plant regeneration system was developed for azuki bean, *Vigna angularis* where adventitious regeneration was observed from hypocotyls segments of cotyledonary node explants by using BAP (Avenido and Hattori, 2000). Ethylene inhibitors such as silver nitrate, 2,5-norbornadiene and cobalt chloride were shown to enhance the organogenic frequency from the cotyledon explants of cowpea, *Vigha unguiculata* (Brar et al, 1999). Regeneration was achieved using hypocotyls and cotyledonary explants excised from green immature pods of cowpea (*Vigna unguiculata*). A primary polyamine putrescine was also used and some somaclonal variation was also observed (Pellegrineschi, 1997). Morphogenic potentials of shoot regeneration from root explants of *Lotus corniculatus* were exploited to regenerate whole plants. Explants were isolated from 3 different parts of the root from 4, 8 and 16-day-old seedlings and buds were formed on the proximal end of explants, and roots on the distal end. Explants located proximally regenerated more shoots than those originating from the distal end. In the presence of BAP the number of regenerated plants was higher because numerous meristematic zones formed in the secondary cortex. In contrast to the explant response on hormone-free medium, disturbances of explant polarity were observed in the presence of BAP. (Rybczynski et al., 1995). The pre-treatment of immature inflorescence with phytohormones, especially BAP, and culturing on hormone free medium, resulted in shoot bud induction and shoot differentiation, of an important pasture legume *Medicago lupulina* (Li and Demarly, 1995). Preconditioned multiple shoots were obtained by

germinating the seeds of pasture legumes, *Lathyrus cicera*, *L. ochrus* and *L. sativus*. Best multiple shoot frequency was obtained on medium containing 50 µM BAP (Malik et al., 1992). Multiple shoots were induced from shoot tip explants of *in vitro* grown seedlings of *Macrotyloma uniflorum*. Effects of adenine sulfate, BAP and IBA were studied (Varisaimohamed et al., 1999). High frequency regeneration of adventitious shoot buds was observed in *Pisum sativum*. Silver nitrate did not show any promotive effect on multiple shoots but resulted in shoots with well-developed tendrils and large stipules (Ozcan et al., 1992). Young inflorescences of *Ceratonia siliqua* were cultured on MS medium supplemented with BAP with various concentrations of casein hydrolysate for obtaining multiple shoots (Bhalerao and Chinchanikar, 1992). *Lathyrus sylvestris* (flatpea) is an important forage legume especially in acidic soils. Hypocotyl derived multiple shoot system was developed for the clonal propagation of this species (Foster et al., 1991). An efficient micropropagation method was developed for *Sesbania rostrata* by optimizing parameters like variations in the basal medium and photoperiod changes (Pellegrineschi and Tepfer, 1993). Multiple shoot regeneration was observed from immature seedling explants of *Lupinus mutabilis* by using TDZ with modified Schenk and Hildebrandt medium (Rahim et al., 1999). Complete plants of *Lupinus luteus* were regenerated from hypocotyls segments and were efficiently nodulated by *Bradyrhizobium* sp. (Daza and Chamber, 1993). *Anthyllis cytisoides*, a legume shrub used for aforestation and reclamation was successfully regenerated from both juvenile (cotyledonary nodes and apical buds) and mature (axillary buds) explants (Gavidia et al., 1997).

Indirect organogenesis: Plant regeneration was achieved via callus phase using hypocotyls explants of an important forage legume *Astragalus adsurzens*. The

combinations and concentrations of different growth regulators such as 2,4-D, BAP and NAA were shown to be critical factors for both the frequency and the type of callus formation as well as for the potential of callus differentiation (Luo and Jia, 1998). Petiole, stem, leaf and cotyledonary explants of *Medicago sativa* were used to induce callus by using 2,4-D and whole plants were regenerated (Moursy et al., 1995). NAA in combination with BAP was used to regenerate plants via callus phase by using stem, rachis and leaf explants of beach pea, *Lathyrus japonicus* (Debnath et al., 2001). Callus, organogenesis and plantlet formation was observed from seeds of *Clitoria ternatea* in the presence of high kinetin and IAA levels (Lakshmanan and Dhanalakshmi, 1990). Canavanine and canaline were detected in the callus cultures induced with BAP and IAA in *Canavalia lineata* (Hwang et al., 1996). Shoot buds were regenerated either directly or through callus phase from leaf explants of fodder legume *Vigna aconitifolia* and various factors affecting regeneration were also studied (Bhargava and Chandra, 1989). Four species of *Sesbania, S. bispinosa*, *S. cannabina, S. Formosa* and *S. sesban* were regenerated in vitro by using root, hypocotyls and cotyledon explants. Callus was induced with 2,4-D and shoots regenerated with BAP (Zhao et al., 1993). Callus was obtained from mature leaves, stems, petioles and roots of young seedlings of *Psoralea corylifolia* and regenerated to whole plants on BAP containing medium (Saxena et al., 1997). Hairy roots that were induced by infection with *A.rhizogenes* were used for the regeneration of multiple shoots in *Crotalarea juncea* followed by complete plant regeneration and confirmation of their transgenic nature through Southern blot analysis (Ohara et al., 2000). Seventeen accessions of *Medicago polymorpha* were screened for their capacity to produce callus. Hypocotyls proved to be the best for the regeneration of whole plants via callus

phase (Scarpa et al., 1992). Direct and indirect multiple shoot regeneration was reported in the winged bean, *Psophocarpus tetragonolobus*. Shoot tip, epicotyl, hypocotyl and internode explants were cultured on Ms media supplemented with different concentrations and combinations of BAP, NAA and IAA. Plant regeneration was achieved from internode and hypocotyl explants via direct organogenesis and from internode and epicotyl explants via indirect organogenesis depending on the growth regulators (Anjumanara et al., 1998). Callus derived shoot regeneration was achieved from root explants of *Lathyrus sativus* by using various concentrations of kinetin and rooting was done on IBA (Roy et al., 1992). *Macrotilium atropurpureum* is a model plant with broad symbiont range for nodulation. First report of in vitro plant regeneration in this species by using hypocotyls explants was reported by Ezura et al. (2000). A novel method of indirect organogenic regeneration was developed for *Pisum sativum* using thin cell layer segments of nodal explants from which leaflets and axillary buds were removed (Nauerby et al., 1991). Indirect regeneration of shoots via callus phase was obtained in pasture legumes *Lotononis bainesii* (Bovo et al., 1986), *Centrosema brasilianum* (Angeloni et al., 1992), *Desmodium affine* and *D. uncinatum* (Rey and Mroginski, 1997). Various explants like hypocotyls, root and leaves of pasture legume *Stylosanthes guyanensis* were used to regenerate shoots via callus phase (Meijer and Broughton, 1981).

2.2.2.2 Somatic embryogenesis

Five species of *Medicago, M. ciliaris, M. merex, M. orbicularis, M. polymorpha* and *M. truncatula* were assessed for somatic embryo induction with 2,4-D. Incorporation of PEG resulted in better maturation of the embryos (Iantcheva et al., 2001). Callus was induced from shoot-tip cultures of eight species of *Trifolium* and whole plants were

regenerated via somatic embryogenesis of *T. rubens* (Parrot and Collins, 1983). Direct somatic embryogenesis and plant regeneration was obtained from protoplasts of red clover, *Trifolium pratense* (Radionenko et al., 1994). Plants were regenerated by the direct somatic embryogenesis from the cultured embryos of genus *Trifolium* (Repkova, 1991). High frequency somatic embryogenesis and plant regeneration was achieved from the callus cultures of *Astragalus adsurgens* (Luo et al., 1999). A protocol for regeneration via somatic embryogenesis pathway in the pasture legume *Clitoria ternatea* was developed. Manipulation of kinetin in combination with IAA was found to be useful (Dhanalaskhmi and Lakshmanan, 1992). Methyl jasmonate and Abscisic acid were used generally for the maturation of induced somatic embryos. However, their application in the somatic embryo induction medium was tested and they were found to be inhibitory to the embryo induction. Somatic embryo induction in *Medicago sativa* was also found to be inhibited by aminoethoxyvinylglycine, amino-oxyacetic acid, 2,4-dintrophenol and salicylic acid (Meijer and Brown, 1988). With a view of obtaining plants free from neurotoxin, a quick and efficient system was developed for regenerating plants by using explants from wide range of tissues of *Lathyrus sativus*. An embryo rescue method was also described to facilitate inter-specific hybridizations (Misra et al., 1994). Immature cotyledons of *Vigna sinensis* gave rise to somatic embryos on 2,4-D and BAP-containing medium, which eventually developed into whole plants (Li et al., 1995). Treatment with 2,4-D followed by BAP treatment regenerated whole plants via an exuberant somatic embryogenesis from leaf sections of *Medicago suffruticosa* (Li and Demarly, 1996).

2.2.2.3 Other methods

Protoplasts were isolated from immature cotyledons of *Glycine soja*. These protoplasts started to divide after 3 days of culture and the division frequency of protoplast-derived cells counted at 12 days was 36.8%. Shoot buds were regenerated on the surface of the nodular calluses with a frequency of 25% when the calluses were placed on MSB medium with IAA and BAP. Whole plants were regenerated upon transfer of 3-4 cm shoots to 50% MS medium with IBA (Wei and Xu, 1990). A breakthrough in the plant regeneration from the protoplasts isolated from *Vicia faba* and *V. narbonensis*. Protoplasts of 10 cultivars were isolated from etiolated shoot tips and tested for their regeneration capacity. After purification, protoplasts were embedded in sodium alginate and cultured in the medium containing 2,4-D, NAA and BAP. Division frequencies of up to 40% were obtained. Six weeks after embedding, protoplast-derived calluses were transferred to Gelrite-solidified media with different combinations of growth regulators (Tegeder et al., 1995). Low voltage treatment and nurse cells from *Medicago sativa* were used to regenerate callus from protoplasts isolated from seedling and suspension cultures of *Trifolium subterraneum* (Li et al., 1990). A protocol for the isolation of root protoplasts from *Vigna radiata* and leaf mesophyll protoplasts of mothbean, *V. aconitifolia* gave a plating efficiency of 1.3% and 2.81% respectively. Shoot meristemoids developed only from mothbean, into shoots and later into whole plants (Avenido et al., 1993). Genotype dependent protoplast regeneration into whole plants was observed in red clover (*Trifolium pratense*). Protoplasts were derived from leaf and suspension cultures of the cultivar (Myers et al., 1989). Cell suspension cultures were grown and plantlets were regenerated in *Indigofera enneaphylla* (Bharal and Rashid, 1984). In vitro conditions for plant

regeneration from protoplasts and callus cultures of *Hedysarum coronarium* were optimized (Arcioni et a., 1985). Protoplasts were isolated from mesophylls of *Medicago sativa* and various phytohormones tested for a better frequency of regeneration (Kim and Choi, 1989). Protoplasts of *Lotus corniculatus* were isolated using pre-plasmolysation of green cotyledons in CPW salts containing 13% mannitol. Plantlets were also regenerated with two lines being somaclonal variants (Vessabutr and Grant, 1995). Callus and protoplast cultures were used to regenerate plantlets of *Medicago* and leaves of *Medicago littoralis*, an annual legume resistant to fungus *Pseudopeziza medicaginis*. Plantlets were regenerated on medium containing 2-iP combined with IAA, and/or BAP with NAA (Zafar et al., 1995). Plant regeneration from cotyledon protoplasts was achieved in *Medicago sativa* cv. Krasnovodopadskaya 8 by culturing cell aggregates on agar medium after the immobilization of protoplasts in agarose. Regeneration was also achieved from tissue cultures of the wild species *M. prostrata, M. orbicularis, M. trautvetteri, M. borealis, M. caerulea, M. rigidulla* and *M. falcata* (Svanbaev, 1991). Cotyledon protoplasts served as useful tools for regeneration of *Sesbania bispinosa.* In a liquid-over-agar culture system with MS medium supplemented with 2,4-D, BAP, glutamine and mannitol, 84% of these protoplasts divided and formed callus. Callus formed from the protoplasts differentiated shoots on MS medium supplemented with IBA and BAP. These shoots developed into complete plantlets when excised and cultured on MS containing IBA (Zhao et al., 1995). Protoplasts derived from 3 species of *Stylosanthes* were cultured in K8P medium at densities of 5 X 104/ml and 1 X 105/ml Protoplast-derived colonies transferred to MS medium supplemented with combinations of NAA and BAP formed compact, green microcalluses. Shoot regeneration, which occurred after 28-56 days of culture, was by

organogenesis rather than somatic embryogenesis, with leaves and stems developing directly on the callus surface (Vieira et al., 1990). Protoplasts were isolated from epicotyls and shoot tips of *Vicia narbonersis* etiolated seedlings. They developed into plantlets via somatic embryogenic pathway when cultured on less auxin medium, and via organogenic pathway when cultured on TDZ containing medium (Tegeder et al., 1996).

Cotyledonary explants of *Sesbania grandifolia* were irradiated with gamma rays for callus growth and regeneration. Cytogenetic studies showed distinct chromosomal aberrations (Sinha and Mallick, 1993). Enhanced shoot regeneration was observed using homogenized callus of *Lotus corniculatus* (Orshinsky et al., 1983).

2.2.2.4 Genetic transformation

Agrobacterium-mediated transformation: A rapid and reproducible protocol for transformation of *Lotus corniculatus* by using cinnamyl-alcohol dehydrogenase (CAD) was developed for the purpose of lignin reduction. The transgenics were confirmed by the polymerase chain reaction (PCR) and CAD activity. The gene was derived from *Aralia cordata* (Akashi et al., 1998). Three cultivars of *Medicago sativa* and one cultivar of *Onobrychis viciifolia* were evaluated for their response to inoculation with *A. rhizogenes* strain A4T (containing pRiA4b). A cultivar-dependent response was observed in *M. sativa* with 94%, 25%, and 4% of infected stem explants producing transformed roots in the cultivars Vertus, Regen-S, and Rangelander, respectively. In *O. viciifolia* cv. Hampshire Giant, an explant-dependent response was observed with 78% and 50% of seedling cotyledon and hypocotyl explants responding, respectively (Golds et al., 1991). An accession-dependent genetic transformation was observed when *Glycine canescens* and *Glycine clandestina* were transformed with *A. rhizogenes* (Rech et al., 1988). The

transformants were identified by synthesis of opines in the shoots. An important fodder legume, *Astragalus sinicus* was transformed by *A. rhizogenes* and the *uid*A gene activity was confirmed (Cho et al., 1998). The regenerants exhibited Ri-plasmid syndrome (small thin leaves and short internodes) and 55% of the seedlings showed GUS activity and positive gene integrations. Genetic transformation of the pasture legume and the model plant for studying *Rhizobium* legume symbiosis, *Medicago truncatula* was reliably transformed with binary vector harboring *npt*II and *uidA* and NoS and CaMV 35S promoters respectively. The T2 generation of the plants showed Mendelian inheritance of the integrated transgenes (Wang et al., 1996). A rapid regeneration system from cotyledonary node explants was used for stabbing and injection co-cultivation of *Agrobacterium*, in *Pisum sativum* (Jordan and Hobbs, 1993). Genetic transformation via somatic embryogenesis pathway with immature leaflets and nodal explants of pasture legume *Lathyrus sativus* was reported by Barna and Mehta (1995). Mature de-embryonated cotyledons with intact proximal end of *Vigna unguiculata* were used for *Agrobacterium* transformation. Over 15% of the shoots were selected on selection medium. Integration of *hpt* gene was detected by using Southern blot analysis (Muthukumar et al., 1996). Ri plasmid and disarmed Ti plasmids were used to transform *Medicago truncatula*. Genes of Ri plasmid negatively interfered with somatic embryogenesis. Only Ri plasmid with an inactivated *rol A* gene regenerated transgenic plants (Thomas et al., 1992). *Lotus japonicus* is a model plant for *Rhizobium* host interactions. *A. rhizogenes* mediated transformation was performed and hairy root formation was observed. Most virulent strains *A. rhizogenes* for this species were also found (Stiller et al., 1997). *Lotus corniculatus* and *L. tenuis* were transformed with *A.*

rhizogenes for the integration of *hpt* gene (Damiani et al., 1993). An efficient protocol for *A. rhizogenes* transformation was developed for *Lotus angustissimus*. This was receded by an efficient regeneration system of indirect organogenesis using hypocotyl, leaf, stem, cotyledon and root explants (Nenz et al., 1996). Bean yellow mosaic virus resistant transgenics were obtained by transformation of *Trifolium subterraneum* with various segments of virus coat protein (Chu et al., 1999). Rapid and efficient transformation of *Medicago truncatula* and *M. sativa* was done with two early nodulin and late nodulin genes where plant regeneration occurred through somatic embryogenesis (Trinh et al., 1998). Two different regeneration systems were used to obtain transformed plants of *Medicago falcata*. The *A. tumefaciens* inoculated plants were regenerated via direct and indirect somatic embryogenesis pathways (Shao et al., 2000). *Medicago truncatula* was transformed with *A. tumefaciens* using *pat* selection marker. Transformants could be obtained in just 2.5 months (Trieu and Harrison, 1996).

Biolistics: Plasmids containing *nptII* gene under CaMV35S promoter were used for particle bombardment experiments into Lucerne calluses derived from petiole and stem sections of *Medicago sativa*. Analysis of transgenic plants showed integration while progeny showed 1:1 Mendelian segregation ratio (Pereira and Erickson, 1995).

2.3 Insect resistance management

World-wide crop losses without the use of pesticides and other non-chemical control strategies is estimated to be about 70% of crop production, amounting to US $ 400 billion. The world-wide pre-harvest losses due to insect pests, despite, the use of insecticides is 15% of total production representing over US $ 100 billion (Krattiger, 1997). The annual cost of insect control itself amounts to US $ 8 billion, thus warranting

urgent economical control measures. Many of the crop varieties developed in the past 30 years were high yielders, but had poor storage characteristics (Kerin, 1994). Insect pests are capable of evolving to biotypes that can adapt to new situations; for instance, they overcome the effect of toxic materials or bypass natural or artificial plant resistance, which further confounds the problem (Roush and McKenzie, 1987). An integrated pest management (IPM) program, comprising a combination of practices including the judicious use of pesticides, crop rotation, field sanitation and above all exploitation of inherently resistant plant varieties would provide the best option (Meiners and Elden, 1978). The last option includes the use of transgenic crops, expressing foreign insecticidal genes, which could make a significant contribution to sustainable agriculture and thus could be an important component of IPM.

2.3.1 Methods of control other than biotechnology

Insect resistance management is not a concept evolved from a single method of operation, but an integrated approach, as opined by many others. Classification of methodologies has taken a different turn with advent of biotechnology. Biotechnological control conceptually is a part of biological control method, however, separated itself from other methods owing to vast literature and logical base it has acquired. Hence this section describes all the methods other than biotechnology, which have been an integral part of Integrated Pest Management.

2.3.1.1 Ecological control:

This is a strategy in which various methods are employed. It is somewhat similar to the "biointensive" control described by Frisbie and Smith (1991) that rely mostly on

natural biological controls with prescriptive chemical input as last resort. The strategy is primarily based on understanding the interaction of pest with its environment, in the present context, agro-ecosystem, defined as the effective environment at the crop level (Altieri, 1994), or at the level of local landscape (Duelli, 1997; Collins and Qualset, 1999). It is defined that an insect becomes a pest when general equilibrium population exceeds economic injury level (EIL) (Higley and Pedigo, 1996). Exploitation of species diversity to decrease the herbivore population was done effectively (Altieri, 1994). Biodiversity increase in agro-ecosystems results in development of more internal links within the food webs resulting in fewer pest outbreaks (Altieri and Nicholls, 1999). These understandings led to some practical approaches such as poly harvesting (Francis, 1990) and interplanting (Altieri and Whitcomb, 1980) are potential eco-control measures.

2.3.1.2 Physical control:

The methods in this strategy are relatively ancient, but stood for ages, as they are farmer and eco-friendly while being easy-to-use. They require lesser scientific knowledge and are based on physical destruction of insect populations. These have attracted considerable attention in the recent past because of the development of effective food and visual attractants (Lindgren and Fraser, 1994). Understanding the insect behavior is a crucial prerequisite for making traps (Alm et al, 1994). Usage of baits such as pheromones and novel trap designs made insect trapping devices attractive tools in the industrial point of view (Dowdy and Mullen, 1998). Usage of some sort of banding material especially around the trunks of the trees had some degree of antagonist effect on insects (Raupp et al, 1992). Live stock insect traps (Tozer and Sutherst, 1996), colored traps (Vernon and Broatch, 1996), and fermentation traps (Norris, 1933) are some of the effective

applications of traps. Technically more advanced methods such as light traps (Pickens and Thimijan, 1986), electrocuting traps (Gilbert, 1984) micro irradiations (Biron et al., 1996), gamma irradiation (Metcalf and Metcalf, 1993) and other irradiations, temperature, controlled atmospheres with CO_2 in combination with other gasses and various other physical methods designed at the convenience of the user and type of insect pest are being widely used in various industrial and domestic environments.

2.3.1.3 Chemical control:

Use of chemicals to fight insects, dates back to 1200 BC. This is the most popular but eco-unfriendly strategy. Global market for insecticides was estimated to be over 20 billion in 1989 (Environment Protection Agency, 1989). Wide varieties of insecticides were discovered due to their instant results despite their adverse effects on the integrity of the environment. To name a few, organochlorines (DDT, BHC), organophosphates (Carbophenothion), Carbamates (aldicarb, carbaryl), pyrethroids (allethrin, cyalothrin), avermectins are some of the widely used insecticides. Many plant based insecticides have been used for ages, such as pyrethrin, rotenone, azadirachtin etc. Keeping aside their popularity, insecticides usage is highly alarming as they negatively affect many of the living things living in their respective ecosystems. These concerns have been published by many authors, emphasizing the adverse effects of the insecticide use (Rand, 1995; Edwards et al., 1996). Their effect on soil microorganisms (Domsch, 1983) microorganisms of the aquatic environments (Parr, 1974; Gurney and Robinson, 1989), soil inhabiting invertebrates (Edwards and Thompson, 1973), nematodes (Yardim and Edwards, 1998), alukatic invertebrates (Brown, 1978), fish, amphibians, birds (Hardy, 1990) and many other loving components has been a point of serious concern. Despite many precautions

based on these reports, an estimate of over 1 million people worldwide is poisoned, and over 15,000 eventually die.

2.3.1.4 Biological control:

Various pest control strategies have been developed where each one providing partial answer to the singular or a set of pest problems. 1940s witnessed discovery and application of wide array of chemical pesticides and people thought a "panacea" has evolved for all pest problems. This seemed true till it was logically disproved firstly by Carson (1962) and was repeatedly emphasized by various authors in the following years. For example, an estimated 7% of crops were destroyed by insect pests prior to 1940s, and by late 1980s crop destruction has risen to 13% despite a 12-fold increase in the pesticide use (Environmental Protection Agency, 1989). These alarming statistics diverted the pest management strategies to opt for age-old control practices such as physical and biological methods.

Definitions of biological control varied through ages, and one of the latest included the use of predators, parasitoids, pathogens, pheromones, and natural plant products. According to this definition biotechnological approach becomes an integral part of biological control. Oldest known method was colonization of ants in china and Yemen (Coulson et al., 1982). There are three basic types of biological control, namely, conservation, introduction and augmentation (Waage and Mills, 1992). Introduction of natural enemies becomes a necessity when there is a sudden outburst of foreign insect pests, which are not native to a particular region. In such cases their natural enemies are imported, multiplied and introduced (Waage, 1996). Augmentation is increasing the natural enemy numbers from the existing populations, by producing and multiplying them

in the laboratory and releasing them (Hoffmann et al., 1998). Pathogens used for biological control include bacteria, fungi, viruses, protozoans and nematodes. Mass production and marketing of these agents are relatively easy compared to the other two (Cook et al., 1996). Over 700 species of fungi were believed to infect insects (Jaronski, 1997); for example, *Beauveria bassiana* is a potential biological control agent of many arthropod insects (Maddox, 1987; Georgis, 1992). The method of using bacteria for biological control is relatively dominated by *Bacillus thuringiensis* species and the δ-endotoxins released by them, molecular genetics of the resistance mechanism has been the order of the day in the recent biotechnological approaches.

2.3.2 Biotechnology for insect resistance

One of the methods of biotechnology, marker assisted selection could not cater to the imminent needs of the IPM specialists, as resistance phenotypes and their QTLs could not be conclusively identified. Hence there was a need of short cut methods to combat the worst biotic constraint, insect pests. Discovery of δ-endotoxins produced by the famous *Bacillus thuringiensis* gained considerable significance in the recent past. Along with *B. thuringiensis* many other genes conferring resistance partially have been discovered out of which protease inhibitors are of significant importance.

2.3.2.1 Bt: An Amazing Concept: Perhaps most widely used entomopathogen is the bacterium *Bacillus thuringiensis* Berliner. Many sub-species of this bacterium release wide array of larvicidal δ-endotoxins that confer resistance to various insects. Other species of *Bacillus*, like *B. sphaericus* (Porter et al., 1993) and some anaerobic bacteria such as

Clostridium (Barloy et al., 1998) were identified to pocess potential mosquito larvicidal properties.

Bacillus thuringiensis subsp. *israeliensis* (Bti) was the first subspecies of Bt that was found to be toxic to Deptera larvae (Margalith, 1990). It was also found to be effective against many species of mosquitoes and black fly larvae. Extensive studies were carried out to demonstrate the toxicity of these organisms towards mammals and it was not found to be highly host specific thus ensuring its safety (Murthy, 1997). It was also proven to have an expanded host range such as snails, some human and avian parasites and few other insect pests (Saraswati and Ranganathan, 1996).

The toxin proteins and their genes: The family of insecticidal crystal proteins (ICPs) is normally associated with larger plasmids of various sizes ranging between 5 to 210 kilobase pairs that have been broadly classified as Cry and Cyt δ-endotoxins(Lereclus et al., 1993). Though these toxins are not related structurally, they are functionally related in their membrane permeating activities. All insecticidal δ-endotoxins are initially synthesized as a larger protoxin that is eventually cleaved at specific sites to form the actual toxin. In total there are seven classes of Bt insecticidal proteins, but four proteins and few accessory proteins and their molecular genetics associated with Bti are well characterized. The major four proteins are localized in parasporal crystalline endotoxins synthesized during sporulation and they are synthesized by respective genes: *BtCry1Aa*, *BtCry11A*, *BtCry4B* and *BtCry4A* (Federici et al., 1990). Much of the mosquitocidal properties is attributed to the synergistic interactions of these four proteins, but the whole crystal was found to be much more toxic (Crickmore et al., 1995). Wealth of information was accumulated regarding the molecular biology, biochemistry and structural biology and

have been exhaustively reviewd (Dai and Gill, 1993; Douek et al, 1992; Wirth et al, 1997; Guerchicoff et al, 1997; Purcell and Ellar, 1997). Two accessory proteins namely P19 and P20 that are required in the assembly of an inclusion body have also been characterized (Wu and Federici, 1993; Thiery et al, 1997; Manasherob et al, 1997).

Mode of action: Early studies showed that the primary target of Bti toxicity is the midgut epithelium, where enzymatic systems transforms the protoxin tnto an active toxin under alkaline conditions (Al-yahyaee and Ellar, 1995). These toxins act coordinately and synergistically to distupt the epithelial cells of the larval gut where midgut cells vacuolise and lyse (Lahkim-Tsror et al., 1983). These symptoms are more or less same for toxins of all Bt strains other than Bti. Though Cry and Cyt toxins are structurally dissimilar, they have the similar membrane-permeating ability, where Cry toxins bind to membranal proteins and *BtCyt1Aa* binds to unsaturated phospholipids acting as "binding sites" (Feldmann et al., 1995; Gill et al., 1992).

Mode of action takes place in two steps; binding to a cell receptor and subsequent pore formation (Knowles and Ellar, 1987). Soon after conversion of proto-toxin into active toxin by gut proteases, the toxin is distinguished into two domains (Domain I and Domain II) where Domain II binds to a brush border membrane receptor, acts as an anchor, while Domain I inserts itself into the membrane forming a pore (Dean et al., 1996; Flores et al., 1997). Binding of the toxin becomes irreversible after the insertion of Domain I into membrane (Chen et al., 1995). α4 and α5 helices insert into the membrane and α7 serves as sensor to initiate the structural rearrangement of the inserting domain (Gazit and Shai, 1998). Many other functions of segments of the toxin have been elucidated. It was found that substitution of glutamine at 149 by proline in the center of helix 4 results in complete

loss of toxin activity (Uawithya et al., 1998). With reference to cytosolic (Cyt) toxins Cyt 1Aa was studied in detail. It was found that plasma membrane liposomes containing phospholipids are the target of this toxin (Thomas and Ellar, 1983). Toxin binding leads to a detergent-like rearrangement of the bound lipids, resulting in hypertrophy disruption of the membrane integrity and eventually cytolysis. Pore formation was observed prior to cytolysis (Gill et al., 1992). The 24 kDA active toxin was found to be three times active than the protoxin (Butko et al., 1996). Another interesting aspect of the mode of action is that the toxin monomers display a synergistic action towards specific insects. Different combinations of monomers of respective δ-endotoxins rearrange among themselves synergistically exerting some host specific action (Crickmore et al., 1995) and this was demonstrated by cloning a combination of *cry* 4A and *cry* 11A of Bti into *E.coli* (Ben-Dov et al., 1995).

2.3.2.2 Genes employed for insect resistance other than Bt:

Protease Inhibitors:

The production of transgenic crops has seen rapid advances during the last decade with the commercial introduction of *Bt* transgenics. However, the major concern with these crops has been the development of resistance by pest and public acceptability. Hence, there has been a need to discover new effective plant genes, which would offer resistance and protection against these pests. Protease inhibitors (PIs) are one of the prime candidates with highly proven inhibitory activity against insect pests.

Plant protease inhibitors: The possible role of protease inhibitors (PIs) in plant protection was investigated as early as 1947 when, Mickel and Standish observed that the

larvae of certain insects were unable to develop normally on soybean products. Subsequently the trypsin inhibitors present in soybean were shown to be toxic to the larvae of flour beetle, *Tribolium confusum* (Lipke et al., 1954). Following these early studies, there have been many examples of protease inhibitors active against certain insect species, both in in vitro assays against insect gut proteases (Pannetier et al. 1997; Koiwa et al, 1998) and in in vivo artificial diet bioassays (Urwin et al. 1997; Vain et al. 1998). The term "protease" includes both "endopeptidases" and "exopeptidases" whereas, the term "proteinase" is used to describe only "endopeptidases" (Ryan, 1990). Several non-homologous families of proteinase inhibitors are recognized among the animal, microorganisms and plant kingdom. Majority of proteinase inhibitors studied in plant kingdom originates from three main families namely leguminosae, solanaceae and gramineae (Richardson, 1991).

A large number of protease inhibitor genes with distinct modes of action have been isolated from a wide range of crop species. Development of transgenic crops have come a long way from the first transgenic developed by Hilder et al. (1987). Considering the high complexity of protease/inhibitor interactions in host pest systems and the diversity of proteolytic enzymes used by pests and pathogens to hydrolyze dietary proteins or to cleave peptide bonds in more specific processes (Graham et al. 1997), the choice of an appropriate proteinase inhibitor (PI) or set of PIs represents a primary determinant in the success or failure of any pest control strategy relying on protease inhibition. Firstly, the choice of suitable PIs should be based on a detailed understanding of the biological system assessed. Resistant biotypes of insects may evolve after prolonged exposure to selection pressure that is mediated by an insecticidal protein or plant resistance gene (Sparber, 1985). Second

point to consider would be the targeted expession of PIs in response to pest attack. This will be controlled by using inducible promoters, such as those of PI-I185 and TobRB7, that are activated at the site of invasion by pests, pathogen and nematodes, respectively (Opperman et al.1994). An ideal promoter should be highly responsive to invasion of the host plant by a pest, or regulated by inducers just prior to pest attack. The promoter should be sufficiently active to mediate a substantial defense, specially localized to the site of pest invasion (Babiychuk et al. 1997). Despite these promising developments, the general usefulness of recombinant PIs in plant protection still remains to be demonstrated.

Insect resistance genes other than *Cry* class of genes transferred to crop species

alpha-Amylase inhibitors	*Lectins*
Bean	Snowdrop lectin—GNA
Cereal	Pea lectin
Protease inhibitors	Wheat germ agglutinin—WGA
Soybean (serine protease)	Jacalin
Barley (trypsin)	Rice lectin
Squash (trypsin)	*Others*
Cowpea (trypsin)	Bean chitinase
Mustard (serine protease)	Tobacco peroxidase
Rice (cysteine protease)	Tomato chitinase
Potato (protease inhibitors I and II)	Tryptophan decarboxylase
Soybean (Kunitz trypsin inhibitor)	*Animal genes*
Tomato (protease inhibitors I and II)	Various enzyme inhibitors

3.0 MATERIALS AND METHODS

3.1 Plant material and culture conditions:

Mature seeds of chickpea (*Cicer arietinum* L.) cultivar C-235, a widely grown cultivar in India, were surface sterilized with 70% (v/v) ethanol for 1 min., 0.1% mercuric chloride for 10 min., and rinsed 5 times in sterile distilled water prior to soaking overnight. The de-coated seeds were kept for germination on Murashige and Skoog's (1962) medium (MS; see appendix I), or on a medium specified for the explant preparation. Culture media were used as liquid or as semi-solid having 0.8% (w/v) Difco-Bacto agar as required and pH was adjusted to 5.8 unless otherwise mentioned. All the tissue cultures were maintained at 26±1°C under continuous cool white light provided by fluorescent lamps (30 $\mu Em^{-2}S^{-1}$).

3.2 Regeneration

Experiments were conducted for the development of efficient protocols for regeneration of whole plants via somatic embryogenic and organogenic pathways. Adventitious regeneration of multiple shoots from the selected explants was the main aim of the conducted experiments that could eventually be applied for genetic transformation.

3.2.1 Somatic embryogenesis:

3.2.1.1 Preparation of various explants:

Various explants were derived from seedlings of different ages and conditions and the mature embryo axis was obtained from the overnight soaked and de-coated seeds, which was considered as 0-day-old seedling.

Mature embryo axis: This can be considered as the 0-day-old seedling. The seeds were soaked overnight and the seed coat was removed the following morning. The cotyledons were split open and the embryo axis was removed carefully and cultured on the embryo induction medium (EIM) that consisted of 10 µM 2,4,5-T and 2 µM kinetin.

Parts of mature embryo axis: Mature embryo axis was isolated as described above and five parts namely plumule, radicle, side arms and the middle portion of it were excised as shown in the figure (Fig. 3.1) and cultured on (EIM).

Leaflets: Young and juvenile leaflets, semi-mature leaflets from the middle portion of the seedling and mature leaflets from the basal parts of the seedling were excise from 7-day-old seedlings and cultured on the induction medium (EIM).

Stem segments: The overnight grown seeds were de-coated on the following morning and the seeds were germinated on MS medium. Stem segments devoid of meristematic zones from 7-day-old seedlings were excised and cultured on the induction medium.

Root segments and root tip: Root segments and root tip were excised from 7-day-old seedlings and cultured separately on the induction medium.

3.2.1.2 Induction of somatic embryos:

Synthetic auxins, 2,4,5-T and 2,4-D are the basic components of the embryogenic induction media. All the media were tested on mature embryo axis explant for optimization. The induction media were two types. One 2,4,5-T based and the other 2,4-D based. The former was named as JEM series and the later was named as JDM series. In JEM series the 2,4,5-T was added at 2, 5, 10 and 15 µM and in each treatment variable concentrations of TDZ at 0.5 and 1.0 µM, BAP at 0.5 and 1.0 µM, zeatin at 0.5, 2.0 and 5.0 µM and kinetin at

0.5, 2.0 and 5.0 µM were added. In JDM series, 2,4-D was added at 5, 10, 15 and 20 µM and in each treatment variable concentrations of TDZ at 0.5 and 1.0 µM, BAP at 0.5 and 1.0 µM, zeatin at 0.5, 2.0 and 5.0 µM and kinetin at 0.5, 2.0 and 5.0 µM were added.

Best medium for induction of maximum number of explants was MS with 10 µM 2,4,5-T and 2 µM kinetin (JEM 29; Table 4.2). All the explants described above were cultured on JEM 29 medium to test their efficacy for induction of embryos. The medium was prepared either in a solid or liquid form. Solid medium was poured in the petri plates, and the induction with liquid medium was done in culture tubes. For the later a sterile filter paper bridge was immersed in the liquid medium and the explants were placed on the bridge.

3.2.1.3 Maturation of somatic embryos:

Prolific induction of embryos was achieved in chickpea with various explants. However, a successful somatic embryogenesis protocol is characterized by good maturation frequency. For this purpose, various media and methods were attempted.

The induced embryos were transferred to the maturation media either singly or in bunch. Often the whole explant bearing the immature embryos were transferred to the embryo maturation medium. A number of maturation media were tested that included various plant growth regulators, ABA (5, 10, 15 and 20 µM), GA_3 (5, 10, 15 and 20 µM), TDZ (2, 5 and 10 µM), BAP (2, 5 and 10 µM), zeatin (5, 10 and 15 µM), and kinetin (5, 10 and 15 µM). The combinations were termed as JCM series and all the media are described in detail in the results section.

Since most of the efforts for maturation and conversion of the induced embryos were unsuccessful some additional mechanical methods were also tested.

Cold shock: The explants bearing the embryos were placed on MS containing 5 µM ABA and 10 µM GA_3 and the plates were placed at 4 °C for 1, 3, 5, 7 and 9 hours on fresh medium with same components.

Heat shock: The explants bearing embryos were placed on the medium as mentioned above and were incubated as separate batches at 40 °C and 50 °C for 30, 60, 90 and 120 minutes followed by their transfer to fresh medium and incubation in the culture room.

Liquid agitation: The above-mentioned media were prepared in liquid form and the explants were gently placed in the liquid medium with a density of 10 explants per 100 ml flask. They were agitated for 2, 4, 8, 10 hours, blotted on sterilized filter paper and were subsequently cultured on the solid medium having same composition.

3.2.2 Direct and Indirect Organogenesis:

3.2.2.1 Preparation of explants:

Type of explant is crucial factor for the achievement of suitable regeneration for genetic transformation experiments. Various explants from seedlings of different age, culture and processing conditions were prepared.

Mature embryo axis: The seeds were soaked overnight and the seed coat was removed the following morning. The cotyledons were split open and the embryo axis was surgically excised and cultured on multiple shoot induction medium that had MS with BAP within a range of 5 to 100 µM in combination with 2 or 5 µM kinetin.

Shoot tip: The de-coated seeds were germinated on MS for 2 days, the shoot tip (2 mm) was then excised surgically and cultured on the shoot induction medium (SIM) that consisted of MS with 4 µM TDZ, 10 µM 2-iP and 2 µM kinetin.

Leaflets: Young and juvenile leaflets, semi-mature leaflets from the middle portion of the seedling and mature leaflets from the basal parts of 7-day-old seedling were separated and cultured on SIM.

Leaf base: The de-coated seeds were germinated on MS for 7 days prior to obtaining the leaf explant. Leaf base (petiole base) that measured about 3 mm was excised and cultured on the SIM.

Stem segments: The overnight grown seeds were de-coated on the following morning and germinated on MS. Stem segments devoid of meristematic zones from 7-day-old seedlings were excised and cultured on SIM.

Hypocotyl: The overnight soaked and de-coated seeds were germinated on MS and 4 to 5-day-old seedlings were selected for deriving the explant. The stem part that was just above the cotyledonary node junction was excised and the shoot tip region was removed. The explant that measured about 4 mm was cultured on SIM.

Epicotyl: The overnight soaked and de-coated seeds were germinated on MS and 4 to 5-day-old seeds were selected for obtaining the explant. The upper most part of the tap root, just below the cotyledonary node junction was excised and the explant measuring 4 mm devoid of root tip region was cultured on the induction medium.

Root segments and root tip: Root segments and root tip were excised from 7-day-old seedlings and cultured separately on the induction medium.

Axillary bud: Two types axillary bud explants were prepared. The major variation for preparation of these explants was their germination pattern. The first one, designated as AB1, was prepared by excising the axillary bud from a 7-day-old seedling that was germinated on MS medium. This axillary bud measured around 1 to 1.5 mm in length. The second one,

designated as AB2, was prepared by excising the axillary bud from a 6 to 7-day-old seedlings that were germinated on MS containing 5 μM thidiazuron (TDZ). This axillary bud measured approximately 2 to 3 mm

Cotyledonary node: The overnight soaked and de-coated seeds were germinated on MS. 3-day-old seedling was taken and the shoot tip, root tip and cotyledons were removed. The cotyledonary node junction that measured 3 to 4 mm was cultured on SIM.

Axillary meristem explants: Explant preparation and orientation on the culture medium is crucial for optimum organogenic response. Owing to the differences in the germination pattern, age of the seedling and processing, four types of the axillary meristem explants were prepared and they were named as AM1, AM2, AM3 and AM4 (see Fig 3.2 for diagrammatic representation of explant preparation). Their preparation is as follows.

AM1 explant: Seed coat of the surface sterilized and overnight soaked seeds was removed and the de-coated seeds were germinated on plain MS. 2-day-old seedlings were selected and root portion was removed by leaving some of the hypocotyl region. Then two cuts were made through the axillary meristem. This resulted in three explants that included two axillary meristem, one shoot tip with epicotyl. The axillary meristem explants with cotyledon intact was named as AM1 and was cultured on the induction medium.

AM2 explant: The de-coated seeds were germinated on SIM and after prominent appearance of axillary buds in about 4 to 5 days, they were carefully removed and two cuts were given through the axillary meristem, discarding the root portion. The resultant axillary meristem explants with cotyledons intact (AM2) were cultured on the above-mentioned SIM again.

AM3 explant: This was a by-product during the preparation of AM2 explant as indicated above. Removal of AM2 explants resulted in shoot tip with epicotyl region. Further removal of shoot tip provided the AM3 explant that contained axillary meristem on either side of the epicotyl and was cultured on SIM.

AM4 explant: The de-coated seeds were germinated on SIM at a density of 10 to 15 per plate. They were grown for about a week until axillary bud was prominent. Then the axillary bud was removed up to the base two cuts were given as in the case of preparation of AM2 explant. The resultant axillary meristem explants were sub cultured on the same medium for another 6-7 days. The base of axillary bud enlarged and some multiple shoot buds emerged. The emerging shoot buds were removed again by carefully scrapping the shoot buds with a sharp blade. The resultant explant containing cotyledon with a bulge on the cotyledonary node region was cultured on hormone free MS.

3.2.2.2 Induction of multiple shoots:

The media for induction of multiple shoots from the given explants can be divided in two major classes. 1. TDZ-based and 2. BAP-based. These consists of either TDZ or BAP as principal multiple shoot inducting cytokinin and some accessory cytokinins and auxins were included in the media as per the requirement. The other cytokinins and auxins added were 2-iP (5 and 10 μM in TDZ-based media), kinetin (2 and 5 μM in TDZ-based and 2 and 4 μM in BAP-based media combinations). Optimization of the induction medium was done initially with mature embryo axis and later with axillary meristem explant, AM2. Unless otherwise mentioned, the explants were cultured on the induction medium for about 18 to 20 days after which they were transferred to the elongation medium. An average of 6 to 8 explants were cultured per plate and the responses were studied.

Effect of pH on multiple shoot induction: The shoot induction medium (SIM) was prepared with pH variations of 4.0, 4.5, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0 prior to autoclaving. Solidification of the medium was carefully monitored and the prepared AM2 explants were cultured on the SIM with above-mentioned pH variations.

Role of cotyledonary tissue: Complete or partial inclusion of cotyledon with the regenerating explant, on multiple shoot induction frequency was studied. The AM2 explants were prepared according to the procedure mentioned above and the cotyledon portion was surgically excised either completely or partially (half) and one set of explants were cultured with inclusion of complete cotyledon. The resulting explants were cultured on SIM with a density of 6 to 8 explants per plate.

Comparative multiple shoot induction from different explants: Mature embryo axis, shoot tip, axillary bud, AM1, AM2, AM3 and AM4 explants were prepared according to the procedures mentioned in the section 3.2.2.1 and cultured on SIM. Number of responding explants and number of multiple shoots per explant were recorded in the third week just before their transfer to the shoot elongation medium.

Age of the seedling: Basing on the explant preparation strategy, different explants were derived from seedlings of various ages. AM1, AM2, AM3 and AM4 explants were prepared according to their respective procedures mentioned above from 2, 4, 6, 8, 10, 12, 14 and 16 day-old seedlings. The resulting explants were cultured on SIM.

3.2.2.3 Shoot elongation:

The shoot elongation medium (SEM) basically consisted of lowered concentration of plant growth regulators, when compared to the induction medium. Various plant growth regulators used for this purpose were 2-iP (2 and 5 μM), BAP (2 and 5 μM), kinetin (2 and 5

μM) and GA_3 (2 and 5 μM), either singly or in combination with each other. The induced multiple shoot buds were carefully excised from the explants as a bunch, without any extra growths of callus or globular structures and cultured on the elongation medium initially for about 10 days. They were sub-cultured for 2 to 3 passages of 10 days each on fresh medium. During each passage few shoots elongated and the un-elongated buds were sub cultured on the fresh medium. The bunch of multiple shoot buds grew thin with respect to the number of shoot buds through each passage. Preferably, the elongating shoots and shoot buds from the second passage were cultured on MS with GA_3 (2 μM). Elongated shoots to about 5 cm in length in each passage were further proceeded to rooting phase.

3.2.2.4 Rooting of shoots:

The medium used was either in semi-solid form or liquid form. The elongated shoots were used to optimize the rooting. Media variations were tried in the liquid medium as the semisolid medium resulted in a very low frequency of rooting. Rooting in the liquid medium was done in two phases, phase 1 and phase 2. Both the phases were maintained in the culture room under aseptic environment. The shoots that did not root in both phases were carried to the phase 3. Dark green and healthy shoots of around 5 cm length were ideal for rooting. The rootable shoots were cultured in culture tubes (25x200 mm) containing filter paper bridges immersed in liquid root induction medium (RIM) that consisted of MS with modified levels of KNO_3 (9.4 mM; half of the concentration in MS). IBA was filter sterilized and added at 5 μM. While 60 to 80% of the elongated shoots rooted in the phase 1 the shoots (>8 cm) devoid of roots were carried on to phase 2. Such shoots were briefly dipped in filter sterilized solution of 100 μM IBA and placed on filter paper bridge in culture tubes containing hormone-free liquid MS. Effect of half MS and MS devoid of any growth regulators and

effect of various concentrations of NAA (5 and 10 μM) and IBA (5 and 10 μM). Effect of sucrose on rooting was studied by adding sucrose at 0.0, 1.0, 1.5, 2.0, 2.5 and 3.0% while all the other constituents of RIM were maintained same as mentioned above. The root inducing growth regulator in these media was IBA added at 5 μM concentration.

Rooting in the hydroponics system: This system is termed as phase 3 of rooting and this step is an optional one. About 10 to 20% of the rootable shoots, which did not root even after 2 to 3 sub-cultures on RIM were carried to the hydroponic system that was generally used for hardening during the transplantation process. ¼ strength Arnon's solution was filled in 8 cm Magenta jar and the shoot was suspended with support such that 1 cm of the shoot base was immersed in the solution that contained 3 μM IBA. The medium was changed every 4 to 5 days until the root primordia appeared. The shoots with roots were transferred to the hormone-free Arnon's solution for further growth and hardening.

3.2.2.5 Hardening and transplantation of the rooted plants:

Hardening and transplantation process was broadly divided into three stages; stage 1: initial transfer into 8 cm (dia) pots with cover (7 to 10 days); stage 2: acclimatization phase in which the plants were gradually exposed to the ambience by pinching holes and removal of the corners of the cover (15 to 20 days); stage 3: transfer of the plants to the 20 cm (dia) pots and maintenance in glasshouse for further growth.

Hardening started with the removal of cotton plugs of the culture tubes for 1 to 2 days. The plants were carefully taken out of the tube and the roots were thoroughly washed, dipped in diluted thiram™ (fungicide) solution and transferred to 8 cm (dia) containing coarse sand (2 to 4 mm dia) as the potting medium. They were completely covered with transparent polypropylene bags and allowed to grow for 7 to 10 days. Condensation on the inner surface

of the plastic bag was removed twice daily. The plants were exposed to the ambient conditions gradually by pinching holes and cutting the corners of the polypropylene bags. Finally after 10 days the cover was opened on top and allowed to stay for about a week following which the plant was carefully transferred to 20 cm (dia) pot having the potting mix. The potting mix comprised of a mixture of smooth and coarse sand mixed with black farm soil at 3:2 ratio and having 10% organic matter (equal amounts of Cell Rich[TM] and rice straw compost). Effect of potting medium composition (black soil, red soil, smooth sand, coarse sand and vermiculite) either singly or in combination with each other was studied both in stage 1 and stage 2, for testing the survival frequency of the transplanted plants. Temperature variations (20 to 30 °C in the day time and 12 to 17 °C in the night), humidity variations in the culture room as well as walk in type (Conviron[TM]) growth chamber (50 to 90% in the day and night times), light intensity (2000 lux to 12000 lux) and irrigation were also studied.

Hydroponics system: this system can also be used directly for hardening of the rooted shoots or at the stage 1 of the above proves, when little or no growth of the transplanted plants is observed. In some cases, especially with the plants rooted in the phase 2 of the rooting, there was little no growth of the plant after transfer to the 8 cm (dia) pots. This was found due to the lack of growth of the rooting system. Such plants at the end of the stage 1 were removed from the small pots, washed off the sand completely and were placed into the Magenta jars containing ¼ strength Arnon's solution (Arnon, 1938). Care was taken that the whole root system is immersed in the solution (Fig 4.9 B). After about 2 to 3 weeks when the root system attained sufficient growth they were transferred to 20 cm (dia) pots having sand and black soil (3:2) for further growth and development of plants.

3.3 Histological studies on multiple shoot initiation from AM4 explant:

Preparation of AM4 explant is described in the section 3.2.2.1. The histological studies were carried out from the stage when the axillary bud was removed. The day of axillary bud removal was considered as day-1. The development of AM4 explant was studied upto 8 days after the removal of axillary bud i.e., up to day-8.

Preparation, fixation and dehydration of tissue samples: The explants were prepared and before fixation cotyledon was removed. Basal tissue of the regenerating area was also removed making the sample into a block of 4 to 5 mm size. The sample was immediately placed in fixative solution (see Appendix for composition) and stored overnight at 4 °C. The fixative was discarded and the samples were dehydrated with 10%, 30%, 40%, 50%, and 70% alcohol sequentially for ½ hour each and finally they were stored in 70% alcohol.

Tissue processing and section cutting: This involves dehydration, clearing and infiltration of the tissue with paraffin. The usual dehydrating agent is ethyl alcohol; acetone and isopropyl alcohol can also be used. Following dehydration, the tissue was transferred to a paraffin solvent, which is miscible with the dehydrating agent as well. These are known as clearing agents eg. Chloroform, xylene etc.

The specimen was marked with an identifying number. This number was kept with the tissue block throughout processing. The tissue block was conveyed through a series of following solvents as per the schedule for dehydration, clearing and paraffin infiltration.

Step number	Solvent	Concentration (%)	Time
1.	Isopropyl alcohol	80	1 hour
2.	Isopropyl alcohol	90	1 hour
3.	Isopropyl alcohol	95	1 hour
4.	Isopropyl alcohol	95	1 hour
5.	Isopropyl alcohol	Absolute	1 hour
6.	Isopropyl alcohol	Absolute	1 hour
7.	Isopropyl alcohol	Absolute	1 hour
8.	Chloroform	-	1 hour
9.	Chloroform	-	1 hour
10.	Chloroform	-	1 hour
11.	Parafin-1	-	3 hours
12.	Parafin-2	-	3 hours

During the process of embedding, the tissue blocks were oriented so that sections were cut in the desired plane of the tissue. Two L-shaped metal moulds were laid on metal plate so as to enclose a rectangular or square space. This is then partly filled with melted paraffin (58 to 60 °C melted paraffin was used) and the tissue was placed in the desired position. The container was then filled with melted paraffin and allowed to cool until reasonably firm so that the set block of paraffin with the tissue can be removed from the moulds. The block was the trimmed to a suitable size and fixed on a metal object holder. The

block was further trimmed so that paraffin overlying the piece of tissue was excluded and an adequate area of the tissue facing the knife was exposed. The block was then kept for cooling at 0 °C.

Usually sections are cut at 5 µm thickness and floated in a water bath between 38-49 °C. The sections were cut using Leica RM 2155^{TM}, India microtome device. The sections from the water were mounted on clean glass slides, which have been smeared with a drop of Mayer's egg albumin. They were then dried on a hot plate at about 50 °for 30 min. The sections on the slides were then ready for staining.

Staining of the samples on slides: Staining of the slides was done using Hematoxylin-Eosin stain (see Appendix for composition). The slide containing the section was serially processed as follows:

Xylol I	3 min.
Xylol II	3 min.
Acetone	3 min.
95% alcohol	3 min.
Running water	3 min.
Hematoxylin stain	12 min.
Wash in running tap water	12 min.
Eosin working solution	1 min.
95% alcohol	2 to 3 dips
95% alcohol – 2 changes	3 min each
Acetone – 2 changes	1 to 2 min each
Xylol – 2 changes	3 min each

The slides were then mounted and viewed under microscope. The nuclei stained bluish violet and cytoplasm in various shades of pink.

3.4 Genetic transformation

Genetic transformation of the AM4 explants was done via biolistic as well as *Agrobacterium*-mediated methods. Biolistic transformation was done by using the plasmid pRT99:GUS-Int (6.7 kb) (Fig 3.3) (Vector was kindly provided by Dr. R. Topfer). This plasmid has *npt*II gene as selectable marker, *uid*A gene with an interspersed intron (GUS-Int) as the reporter and ampicillin resistance gene for bacterial selection. Both *npt*II and *uid*A genes were under the regulation of CaMV-35S promoter. A variety of single and multiple cloning sites were present at different locations on the plasmid. The *Agrobacterium*-mediated method was performed by using two binary vectors, namely, pHS 723:Bt (Fig 3.4) and pHS 737:*SBTI* (Fig 3.5) (both the vectors were kindly provided by Dr. G. Selvaraj, Plant Biotechnology Institute, Saskatoon, Canada) which harbored *BtCrylAb* and *SBTI* as agronomically important genes respectively. Both the vectors have *uid*A and *npt*II genes fused into single unit driven by CaMV 35S promoter. *BtCrylAb* gene was driven by double 35S promoter where as *SBTI* gene was driven by single 35S promoter. The selectable marker and reporter fusion gene (*gus*::*npt*II) and *BtCrylAb*/*SBTI* genes were cloned in the T-DNA region between right and left borders. The binary vectors have origin of replication that can operate both in *E. coli* as well as *Agrobacterium* and other attributes such as multiple cloning sites.

In both biolistic as well as *Agrobacterium*-mediated processes procedures the putative transformants were obtained by selecting the transformants using *npt*II as the selecting gene and kanamycin as the antibiotic for selection. Control explants were used to test the lethal

dose (LD-50). This experiment was done by culturing AM4 explants on MS with kanamycin (5, 10, 15, 20, 25 and 30 mg/L) and with varying concentrations of TDZ (0, 2, 4, 10 μM). 6 to 8 explants were cultured per plate.

3.4.1 Transformation by biolistics method:

Candidate plasmid isolation and purification is the first step for biolistics method of transformation followed by coating of the plasmid onto the microcarriers and bombardment of the explants with the microcarriers. Bombardment events were performed by BioRad 1000/He PDS system (BioRad, USA).

3.4.1.1 Mini Preparation of Plasmid DNA:

Mini-preparation of plasmid DNA was done following the method optimized by Birnboim and Doly (1979).

1. A single colony of bacterial culture was grown in about 10-20 ml liquid LB medium with appropriate selection overnight at 37 °C. 1.5 ml of the bacterial culture was taken in an eppendorf tube and centrifuged at 12,000 rpm for 30-60 sec at room temperature.
2. Supernatent was removed and pellet was suspended in 100 μL GTE (Glucose-Tris-EDTA) buffer and was kept on ice for 5 min.
3. 200 μL lysis buffer was added; tube was inverted several times to mix the contents and left for 5 min on ice.
4. 150 μL 5 M potassium acetate was added, vortexed and left on ice for 5 min.
5. The reaction mixture was centrifuged for about 5 min at 14000 rpm and the supernatant was transferred to a fresh tube (care was taken not to carry over the precipitate or floating material).

6. The supernatant was taken and the DNA was precipitated with 0.8 vol. (400 µL) of isopropanol. The mixture was allowed to stand at room temp. for 2 min and centrifuged at 12,000 rpm for 10 min at room temperature.
7. The pellet was washed with ice-cold 70% ethanol. Ethanol was removed (by decantation or aspiration) and the pellet was air-dried.
8. The pellet was dissolved in 30-50 µL water or TE 8 buffer (It should contain approximately 1 µg/10 µL). This DNA can be used for restriction analysis or for RNase treatment.
9. RNase treatment:
10. The pellet was resuspended in 500 µL TE 8 and 10 µL of RNase A solution was added (5 mg/ml).

The sample was incubated at 37^0C for 30 min. 1 vol (500 µL) Phenol: chloroform mixture was added, mixed vigorously and centrifuged at 12,000 rpm for 2 min. The supernatent was taken (aqueous phase) and 1 vol (500 µL) of chloroform was added, mixed and centrifuged at 12,000 rpm for 2 min. 0.1 vol (50 µL) of 3M sodium acetate (pH 5.2) was added to the supernatent and the DNA was precipitated with 0.8 vol (400 µL) isopropanol. The sample was allowed to stand at room temp for 2 min and centrifuged at 12,000 rpm for 10-15 min. The supernatent was removed and the pellet was washed briefly with ice-cold 70% ethanol. The pellet was air-dried prior to dissolving the DNA in 30-50 µL water or TE 8.

3.4.3.1 Microcarrier preparation

Microcarrier preparation is the preparation of particles (gold or tungsten) for coating of plasmid DNA that was used for transformation and the method was by Sanford, (1993).

1. In a 1.5 ml microfuge tube, 60 mg of microprojectiles were weighed out.

2. 1 ml of freshly prepared 70% ethanol was added.
3. The sample was vortexed on platform vortexer for 3-5 minutes and incubated for 15 minutes.
4. Microparticles were pelletted by spinning for 5 seconds in a microfuge.
5. The liquid was removed and discarded.
6. The following steps were repeated 3 times:
   a. 1 ml of sterile water was added.
   b. Vortexed for 1 min.
   c. The particles were allowed to settle for 1 minute.
   d. Microparticles were pelletted by spinning for 2 seconds in a microfuge.
   e. Liquid was removed and discarded.
7. 1 ml sterile 50% glycerol was added to bring the micro particle concentration to 60 mg/l.
8. The microparticles were stored at room temperature for up to 2 weeks.

3.4.3.2 Coating DNA onto microcarriers

Coating of DNA onto microcarriers was done by using the protocol developed by Sanford (1993).

1. The microcarriers were vortexed for 5 minutes on a platform vortexer to resuspend and disrupt agglomerated particles.
2. 50 µL (3 mg) of microcarriers were taken into a 1.5 ml microfuge tube.
3. While vortexing vigorously, the following were added in order:
   i. 5 µL DNA (1 µg/µl.)
   ii. 50 µL $CaCl_2$ (2.5 M)
   iii. 20 µL spermidine (0.1 M)
4. Vortexing was continued for 2-3 minutes.

5. Microcarriers were allowed to settle for 1 min.
6. Microcarriers were pelletted by spinning for 2 seconds in a microfuge tube.
7. Liquid was removed and discarded.
8. 140 µL of 70% ethanol was added without disturbing the pellet
9. Liquid was removed and discarded.
10. 140 µL of 100% ethanol was added without disturbing the pellet
11. Liquid was removed and discarded.
12. 48 µL of 100% ethanol was added.
13. The pellet was gently resuspended by tapping the side of the tube several times, and then by vortexing at low speed for 2-3 seconds.

Six aliquots of 6 µL each of microcarriers were collected and transferred to the center of a macrocarrier. Equal amounts (500 µg) of microcarriers each time and to spread evenly over the central 1 cm of the macrocarrier using the pipette tip and then desiccated immediately.

3.4.3.3 Explant preparation and procedure for bombardment:

The transformation by biolistics method was done by using epicotyl and hypocotyl explant as 30 to 40 explants could be grouped at the center of the petri plate. The explants were arranged at the center of petri plate as a group within a diameter of 1.5 inch. The laminar hood consisting of the biolistics gene gun was thoroughly cleaned with 100% ethanol. The petri plate containing the explants was placed at an appropriate distance from the device consisting of the macrocarriers. The explants were bombarded with the microcarriers by applying appropriate pressure in the range of 900 to 1100 psi. Spreading of the microcarriers was ensured and the explants were separated and were cultured on shoot induction medium (SIM) containing MS with 4 µM TDZ, 10 µM 2-iP and 2 µM kinetin. They were incubated

for about 3 to 4 days and were sub-cultured on the SIM consisting of 25 mg/L kanamycin for about two weeks. The regenerating shoot buds were cultured on the shoot elongation medium (SEM1). Rest of the procedure from elongation phase was same as the described in the following section of *Agrobacterium*-mediated transformation method.

3.4.2 *Agrobacterium*-mediated transformation method:

The transformation experiments were conducted by using the basic *Agrobacterium* strain, C-58 harboring binary vectors pHS 723:Bt or pHS 737:*SBTI* containing *BtCryIAb* and *SBTI* respectively. Selectable marker (*npt*II) and reporter (*uid*A) genes were fused into a single gene and all the genes were under the regulation of CaMV 35S promoter. A single colony of *Agrobacterium* culture of interest (pHS 723:Bt or pHS 737:SBTI) harboring the gene of interest was inoculated in the 25 ml yeast extract broth (YEB) that contained 50 mg/ml of kanamycin and grown at 175 rpm overnight at 28 °C in an incubator-shaker. The OD of the overnight grown culture was ensured to be between 0.6 to 1.0 and 12.5 ml. Aliquots of the culture solution was collected in the 30 ml. centrifuge tubes and centrifuged at 5000 rpm for 5 min. The supernatant was discarded and the precipitated cells were washed with 10 ml. of sterile ½ MS medium and were centrifuged at 5000 rpm to collect the cell precipitate. The precipitated cells were suspended in 25 ml. of sterile ½ MS. This culture was poured in a sterile petri plate that was kept inclined. The AM4 explants were prepared as described in the previous section (see 3.2.2.1). The explants were briefly dipped into the *Agrobacterium* culture poured in the petri plate for 1 to 2 sec. They were cultured in MS medium containing 4 µM TDZ, 10 µM 2-iP and 2 µM kinetin (SIM). The explants were co-cultivated with the bacteria for 48 hours. and were cultured on MS medium containing 250 mg/L cefotaxime. The antibiotic cefotaxime was used to terminate the growth of the agrobacterial cells. Inclusion of cefotaxime in the culture media continued for 1 to 2 passages

till the growth of bacterial cells was completely terminated. The cultured explants were maintained in the non-selection induction medium that did not consist of kanamycin, for about 3 to 4 days. The explants were then transferred to the MS medium containing 25 mg/L kanamycin and maintained for one week. Then the explants were cultured on MS medium containing 50 mg/L kanamycin and were maintained for about 7 to 10 days. The shoot buds emerging as bunches were carefully separated from the cotyledon part with some basal callus intact and they were transferred to the shoot elongation medium containing 5 μM 2-iP and 2 μM kinetin (SEM1) and 75 mg/L kanamycin. After about 10 days the elongated as well as the un-elongated shoots were transferred to the shoot elongation medium that contained MS with 2 μM GA_3 (SEM2) and 100 mg/L kanamycin. The un-transformed shoots bleached at every stage of the induction and elongation. The selected shoots that were growing healthily were carefully separated from the elongating shoot bunch and were cultured in the root induction medium which was prepared without adding the selection antibiotic kanamycin as kanamycin was found to negatively interfere rooting frequency. Root induction medium was prepared in liquid form without adding any agar and it consisted of MS with 5 μM IBA (RIM) where the pH was adjusted to 5.9-6.0. Basal stem segment of the elongated shoot was cut into half so that it does not contain any nodal meristem and the shoot was cultured on the filter paper bridges immersed in the RIM. The un-elongated shoots were repeatedly sub-cultured on the SEM2 for 2 to 3 passages and the elongating shoots at every stage were proceeded further for rooting. Rooting was observed in about one week and the roots grew to 5 to 7 cm length in about 2 more weeks. The shoots that did not root were briefly pulse treated with 100 μM IBA and were cultured on the filter papers immersed in the liquid MS medium. The rooted plantlets were hardened preferably with the hydroponics system described in the previous

section and established plants were transferred to the 20 cm (dia) pots for further growth and maintenance. These plants are termed as putatively transformed plants.

3.4.4 Molecular analysis of the putative transformants

Molecular analysis of the putative transformants was done initially by GUS histochemical assay followed by PCR for the genes used in the transformation experiments and Southern blot hybridization using PCR amplified fragments as the probes.

3.4.4.1 GUS histochemistry

GUS histochemical assay was done by the method of Jefferson et al. (1987) and the assay mixture was prepared by the following procedure.

(for 5 ml)

1. 5 mg X-gluc was dissolved in 50 µl. Dimethyl formamide and the following reagents were added.
2. 5 ml Phosphate buffer (0.05M, pH 7.0)
3. 1 mM Potassium ferrocyanide (25 µL of 200 mM stock)
4. 1 mM Potassium ferricyanide (25 µL of 200 mM stock)
5. 10 mM Na2EDTA (50 µl of 1 M stock)
6. 0.1% Triton-X (100 µL of 1:10 diluted stock)
7. 0.2% Sodium azide (200 µL of 50 mg/ml stock)

Note: The assay mixture can be stored for several months at 0 °C In a dark bottle. Usually there is no need to add Items 3,4, and 7.

ASSAY

200 µL (or sufficient to dip the tissue) of assay mixture was added to tissue sample (sections or discs or strips) and vacuum infiltrated for 3-5 minutes. The sample was incubated

at 37 °C for 3-24 hours in dark. The assay mixture was removed and the tissue was cleared off chlorophyll by sequential changes in 70-100% ethanol until tissue had no chlorophyll. Alternatively, for difficult to clear tissue add 75% lactic acid was added and autoclaved for 15 minutes. This gives a very good image for photography. The tissue was mounted in glycerol and observed under a microscope.

3.4.4.2 Nucleic acid extraction:

Genomic DNA of the putative transformants was extracted following the protocol published by Dellaporta et al., (1983). Young leaflets were ideal for DNA extraction.

1. 1 gram of tissue was transferred in liquid Nitrogen and ground to a fine powder and transferred into 30 ml tube.

2. 15 ml of extraction buffer and 1.0 ml of 20% SDS were added, shaken well for 10 sec and the tubes were incubated at 65 degrees for 10 min

3. 5.0 ml 5M Potassium acetate was added, vortexed and incubated at 0 degrees for 20 min

4. Most proteins and polysaccharides removed with the insoluble K-dodecyl sulfate ppt.

5. The sample was centrifuged at 25000g for 20 min and filtered thru Mira cloth into 30 ml tube containing 10 ml isopropanol, mixed and incubated at -20°C for 30 min.

6. DNA was precipitated at 20000g for 15 min. and inverting the tubes on paper towels for 10 min dried pellet.

7. The pellet was resuspended in 500 µL TE 8 and 10 µL of RNase A solution was added (5 mg/ml).

8. The sample was incubated at 37°C for 30 min. 1 vol (500 µL) Phenol: chloroform mixture was added, mixed vigorously and centrifuged at 12,000 rpm for 2 min. The

supernatent was taken (aqueous phase) and 1 vol (500 µL) of chloroform was added, mixed and centrifuged at 12,000 rpm for 2 min. 0.1 vol (50 µL) of 3M sodium acetate (pH 5.2) was added to the supernatent and the DNA was precipitated with 0.8 vol (400 µL) isopropanol. The sample was allowed to stand at room temp for 2 min and centrifuged at 12,000 rpm for 10-15 min. The supernatent was removed and the pellet was washed briefly with ice-cold 70% ethanol. The pellet was air-dried prior to dissolving the DNA in 200-300 µL TE.

3.4.4.3 Confirmation of putative transformants using PCR

Genomic DNA of putative transformants of chickpea was extracted by Dellaporta et al. (1983) from young leaflets and 200 ng was used for PCR amplification reaction. Polymerase Chain Reaction (PCR) was performed using the standard procedures. The basic steps included 1. Denaturation 2. Annealing and 3. Extension. The reaction program was same for amplifications of all the genes, however, the only difference being the annealing temperature. The primers composition used for each of the genes and their respective annealing temperatures are shown in table 3.1. All the PCR reactions were done using Eppendorf Master Cycler Gradient^{TM} machine. All PCR reactions were performed by using ~ 200 ng of purified genomic DNA and the recombinant *Taq* DNA polymerase (Gibco-BRL) according to manufacturer's recommendations. The amplified products were assayed by electrophoresis on 1.2% agarose gels (see Appendix for standard PCR reaction mixture).

PCR for nptII gene: PCR for *nptII* gene was done with reaction conditions of initial denaturation of 94 °C for 3 min (one cycle) and each cycle of amplification with steps of denaturation (94 °C for 1 min), annealing (58 °C for 1 min), and extension (72 °C for 1.5 min) for 30 cycles and a final extension at 72 °C for 5 min (one cycle).

*PCR for uid*A *gene:* PCR for *uid*A gene was done with reaction conditions of initial denaturation of 94 °C for 3 min (one cycle) and each cycle of amplification with steps of denaturation (94 °C for 1 min), annealing (57 °C for 1 min), and extension (72 °C for 1.5 min), and extension (72 °C for 1.5 min) for 30 cycles and a final extension at 72 °C for 5 min (one cycle).

PCR for BtCryIAb gene: PCR for *BtCryIAb* gene was done with reaction conditions of initial denaturation of 94 °C for 3 min (one cycle) and each cycle of amplification with steps of denaturation (94 °C for 1 min), annealing (63.1 °C for 1 min), and extension (72 °C for 1.5 min), and extension (72 °C for 1.5 min) for 30 cycles and a final extension at 72 °C for 5 min (one cycle).

PCR for SBTI gene: PCR for *SBTI* gene was done with reaction conditions of initial denaturation of 94 °C for 3 min (one cycle) and each cycle of amplification with steps of denaturation (94 °C for 1 min), annealing (58.0 °C for 1 min), and extension (72 °C for 1.5 min), and extension (72 °C for 1.5 min) for 30 cycles and a final extension at 72 °C for 5 min (one cycle).

3.4.4.4 Southern blot hybridization:

The extracted and purified DNA chickpea putative transformants used for Southern blot hybridizations with a specific probe following the method given by Southern et al., (1975).

This technique was used to identify the integration of *npt*II and *BtCryIAb* genes from plants transformed with agronomically important *BtCryIAb* and *SBTI* genes. *npt*II

gene was probed with the PCR fragment of 700 bp and *BtCryIAb* gene was probed with 908 bp fragment PCR fragment. The probe was labeled with the commercially available AlkPhos^{R} direct labeling kit provided by Amersham (USA).

Restriction of genomic DNA and electrophoresis

1. The genomic DNA was digested with a suitable restriction enzyme as follows:

   30 µL chromosomal DNA (15-20 µg)

   5 µL 10X restriction buffer

   5 µL restriction enzyme (50 units)

   The final volume was made up to 50 µL with sterile water and was incubated for 2h at 37 C.

2. 2 µL of gel loading buffer was added to each restricted DNA before electrophoresis. The restricted DNA was size-fractionated in 0.8% agarose gel prepared in 1X TBE buffer along with 5 µL plasmid DNA restricted with EcoR1 as standard marker and was electrophoresed overnight at 30 volts in 1X TBE buffer.

3. The gels were stained for 20 min with ethidium bromide at 1 µg/ml buffer and the restricted DNA fragment bands were visualized on a UV transilluminator and photographed.

Processing and cappilary blotting of the gel

1. The size fractionated genomic DNA in the gel was covered with 250 mM HCl and agitated until the bromophenol blue turns to yellow color (5-15 minutes) to depurinate DNA.
2. The gel was washed three times with demineralized water.
3. The gel was incubated with denaturation solution (10X gel volume, 1.5M NaCl + 0.5 M NaOH) for 15 minutes twice, each at room temperature on a shaker.

4. The gel was washed 3 times with demineralized water and covered with neutralizing solution for 2 times 30 minutes each (1.5 M NaCl + 0.5 M Tris-HCl, ph 7.5 adjusted with HCl).
5. Gel was placed on a 3 mm filter paper (on a glass plate) whose edges are dipped in 20X SSC.
6. A nylon membrane was cut to exact size of the gel and presoaked in 2X SSC for 10 min.
7. The membrane was placed on top of the gel and squeezed out of any air bubbles by rolling a pipette, followed by 3 sheets of 3 mm filter paper and a stock of absorbent paper towels with a 1 kg weight on top. The blotting was carried out overnight (20X SSC: 3M NaCl, 0.3 M sodium citrate).
8. The membrane was removed after blotting and marked (by cutting a corner) the side for identification and air-dried.
9. The membrane was cross linked by UV irradiation for 3 min (towards the DNA face) and kept at room temperature until use.

Hybridization and developing of the membrane for signal identification

The hybridization and detection process was done using the commercially available AlkPhos direct system from Amersham Pharmacia (USA). This is a non-radioactive electrochemiluminiscent (ECL) system in which the probe is labeled with alkaline phosphatase enzyme which reacts with the added substrate (CDP-Star^{TM}) and emits photon signals that are identified by an X-ray film.

1. Pre-hybridization buffer was prepared by dissolving 4% blocking reagent and 0.7305 g. of NaCl in 25 ml./blot of Alkphos buffer. The pre-hyb buffer was kept at 60°C for about 1 hour, prior to use.

2. The membrane was carefully inserted in the hybridization tube and the pre-hyb buffer carefully added. The pre-hybridization was done for about 1 hour. at 60°C.
3. While the pre-hybridization is in progress, the probe was boiled for about 10 min. at 100°C for denaturation. It was kept in ice immediately. Following reaction constituents were added to the probe.
    a. 10 µl. of Reaction buffer
    b. 2 µl. labeling reagent
    c. 10 µl. cross-linker (prepared freshly with 2 µl cross-linker with 8 µl water supplied by the company).
4. The reaction mixture was incubated at 37°C for 30 min. and it was added to the hybridization bottle.
5. The hybridization was done overnight.
6. The membrane was taken out on the following morning and was washed with primary wash buffer (for buffer composition, see Appendix) twice for 10 min. each and with secondary wash buffer (for buffer composition, see Appendix) twice for 5 min. each.
7. The blot was treated with CDP-Star™ for about three minutes and was packed in the development cassette.
8. An X-ray film was kept on the blot and exposure was done as per the signal recorded.
9. The X-ray film was washed in the following order.
    a. Developer for 2 min.
    b. Water for 30 sec.
    c. Fixer for 2 min.
    d. Water for 2- 3 min.
10. The developed X-ray film was dried and viewed on the slide-viewer.

4.0 RESULTS

4.1 Regeneration:

4.1.1 Somatic embryogenesis:

Regeneration via somatic embryogenesis protocol in chickpea was a serious problem in our experimentations. Although a high frequency of induction of well-defined embryos was observed, the efforts to mature and regenerate complete bipolar embryos was not possible. Very low frequency of conversion of globular embryos into torpedo shaped ones was observed to occur simultaneously.

4.1.1.1 Induction of embryos:

Most of the experiments on somatic embryogenesis were done using the embryo axis explant. Standardization of appropriate medium for induction of embryos was done using 2,4,5-T and 2,4-D as principal plant growth regulators. Fig 4.1 shows the induction of embryos from mature embryo axis explant for using 2,4,5-T as primary growth regulator and Fig 4.2 shows the induction of embryos using 2,4-D. Additional growth regulators such as TDZ, BAP, zeatin, and kinetin were also used in combination with the 2,4,5-T and 2,4-D. 2,4,5-T containing media (JEM series). Table 4.1 shows the effect of 2 and 5 μM 2,4,5-T in combination with TDZ, BAP, kinetin or zeatin on somatic embryo induction. Combinations containing TDZ and BAP showed very low frequency of embryogenesis in terms of the number of explants responding and average number of embryos per explant. The explants on these media combinations produced a soft green mass of callus on which two to four embryos appeared. The embryos appeared as large globular entities and they did not look like well-defined embryos that appeared in culture media containing 2,4,5-T

with kinetin. More number of explants responded when zeatin was added with 2,4,5-T, but the number of embryos per explant was very low. Embryo axis grew into a swollen mass on which embryos appeared little larger with well-defined globular shape. Media with kinetin in combination with 2,4,5-T were found to be best amongst all the combinations used. An average of 7.6 embryos were induced per explant from the best responding combination i.e., 2 µM 2,4,5-T along with 2 µM kinetin. Table 4.2 shows increase in the concentration of 2,4,5-T to 10 and 15 µM while applying the other hormones as in the case of table 4.1 where there was gradual increase in the frequency of embryo induction with increase in 2,4,5-T, and 10 µM 2,4,5-T was found to be best. Pattern of induction and morphology of callus and embryos was found to be similar in all the four sets of treatments i.e., low frequency induction and green callus with TDZ and BAP containing combinations, slightly increased frequency and white callus with zeatin and best induction frequency with brown callus in the case of kinetin containing media. Amongst all the combinations used, combination of 10 µM 2,4,5-T with 2 µM kinetin induced an average of 19.0 embryos per explant. There was enlargement of the embryo axis in 2,4,5-T and kinetin combinations where the embryos originated directly from the apical and axillary regions. This set of embryos appeared creamy white with well-defined globular head and a stalk. All the other combinations containing 2,4,5-T with TDZ, BAP and zeatin showed embryos with no stalks and looked little larger than the former. Prolonged culture of the embryogenic explants for 6 to 8 weeks showed induction of secondary embryos. The secondary embryos originated from the surface of the globular head of primary embryos.

Tables 4.3 and 4.4 shows the induction frequencies on the media containing 2,4-D as principal growth regulator applied at 10, 15, 20 and 25 µM concentrations (JDM series).

These combinations showed more callus compared to the 2,4,5-T-containing media where the embryos originated from the callus indirectly. The number of explants responding was similar but the average number of embryos per explant was comparatively lower than the above-mentioned JEM series of media. The embryos appeared larger than the ones in JEM series and they lacked any stalks. Fig. 4.2 shows induction of embryos from mature embryo axis explant. Best responding combination was 20 µM 2,4-D with 2 µM kinetin that induced 9.3 embryos per explant. No secondary embryos were observed even on prolonged culture of the explants in the same medium while the induced embryos gradually entered callus phase.

Various other explants such as parts of mature embryo axis (plumule, radicle, side arms and middle portion), leaflets, stem segments and root segments were cultured on best embryogenic combination of JEM29 that contained 10 µM 2,4,5-T and 2 µM kinetin. The results are shown in the table 4.5. Plumule from mature embryo axis and leaflets gave best embryo induction frequency of 70 and 40% respectively. All the other explants except radicle from embryo axis showed higher frequency of induction where a time dependent induction was observed. In general, embryo induction was observed within 4 to 5 weeks and such phenomenon was observed with mature embryo axis derived explants and leaflets. However, stem and root segments showed induction on a prolonged culture on the same medium for about 8 to 9 weeks. The embryos appeared similar to the ones on embryo axis and leaflet explants. The epidermal layer of stem and root segments broke open into crevasses and embryos appeared originating from the inner tissue.

4.1.1.2 Maturation of embryos:

Maturation of the induced embryos was found to be very difficult despite several media combinations and methods like single embryo culture, culture of bunches of embryos and whole explant culture were attempted. Most of the redundant combinations were excluded from tabulations and the results of some combinations are shown in the table 4.6. Only ABA and GA_3 combinations showed signs of maturation of embryos that resulted in conversion frequency of 1 to 2%. The maturing embryos attained torpedo shape and failed to grow further. Most of the trials were found to be futile and usage of cytokinins like TDZ and BAP showed an irreversible entry of embryos into green callus phase. Sub-culture of such callus in 2,4,5-T showed no embryogenesis. Various physical treatments such as cold shock, heat shock and liquid medium agitations were tested after which the explants were cultured on the media with either 5 µM ABA or 10 µM GA_3. These treatments showed some initial indications of conversion but the final result was not promising.

4.1.2 Direct and Indirect Organogenesis:

The experiments on shoot organogenesis were broadly divided into two types namely direct and indirect methods. Basing on this concept the explants used were catagorized into two groups. 1. Explants that gave direct shoot organogenesis and 2. Explants that gave indirect shoot organogenesis. Explants having pre-existing meristems or any traces of meristems come under first group and the others like leaflets, leaf base, stem segments, epicotyl, hypocotyl, root segments and root tip that were not associated with any meristematic tissue comes under the second class. The observations showed that indirect

regeneration was difficult and could not be achieved with the tested media. Mature embryo axis, shoot tip, axillary bud, cotyledonary node and axillary meristem (AM) explants showed shoot regeneration amongst which axillary meristem explants AM2 and AM4 were selected as the best ones (see Fig 4.4).

4.1.2.1 Factors affecting multiple shoot induction:

Standardization of induction medium: Standardization of an appropriate medium for induction of multiple shoots was initially done with mature embryo axis explant. These experiments were done using BAP containing media that included MS with various concentrations of hormones like kinetin, 2-iP and NAA (Table 4.7). It can be observed that the best combination was found to be JBC2 that contained 10 µM BAP with 2 µM kinetin while increase in the concentration of kinetin decreased the number of shoots per responding explant. Fig 4.3 shows multiple shoot induction from mature embryo axis by using BAP as the principal growth regulator. Multiple shoots originated one after the other asynchronously at the shoot tip and axillary bud regions of mature embryo axes. The explant grew three to four times in size and meristematic regions branched into multiple shoots. The other combinations containing 2-iP and NAA with BAP in general showed encouraging results. Many plant growth regulators like zeatin, picloram, IAA and IBA were used either individually or in combination with BAP showed some redundant results of low frequency multiple shoot induction and they were not tabulated due to their insignificant response for shoot induction.

Separate experiments for the standardization of shoot induction medium were done using TDZ as principal multiple shoot inducing growth regulator in combination with 2-iP

and kinetin by using AM2 and AM4 explants. Both the explants showed more or less similar number of multiple shoots per explant (Table 4.8). TDZ was used at various concentrations ranging from 2 to 100 µM. The media combinations were named as JCR series which also consisted of TDZ with 2-iP and kinetin. Standardization of the medium was done keeping the later stages of elongation and rooting into consideration as concentration and time of culture of explants on TDZ-containing media show significant effect on subsequent shoot elongation. Inclusion of 2-iP and kinetin was also found essential as they were also included in the shoot elongation medium (SEM) at lower concentrations. A low level of TDZ (4 µM; JCR2) was most effective and the levels of TDZ up to 10 µM resulted in more or less similar number of shoots per responding explant. Increase in the TDZ concentration resulted in stunted growth of the shoot buds. Shoot buds that appeared with 50 µM and 100 µM TDZ failed to undergo elongation. JCR13 medium, which consisted of a combination of TDZ (4 µM), 2-iP (10 µM) and kinetin (2 µM) showed best results by giving rise to 23 shoots per explant at the end of two weeks and 43 shoots at the end of four weeks (Table 4.8) which were healthy and elongated well on SEM.

Effect of pH on multiple shoot induction: The pH of culture media proved to have significant effect on the regeneration of shoots from axillary meristem explants, as it was observed with mature embryo axis explant (Table 4.9). Effect of pH within a range of 4.0 to 8.0 in the induction medium was tested where the acidic pH of 5.0 clearly showed better multiple shoot induction with 27 to 30 shoots per responding explant (Table 4.9) when compared with the generally used pH of 5.8 to 6.0. The number of multiple shoots

decreased at alkaline pH while the shoot buds induced on media with pH of 5.0 to 5.5 elongated well on the shoot elongation medium.

Effect of cotyledonary tissue on shoot induction: The effect of inclusion of the intact cotyledon or a portion of it along with the regenerating tissue is shown in the Table 4.10. Inclusion of cotyledon has a considerable advantage for regeneration of multiple shoots from axillary meristem region since complete exclusion of the cotyledon showed delayed response and a significant decrease in the number of shoots per responding explant. Exclusion of cotyledon in parts i.e., culturing of axillary meristem tissue with half and zero cotyledon showed time dependent changes in the number of shoots per explant. Explants with intact cotyledon showed much earlier appearance of shoot buds when compared to the explant with half cotyledon followed by zero cotyledon. However, prolonged culture of the explants up to 5 weeks resulted in a decrease in this difference with respect to the number of shoots per explant in explants with full and half cotyledon. Sub-culturing of the regenerating shoot buds in clusters, however, showed an exponential increase in number of multiple shoots per explants. This increase was found to be two to three times superior in the explants with intact cotyledon.

Effect of explant donor seedling age: Age of the seedling from which the explants were derived, plays an important role in regeneration of multiple shoots (Table 4.11). All axillary meristem explants showed age dependent responses. AM1 explant differed from other three basically due to its germination pattern. 2-day-old seedlings on MS medium were ideal for AM1 explant while 6 to 8 day-old seedlings were found to be ideal for removal of axillary bud and regeneration on shoot induction medium (SIM) with respect to AM2, AM3 and AM4 explants.

Shoot regeneration in different explants: All variations of the explants consisted of meristematic zones through which direct regeneration was achieved. Fig 4.4 shows regeneration pattern from different explants and Table 4.12 shows the regenerating abilities of various explants. All the explants expect AM3 showed 100% frequency of responding explants, with varied number of shoots per explant. Embryo axis explant that was frequently used in regeneration experiments showed asynchronous multiple shoots originating sequentially from shoot tip and axillary bud portions. Total number of shoots originating from shoot tip and two axillary bud positions were counted and grouped together in recording the results. A near synchrony of regenerating multiple shoots from shoot tip was observed. Prolonged culture of the explants on SIM resulted in decreased frequency of the shoot elongation. Axillary bud was taken as a by-product of AM1 and AM2 explants. Near synchronous multiple shoots were produced by this explant. Number of multiple shoots per explant was better than embryo axis and shoot tip. However, the axillary bud explant obtained as a byproduct of AM1 explant showed much lower frequency of multiple shoots. The number of multiple shoots was lower than embryo axis and shoot tip. About 60% of the axillary buds showed multiple shoots originating from the base of the axillary bud. This type of regeneration was assumed to be useful for genetic transformation. The axillary bud portion enlarged and the multiple shoots originated from swollen portion of AM1 explant. AM2 explant that gave rise to multiple shoots from one or both sides of the area of the amputated axillary bud. In most of the cases multiple shoots were produced from one side of the axillary bud area where synchronous multiple shoots were observed from this explant. In AM3 explant the regeneration frequency was very low when compared to other tested explants. A very low number of multiple shoots, came from

either side of basal portion of the explant but did not elongate well. AM4 explant that can be considered as ideal for gene transfer experiments gave rise to multiple shoots as multiple clusters where the shoots are formed from cut portions on the swollen part of axillary meristem. Fig 4.5 shows regenerating multiple shoots from AM4 explant where many shoots originated from the basal portions of the removed shoot buds. Prolonged culture of all the above-mentioned explants on shoot induction medium resulted in exponential increase in the number of shoots per explant. It was observed that multiple branching of the regenerating shoots increased the number of shoots per explant with each shoot bud having the ability to develop into complete plant.

4.1.2.2 Elongation of the shoot buds:

Plant growth regulators were employed at low concentrations to increase the number of shoots elongating per explant (Table 4.13). BAP, 2-iP, kinetin and GA_3 were the key components for shoot elongation. The shoot elongation media were named as CEL series from which CEL2 containing 5 µM 2-iP and 2 µM kinetin and CEL7 containing 2 µM GA_3 showed best results by elongating 7.3 and 8.7 shoots respectively (Fig 4.6 C). Elongation on kinetin was found to be minimal. Elongation was also tested on basal MS, but the results were not encouraging when compared to the other treatments. However, prolonged culture on MS resulted in the rooting of 5 to 10% of the elongating shoots. All the tested combination showed elongation of 0.1 to 3 shoots per explant in the initial step (elongation 1), which showed a length of 5 to 8 cm in another two weeks. Rest of the explant was placed on fresh elongation medium. Best results were obtained when the elongation was done with CEL2 in the elongation 1 and CEL7 in the later stages. The shoot buds growing in clusters were cultured in CEL7 for two to three passages at 10 to 12

day intervals. GA_3 was found to be playing crucial role in the elongation 2 phase where its application showed increased internodal length and better leaf morphology. The shoots elongated in each passage were transferred to the rooting medium.

4.1.2.3 Rooting of the elongated shoots:

Rooting was done on both semi-solid and liquid media. Fig 4.7 shows rooting in both the kinds of media. Results of the rooting experiments using liquid media are shown in the Table 4.18. The rooting was also carried out in stepwise manner in three phases. The first phase in turn was done in sequential manner where elongated shoots rooted at different times while sub-culturing. The shoots survived for longer times in liquid media when compared to the shoots transferred to the semi-solid root induction media. Hence, rooting was mostly done on filter paper bridges immersed in liquid rooting medium. Roots were observed at different intervals from different shoots. About 20% of the shoots showed roots within 3-4 days. Another 40% showed roots by the end of one week while 20% more showed them in the second or third week, which were called as late roots. The roots grew to full-length roots with lateral roots in another 2-3 weeks. On an average 70 to 80% of elongated shoots rooted well. It was observed that inclusion of any nodal meristem on the surface that was exposed to the rooting media drastically decreased the rooting frequency. Late roots mentioned above, showed slow growth and they contained several root hairs. Shoots, which did not root, were carried to the phase 2 in which they were pulse treated with 100 µM IBA for 2 to 3 sec. Rooting in this phase was highly adventitious with more number of roots originating from different parts of the shoot. However, some abnormalities resulted as roots formed all over the shoot if the shoot length was less than 5 cm. Tissue in the basal part of the shoot, which was exposed to the high concentration of

IBA showed browning and death. Above the dead part, roots were formed in bunches. Shoot growth slowed down until the new roots started to elongate. Prolonged exposure (more than 3 to 5 min) to high concentration of IBA terminated the growth of the whole plant.

Effect of variations in the sucrose concentration on rooting: Variations in the sucrose concentrations showed varied rooting frequencies (Table 4.14). Results were recorded at the end of phase I of rooting (phase I described in the materials and methods section). Sucrose at 1.5% and 2% showed best results in terms of frequency and root morphology. Little or no response was observed in cultures devoid of sucrose.

Effect of medium on rooting: Effect of various concentrations of IBA and NAA, ½ MS and MS were tested and the results are shown in the Table 4.18. IBA at 5 µM concentration was found to be best for rooting. A brief exposure of the shoots to CPR5 prior to placing them on liquid MS showed much faster and efficient rooting.

Rooting in the static hydroponics system was optional and was employed only when there were some plants remained unreacted even after two to three subcultures in the rooting medium. However, this system has an advantage of hardening and acclimatization of the in vitro grown plants while rooting was in progress. Around 60% of the plants that did not root even after two to three subcultures in the phase I or 2 of rooting, showed rooted in this system.

4.1.2.4 Hardening and transplantation of the rooted plants:

Effect of potting medium: Various potting media such as black soil, red soil, smooth sand, coarse sand (2 to 4 mm particle size) and vermiculite, either singly or in

combination with each other were tested in the stage 1 of the hardening process (Fig 4.8). Combinations of processed organic matter such as Cell Rich^{R} and rice straw compost were also used. Initial experiments with smooth sand and black soil at equal proportions showed better results with a survival rate of 20% of the plants for about three weeks. They gradually died after transfer to the 20 cm pots. In this treatment, it was found that there was considerable growth of the stem but not of the root system. However, subsequent experiments employing coarse sand (2 to 4 mm particle size), showed best results for the stage 1 and stage 2. There was around 80% survival at the end of stage 1 and 50 to 60% by the end of stage 2. A profused growth of the root system was observed at the end of stage 2. The stem grew with an optimal growth that was comparable to seedlings germinated in soil with an average addition of one leaf per week.

In the stage 3, sand (smooth and coarse) and black soil were mixed at 3:2 proportions, respectively that showed best results. Approximately 2 to 5% of the organic matter comprising of the mixture of Cell Rich and rice straw compost was added to the potting medium (Fig 4.8).

Temperature: Stage 1 and stage 2 required a day temperature of 20°C±2°C and night temperature of 15°C±1°C. Stage 3 required slightly higher temperature of 25°C±3°C and 15°C±1°C in the day and night respectively.

Humidity: This physical parameter played a very crucial role in the hardening process during transplantation. Opening of the cotton plugs of the tubes containing the rooted plantlets in the culture room, exposed them to a relative humidity of 40 to 50%. The plants elongated and hardened well, but dried in the later stages. Hence the hardening process was done in the walk-in type growth chamber (Conviron^{TM}) which had the relative

humidity of 50% in the daytime and over 80% in the night. The plants were initially exposed to humidity as high as over 90% by virtue of covering them with plastic bags. They were slowly acclimatized to the ambient humidity by gradually opening the cover. Prolonged covering, i.e., over exposure to high humidity caused wilting and decreased exposure caused premature drying, both resulting in death of the plant. Hence the whole humidity acclimatization process required about a month and utmost care before entering into the stage 3.

Light intensity and photoperiod: Plants up to rooting stage were maintained at a light intensity of 2500 to 3500 lux. However, continuation of this condition in the hardening process resulted in an initial long and abnormal growth and premature death of the plant. Hence, the entire hardening process was done at a light intensity of 10,000 to 12,000 lux in the Conviron™ growth chamber. Direct exposure to the sunlight in the green house caused drying of the plant. With respect to the photoperiod, stage 1 was maintained at 10 hour light and 14 hour dark; stage 2 at 12 hour light and 12 hour dark and stage 3 at 14 to 16 hour light and 10 to 8 hour dark.

Irrigation: Tissue culture grown chickpea was found to be very sensitive towards irrigation process. Optimal moisture was maintained all through the transplantation process by irrigating with 10 to 25 ml of nutrient solution as and when required. Stage 1 and stage 2 were irrigated with ¼ strength Arnon's solution and stage 3 was irrigated with soft water. Observations showed that irrigation was a crucial factor especially in the stage 1.

Alternative methods and troubleshooting: These methods were required to enhance the survival frequency of the tissue culture grown chickpea. Figure 4.9A shows one alternative method of embedding the rooting system in the coarse sand by its direct

addition to the tubes containing liquid medium. In this method the top portion of the plant was exposed to the out side conditions and slowly acclimatized while growing. Figure 4.10 B, C shows another method of static hydroponics system in which the rooting system was completely immersed in the ¼ strength Arnon's solution. In this method, the plant was directly exposed to the ambient conditions from the first day. However, drying was prevented because of the liquid nature of the nourishing medium. This method can supplement the stage 1 and 2 described above and the plants can directly go to the stage 3. This method was employed especially when there was no growth of the root system, which directly inhibiting the growth of the plant. This method was important in enhancing the survival frequency by troubleshooting in the hardening process mentioned in the materials and methods section. Growth of the root system, which is a key factor for the hardening process, was well ensured in this system.

The transplanted plantlets showed normal flowering and seed setting. Flowers appeared in about 15 days after the transplantation into 20 cm pots. The flowers were removed as soon they appeared for about 3 weeks so as to increase the vegetative growth and thus increase the number of seeds per plant. Removal of flowers also resulted in the increase of branching and the plants assumed a bushy appearance (Fig 4.10 B). Drying leaflets were removed to negate any fungal growth in the potting medium. Care was taken to keep the growing stem erect as the falling stem dried and died prematurely. Plants established in the growth chamber were transferred to the glasshouse where the rate of flowering and seed production increased. The pods were allowed to mature on the plant and they were removed from the drying plant. They were kept at 37°C for about 3 days to dry them and were stored at 4°C.

4.2 Histological studies on multiple shoot development from AM4 explant:

As described in section 4.1.2.1, AM4 explants showed adventitious multiple shoot induction with shoot buds originating from different parts of the regenerating area. Histological studies of multiple shoot induction from these explants were conducted to confirm the formation of multiple adventitious meristemoids after the removal of axillary bud. The day when axillary bud was removed was considered as day-1 and the studies were conducted up 7 days. Fig 4.13 A to H shows development of meristemoids at different places of axillary meristem area where 15 to 20 meristemoids developed between 4 to 6 days of culture on the shoot induction medium. Fig 4.13 B shows a small portion of axillary bud remaining even after the excision of the growing axillary bud and development of meristemoids at the base of it. This result shows histological evidence for the emergence of multiple adventitious shoot buds from the basal part of the axillary bud as shown in the Fig 4.4 B. Meristematic activity of the actively dividing and growing cells could be observed from the explants obtained from day-5 onwards (Fig 4.13 D, E, F and H). These divisions resulted in the formation of meristematic zones that resulted in the formation of multiple shoot buds as shown in the Fig 4.13 G. Fig 4.14 A to F shows meristematic activity of dividing cells that eventually developed into multiple shoots.

4.3 Genetic transformation:

Genetic transformation was carried out by using biolistic as well as *Agrobacterium*-mediated methods. In both the procedures the putative transformants were obtained by selecting the transformants using *npt*II as the selectable merker gene and kanamycin as the antibiotic for selection. Control explants were used to test the lethal dose (LD 50) by culturing AM4 explants on MS with kanamycin (5, 10, 15, 20, 25 and 30 mg/L) with

varying concentrations of TDZ (0, 2, 4, 10 μM). 6 to 8 explants were cultured per plate (Table 4.15). TDZ was employed so as to test the effect of TDZ on enhancing survival of thecultured explants on kanamycin containing medium. Survival was the measure of any conceivable growth of the explant including multiple shoot induction and persistence of chlorophyll pigment in the explant. Though the explant growth was inhibited even at 20 mg/L kanamycin concentration, chlorophyll pigment persisted for few more days after which explants bleached. Explant growth and shoot bud induction was completely inhibited and explant completely bleached at the concentration of 30 mg/L kanamycin. A gradual decrease in survival rate of the explants was found with increase in kanamycin concentration. Growth lethal dose was fixed at 20 to 25 mg/L kanamycin as explant growth was inhibited and explants bleached within two weeks of culture.

4.3.1 Biolistics method of transformation:

Epicotyl and hypocotyl explants were used for these experiments. These explants showed a very low frequency of shoot regeneration. A vector pRT99:GUS harboring *uid*A gene as reporter and *npt*II as selectable marker genes was used in the transformation experiments (Fig 4.12 A). This method did not result any transformed shoots and relatively few shoots emerging from the used epicotyl and hypocotyl explants bleached at early stages of induction.

4.3.2 Transformation by *Agrobacterium*-mediated method:

Transformation was done by using two binary vectors namely pHS723:Bt and pHS737:SBTI that contained *BtCrylAb* and *SBTI* genes respectively as the genes of agronomic importance. These binary vectors were used in the strain C-58 of

Agrobacterium tumefaciens. Fig 4.12 B shows these two plasmids that were used for the *Agrobacterium*-mediated method of transformation. pHS 723:Bt had *uid*A as reporter gene, *npt*II as selectable marker and *BtCryIAb* as the gene conferring insect resistance while the second one was pHS 737:SBTI that had *uid*A gene as reporter, *npt*II for kanamycin selection and soybeen trypsin inhibitor (*SBTI*) as gene that synthesizes trypsin inhibitor which is antagonistic to lepidopteran class of insects. In both the binary vectors the reporter (*uid*A) and selectable marker (*npt*II) were fusedand driven by CaMV 35S promoter. However, their products showed independent effects. In general the AM4 explants were co-cultivated for 48 hours, however, in some of the transformation events, 72-hour co-cultivation did not show any over growth of the *Agrobacterium*. Emerging shoot buds were cultured on selection medium containing 25 mg/L kanamycin initially with subsequent increase of kanamycin in stepwise manner i.e., 50 mg/L in SEM1 and 100 mg/L in SEM2. This method of selection was assumed to be useful in obtaining the stringently selected putative transformants. The non-selected shoot buds bleached and showed no further growth. The selected shoots growing healthily were transferred to rooting medium that did not have any kanamycin since it was found to decrease the rooting frequency. The rooted putative transformants were hardened and transplanted that showed normal growth and morphology compared to the controls and they were maintained in the specially designed P2 facility for transgenics. The plants flowered in about 15 days after the transplantation into the 20 cm pots. The plants were propagated vegetatively for an extended time by amputating the terminal buds of some branches and removing the emerging flowers. This method could provide sufficient leaf tissue for molecular analysis. Emergence and maturation of pods was found to be normal, however, the number and size

of the seeds was found to be lower compared to controls. The matured pods were dried and stored at 4 °C for further use. The putative transformants that were transformed with *BtCryIAb* gene were named as CB (CB1, CB2, ... *n*) series and the ones transformed with *SBTI* gene were named CS (CS1, CS2, ... *n*) series. A total of 11 CB plants and 9 CS plants were obtained and they were subsequently analyzed at the molecular level.

4.3.2.1 GUS histochemical assay:

Young leaflets from actively growing plant were excised from the plant and were soaked overnight in the freshly prepared X-gluc solution (see 3.3.4.1 section of Materials and Methods). Blue staining of the vascular tissue in the veins and petiole was clearly observed (Fig 4.13). The staining was stable for years and was preserved in 70% ethanol. 7 plants out of 10 analyzed plants transformed with *BtCryIAb* gene and 3 out of 9 plants transformed with SBTI gene showed positive staining in GUS reaction. A negative control of untransformed plant was maintained which did not show any GUS staining.

4.3.2.2 Molecular analysis of T_0 generation putative transgenic plants transformed with pHS723:Bt and pHS737:SBTI binary vectors containing *BtCryIAb* and *SBTI* genes respectively:

Initial set of transformation experiments was done with pHS723:Bt and pHS 737:SBTI binary vectors containing *BtCryIAb* and *SBTI* genes respectively. Putatively transformed plants with *BtCryIAb* were analyzed by using PCR and Southern blotting techniques and putatively transformed plants carrying *SBTI* gene were analyzed with PCR. Molecular analysis was done initially by doing PCR followed by Southern blottings.

Confirmation of putative transformants using polymerase chain reaction (PCR): PCR was found to be one of the most effective and routinely used technique for initial confirmation of transgenic plants. With respect to CB (*BtCryIAb*) plants, amplification of the expected 700 bp fragment specific to *npt*II gene was observed in 5 out of 10 tested plants. Genomic DNA samples of CB1, CB2, CB3, CB5 and CB7 showed prominent amplifications. 1.2 kb amplified fragment specific for *uid*A gene was observed in 8 out of 10 samples. With respect to CS plants with *SBTI* gene, 5 out of 9 plants (CS1, CS2, CS4, CS5 and CS9) showed amplification of expected 700 bp fragment of *npt*II gene and 5 from 9 plants (CS2, CS3, CS5, CS6 and CS9) showed respective 1.2 kb fragment of *uid*A gene amplified. A faint band also appeared in CS8. Results of the PCR amplifications of *npt*II gene fragments from samples of Bt and SBTI plants are shown in the Fig 4.14 and results of the PCR amplification of 1.2 kb fragment specific to *uid*A gene are shown in the Fig 4.15. With respect to *BtCryIAb* gene amplifications two sets of oligonucleotide primers were used and one set showed variable bands with different molecular weights and only 3 samples (CB2, CB3 and CB4) showed amplification of the respective 908 bp fragment with the other set of primers. Results of the PCR amplifications of fragment specific to *BtCryIAb* gene are shown in the Fig 4.16 (B). Fig 4.16 (A) shows results of *SBTI* gene amplification. A 497 bp. fragment was found in CS2 from 9 of the tested samples.

Factors affecting restriction of genomic DNA: Uniform and efficient restriction of genomic DNA is a prerequisite for an effective Southern hybridization and the constituents of the restriction reaction mixture play a direct role in the process. However, during the course of this study, it was found that it was very difficult to obtain large amounts of genomic DNA consistently. Hence, efforts were made to optimize the restriction of the

genomic DNA for Southern analysis. Genomic DNAs of the putatively transformed chickpea plants were digested either with *Eco*R1 or *Bam*H1 enzymes for which addition of BSA was not prescribed. Observations showed that BSA is required in the reaction mixture. Additional quantity of water with respect to enzyme concentration in the reaction mixture showed that quantity of the enzyme can be minimized if quantity of water can be optimized. Fig 4.17 shows the results of variations in the usage of enzyme, BSA and water in the restriction reaction mixtures. 5 µg of chickpea control plant genomic DNA was digested with *Bam*H1 at activity concentration of 3 units/µg of DNA. It was observed that 1% BSA concentration showing best restriction. Excess enzyme with an increased water also showed good digestion. Inclusion of excess water with BSA showed an degradation of the genomic DNA. Genomic DNA was not properly digested without any added water or BSA. These results shows that optimization of water and BSA concentrations as accessories in the reaction mixture can produce a better restriction of chickpea genomic DNA. Fig 4.18 shows the Southern blot results where there was limited restriction of genomic DNA extracted from the putative transformants where DNA was not properly digested. The non-radioactive signal was recorded at the top portion of the blot where the unrestricted DNA had accumulated.

Confirmation of T_0 generation putative transformants transformed with *BtCryIAb* gene by using Southern blotting:

Southern blotting is considered as the most reliable and effective technique to determine the integration and copy number of the transgene. A non-radioactive method by employing AlkPhos direct labeling kit was used in all the experiments. Fig 4.19 shows the results of the Southern blot hybridizations using *npt*II specific probe of non-radioactive

AlkPhos labeled 700 bp PCR fragment. *Eco*R1 provided single restriction within T-DNA region with respect to *npt*II gene (Fig 3.4). Hence, restriction of genomic DNA of putative transformants with *Eco*R1 was expected to give copy number of the integrated transgene. A total of 7 plants out of 11 tested plants showed integration of the *npt*II with a variable copy number. CB1 showed four copies of integrated *npt*II genes. Sample CB7 showed two copies and samples CB2, CB3, CB4, CB5 and CB11 showed single copy insertions. Fig 4.20 shows the results of confirmation of Southern blot hybridization specific for *BtCryIAb* gene. The *BtCryIAb* gene was probed with the non-radioactive AlkPhos direct-labeled 908 bp PCR fragment. With respect to *BtCryIAb* gene integration, the genomic DNA of putative transformants was digested with *Eco*R1 enzyme that results double restriction within the *BtCryIAb* gene and releases 3 kb fragment (Fig 3.4). A total of 8 out of 11 tested plants showed integration of *BtCryIAb* gene by showing the expected 3 kb fragmen. CB1, CB2 and CB11 showed very faint bands while CB3, CB4, CB5, CB7 and CB8 showed very prominent bands.

5.0 DISCUSSION

Modern biotechnology including tissue culture, genetic engineering and genetic transformation techniques has provided new opportunities to enhance the germplasm of crop plants (Sharma and Ortiz, 2000). Until recent times improvement of chickpea has been mainly through breeding approaches. However, highly time consuming nature of breeding approach and restricted applicability with respect to some traits makes it an incomplete strategy for chickpea improvement. Wild relatives of chickpea possess genes for resistance towards fungal disease and cold stress. However, classical breeding attempts by utilizing both cultivated and wild germplasms to introgress inherent resistance in cultivated chickpea have been carried out but could not lead to any breakthrough in the scenario. Alternative approaches especially biotechnology has gained an increased significance recently. Chickpea biotechnology started with elucidating factors affecting micropropagation and regeneration and whole plants from some selected explants. Besides micropropagation various tissue culture methods were worked out so as to facilitate incorporation of some selected monogenic traits through genetic transformation. However, perceived recalcitrant nature of chickpea in in vitro environment posed serious problems to achieve these goals. All direct and indirect methods of regeneration such as somatic embryogenesis and organogenesis were tried to accomplish an efficient protocol for incorporating alien genes into chickpea.

During the last decade, biotechnology has made impressive advancements. Genetic transformation of crop plants is its valuable aspect, leading towards the building of an organized and healthy agriculture system free from the use of polluting insecticides and fungicides. Exploring the possibilities of transferring pest resistance genes in pea

(Shade et al., 1994; Morton et al., 2000) has opened new vistas for transferring agronomically important traits into other crop plants to develop elite cultivars (Birch, 1997). The technology emphatically supports the transfer, integration and expression of foreign genes in heterologous organisms. Agronomically important genes to improve the crop yield qualitatively as well as quantitatively have been isolated and cloned from various sources. These include genes for fungal resistance- chitinases and glucanases; viral resistance- coat protein, replicase and ribosome inhibiting proteins; pest resistance- amylase inhibitors, proteinase inhibitors and Bt toxin genes and genes for nutritional improvement- 2S albumin gene. Biotechnology encompassing genetic engineering offers avenues to overcome the barriers in achieving maximum chickpea production.

5.1 Tissue culture studies

5.1.1 Somatic embryogenesis: Somatic embryogenesis is a potential strategy to incorporate alien genes especially with biolistics method, even when the basic tissue is meristematic. However, this strategy like any other one needs a high frequency of induction and maturation of the somatic embryos in a synchronous manner. Various protocols appeared in chickpea somatic embryogenesis that did not satisfy the requirements for genetic transformation. Early reports of Rao and Chopra (1989), Shankar and Ram (1993) used 2,4-D to induce embryos and cytokinins like BAP to mature them. In a comparative study, the zygotic embryos isolated from immature pods three to fifteen days after pollination were compared with somatic embryos obtained from immature cotyledonary segments and embryo axes induction on MS containing 2,4,5-T (Suhasini et al., 1997; Sagare et al., 1999). Somatic embryogenesis and whole plant regeneration was studied in immature leaflets (Barna and Wakhulu, 1993) and mature

leaflets (Dineshkumar et al., 1994). Zeatin and IAA were used to induce somatic embryos from immature cotyledons. Very high frequency of plantlets were regenerated from these embryos (Hita et al., 1997). However, all these protocols reiterated the difficulty of maturation and regeneration of whole plants via somatic embryogenesis pathway. This was re-confirmed in our experiments. A very good frequency of induction was observed from mature embryo axis and leaflet explants. Embryogenic tissue was observed to induce better embryogenesis. Mesophyll tissue of young leaflets was also found to induce somatic embryos. Reproducibility of the published reports becomes questionable as the media combinations used by them failed to mature the embryos. The published protocols were repeatedly tried with little or no success. These results suggest that regeneration protocol via somatic embryogenesis cannot be a reliable one for genetic transformation experiments.

5.1.2 Oraganogenesis: Micropropagation and regeneration via direct or indirect organogenesis have been extensively studied and several of reports were published. Inclusion of meristematic zones with the explants was found to be crucial for regeneration of shoot buds and whole plants in chickpea. Early reports on chickpea showed callus initiation and growth with no regeneration of plants (Rao and Chopra, 1987; Riazuddin et al., 1988). Mature embryo axis, shoot tip and cotyledonary nodes were the most studied explants. Most of the reports consisted of BAP as the principal multiple shoot inducing growth regulator. Seeds devoid of radicle tip gave rise to as many as 20 shoots with 5 μM BAP (Polisetty et al., 1997). Mature seeds were cultured on TDZ containing medium and an increased number of multiple shoots was observed (Malik and Saxena, 1992). In vitro culture of seeds obviates the complexities regarding

selection and preparation of explants. The technique proves useful when large-scale multiplication of a species is required. In other words this explants is useful for micropropagation alone and zygotic embryo based cultures were not considered as ideal for transformation studies. Other explants like immature cotyledons (Shri and Davis, 1992) cotyledonary node (Subhadra et al., 1998; Sharma et al., 1998) and embryo axis (Fontana et al., 1993) were also used that employed BAP based media for multiple shoot induction. Due to the limited applicability of the multiple shoots that were regenerated by using BAP based media for genetic transformation, various media accessories were used to enhance the frequency and adventitious nature of those shoots. Amino acids such as L-serine and L-asparagine (Riazuddin et al., 1988), L-adenine (Sangvan et al., 1989) and L-glutamine (Vani and Reddy, 1996), ethylene inhibitor silver nitrate (Eapen and George, 1994; Kumar et al., 1995) were employed in the culture media so as to increase the number of multiple shoots. However, the degree of enhancement was very low while most of the treatments gave rise to more or less similar number of multiple shoots. Some unconventional hormones used in organogenesis experiments such as zeatin (Suhasini et al., 1994; Adkins et al., 1995) and picloram (Shankar and Ram, 1993; Eapen and George, 1994) were employed to regenerate multiple shoots at higher frequencies. The results have not been reproducible with some of the interesting reports making their usage in genetic transformation experiments doubtful.

Indirect regeneration via callus phase is not a reality in chickpea and viability of some reports were checked using all the published combinations with some additional variations. Whole cotyledons and shoot tips were cultured on BAP and NAA containing media and regeneration with a prior growth of callus was observed (Prakash et al., 1992).

Hypocotyls and epicotyls were ideal for induction of callus and they were used to give rise to callus on BAP, kinetin and NAA that subsequently regenerated shoot buds (Khan and Ghosh, 1984; Neelam et al., 1986). Barna and Wakhulu (1994) demonstrated muliple shoot induction from immature leaflet derived callus induced by 2,4-D and regeneration promoted by BAP. However, repetition of these experiments confirmed that any traces of meristem in the axillary bud region showed the low frequency of differentiation reported. Cut portions of the explants like hypocotyl, epicotyl, stem segments and root segments showed undifferentiated growth of cells that formed friable callus. Clearly no differentiation and regeneration of shoot buds could be achieved from this callus. Very few explants showed shoot bud induction at ends and this low frequency is a function of traces of axillary or apical meristems left with the explants while processing. Such regeneration was also observed with the AM3 explant in the present study.

Usage of excess cytokinin in the organogenesis experiments negatively interferes with the shoot bud induction and later stages of regeneration. This was exemplified with the observations on embryo axis and axillary meristem explants where BAP and TDZ were used for these explants respectively. Embryo axes showed better regeneration at lower concentrations of BAP while the high concentrations showed stunted growth of the shoot buds that did not elongate well. It can be suggested that excess cytokinin is taking over the activities of all the other types of growth regulators while inhibiting the shoot bud promotion activity of itself. These reasons have to be thoroughly investigated. Similar inhibition of shoot bud differentiating activity of cytokinin at higher concentrations was also observed with TDZ. However, the concentration of TDZ antagonistic to regeneration was found to be lower than that of BAP. Hence, TDZ was

used with an additional care and observation. Shoot buds induced on BAP-containing medium showed a better elongation compared to the ones grown on TDZ. However, number of shoots per explant on TDZ medium was 1.5 to 2 times better than BAP media.

As is mentioned above, additional care should be exercised while using TDZ in the culture media. Ever since 1988, TDZ has been reported to induce adventitious shoot formation in a number of species, especially woody plants (Briggs et al., 1988; Henry and Fooshee, 1990). However, there are several disadvantages in using TDZ that include stunted shoots (Cambecedes et al., 1991), abnormal leaf morphology (Fasolo et al., 1989) and difficulty in elongation and rooting of the regenerated shoots (Meyer and van Staden, 1988). To overcome these problems, explants should be induced with the lowest but effective TDZ concentration and kept on TDZ medium for the least duration that is specific for each species. Shoot quality can be considerably improved by employing a purine cytokinin in combination with TDZ (Briggs et al., 1988). The shoots originated on TDZ containing media were short, but elongated after transfer to the medium containing IBA and 2-iP (Preece and Imel, 1991). In chickpea, multiple shoot regeneration was reported after the application of TDZ to the whole seed (Malik and Saxena, 1992). The use of pre-existing meristems such as shoot tip and cotyledonary nodes, and pre-treatment of whole seed with lower concentrations of TDZ and BAP prior to preparation of explants was found to be an effective technology in difficult crops such as chickpea (Sharma and Amla, 1998). In contrast, the regeneration system described in this study uses tissues that produce fresh meristematic cells that are more amenable to gene incorporation by preferably *Agrobacterium* method. Incorporation and standardization of

appropriate concentration of TDZ in germination and induction media is a significant modification over existing protocols.

As it was reiterated many times throughout this thesis, most of the previous reports used explants having pre-existing meristems such as embryo axis, shoot tip, cotyledonary node etc. However, selection and processing of proper explant is prerequisite for genetic transformation. Serial culturing of mature embryo axis and shoot tip resulted in an exponential increase in the number of multiple shoots. This was achieved using BAP (Sheila et al., 1991). However, Fig 4.4 A and C shows that the regeneration of multiple shoots is not synchronous and shoots originated one after the other. Removal of axillary bud, cut through the axillary meristem and inclusion of cotyledon were the significant variations achieved in the present study. A comprehensive protocol for regeneration and recovery of whole plants of chickpea by using axillary meristem explants was reported (Jayanand et al., 2003). The present study further shows that the AM2 and AM4 explants shows almost similar shoot regeneration efficiencies and either can be used. It was observed that multiple shoots originating from the regions adjacent to the amputated axillary bud were synchronous showing direct relevance to genetic transformation. Removal of axillary bud provides proper wounding through which the *Agrobacterium* can reach the regenerating tissue. Results showed that axillary meristem explants scores over the apical meristem explants like shoot tip and cotyledonary nodes in terms of the number of shoots per explant. The AM2 and AM4 explants can especially be ideal for genetic transformation as they had proper wounding and number of shoots per explant was more. Reason that can be suggested here is a coordinated physiological signal within the apical meristem promotes the organized

tissue to regenerate into lesser number of multiple shoots. In the case of axillary meristem, removal of axillary bud nullifies the apical dominance. In addition to this, the signal provided by the externally applied cytokinin, might be divided by pockets of the organized apical meristem which otherwise will grow into a single shoot on basal MS medium. The tissue adjacent to the axillary bud area that has meristematic tissue receives the signal provided by externally applied cytokinin, regenerates into more number of shoots. Effect of age of the seedling from which the explants were derived shows that there is gradual loss of multiple shoot regenerating capacity of the meristematic tissue with age. We have standardized an optimum age of the seedling from which various explants will be derived. Nodal and axillary meristems have been promising tissues for micropropagation of various economically important plant species. Exploitation of high regenerating capacity with convincingly adventitious nature of the regenerating multiple shoots from the axillary meristem has been a point to ponder in developing in vitro regeneration protocols of various plants. Axillary meristem was cultured under various names and culture conditions preferably in direct micropropagation experiments. Most of the published protocols showed cotyledonary node cultures without intact cotyledon of chickpea (Chandra et al., 1993; Shankar and Ram, 1993). It has been shown that qualitative presence or absence of the cotyledonary lamina along with the regenerative tissue has a direct correlation with shoot or root differentiation potential in *Brassica juncea* (Sharma et al., 1991). Our observations showed that there was 2 to 3 folds increase in the number of shoots per explant with inclusion of cotyledon. The rate at which multiple shoots regenerated and their growth was proportional to the size of

cotyledon included. Axillary meristem explants devoid of cotyledon showed lesser number of shoots that multiplied further on prolonged culturing on the same medium.

pH of the external medium is known to play a significant role in tissue culture as it affects uptake of various nutrient ions. Uptake of nitrate and ammonium are primarily affected by the medium pH (Behrend and Mateles, 1975; Raven, 1986). It was observed that nitrate uptake is favoured at low pH and ammonium at higher pH. Axillary bud multiplication in shoot cultures of Castanea was most satisfactory when the pH of MS was reduced to 4 (Chevre et al., 1983). Results with chickpea showed that lower pH 5.0 was good for induction while 5.5 and 6.0 were better for elongation and rooting respectively. It was observed with chickpea that decrease in the concentration of nitrates in the form of KNO_3 was favorable for rooting. The nitrate uptake is favored at lower pH and ammonium at higher pH, it can be hypothesized that ammonium ions might be favoring rooting at higher pH and nitrates favoring shoot induction.

Standardization of induction medium was done considering all stages of plant regeneration such as elongation and rooting. Firstly, lowest but effective concentration of TDZ was found to be between 4 to 10 µM. In view of the fact that higher concentration of TDZ negatively interferes with the number of shoot buds and their elongation, 4 µM was considered as optimal working concentration. Number of shoot buds also decreased with increase in TDZ concentration. Very high concentration of the TDZ was observed to give stunted shoot buds with little or no elongation. In addition to this extended time of culture on TDZ also has some negative effect on the later stages. Though 4 µM TDZ was optimal working concentration, culturing the explants on the same medium for 5 to 6 weeks decreased the elongation frequency. Application of any growth regulators after the

prolonged culture on TDZ showed no promotive effect on elongation. Hence, the induction time was restricted to 2 to 3 weeks. With respect to AM4 explant, the whole preparation was done on TDZ containing media and hence MS medium was used as induction medium, as the multiple shoot buds were induced by TDZ already taken up by the explant. Elongation of shoot buds decreased from AM4 explant cultured on JCR13 medium when compared to the ones cultured on MS. Inclusion of 2-iP and kinetin was found to be even more effective by providing a faster and efficient elongation. Increase in kinetin concentration in the induction medium was also found to have some negative influence on induction. There was chlorosis of the induced shoot buds in higher concentrations of kinetin more than the ones that are tabulated. TDZ was completely excluded in the elongation medium. Employing the method in stepwise manner considerably enhanced elongation. Elongation of induced shoots generally consists of two phases, initiation and then increase of number and internodal length. CEL2 containing 5 µM 2-iP and 2 µM kinetin medium elongated first batch of shoots in about 2 to 3 weeks. Sub-culturing of shoot buds on the medium with same composition, few more shoots were elongated. However, sub-culturing the shoots on CEL7 containing 2 µM GA_3 after they were cultured on CEL2 containing 5 µM 2-iP and 2 µM kinetin for about 2 weeks considerably increased elongation frequency. GA_3 present in the CEL7 medium is shown to play and important role in enhancing the elongation frequency. For unknown reasons, frequency of shoots elongated on GA_3 containing medium right from the first phase was not as good the frequency obtained in stepwise manner. All the later sub-cultures on CEL7 resulted in notable increase in the number of shoots elongated. Sub-culture of CEL2 elongated shoots on CEL7 again resulted in a better morphology in

terms of leaflet morphology and internodal length. Prolonged culture of shoots on CEL2 medium dried earlier than the ones cultured on CEL7. Hence, elongation was done initially for about 2 weeks on CEL2 followed by CEL7 in all later passages.

Past record of rooting of elongated shoots of chickpea with any of the popular media for rooting did not show considerable frequency. Polisetty et al., (1996) reported very high frequency of rooting, a method which was not reproducible. A novel technique of rooting on filter paper bridges that were immersed in liquid rooting medium was developed. Rooting was also done on semi-solid medium and a comparative study was done. Rooting frequency in liquid medium was 5 to 8 times better than the rooting frequency in semi-solid medium. Frequency was very high in liquid medium at 70 to 90% compared to 5 to 10% on semi-solid medium. The newly developed method not only ensured better frequency but also showed better morphology and increased survival time of the plants. In other words, early desiccation of shoots cultured on semi-solid medium was prevented in liquid medium making them amenable for sub-culturing. However, there were two main disadvantages of the newly developed method. Firstly, the shoots acquired hyperhydricity on serial sub-culturing and secondly, the shoots showed an increased growth rate. The hyperhydricity problem was restricted to the shoots only in the sub-culturing stages and persisting problem could be managed by placing the rooting culture tubes in the sterile environment (laminar flow) with cotton plugs open for about few hours. The second problem could be managed by using longer culture tubes (25x200 mm) and removing the excessive lower part of the stem during the sub-cultures. Additional care was exercised to achieve the high frequency of rooting. Firstly, dark green, healthy shoots with well-developed leaflets were selected for rooting that did not

exceed 5 cm length. Longer shoots posed the above-mentioned problem increased length in the culture tubes. Shoots that did not root should not stay for longer time in the same culture medium and be transferred to the fresh medium after the first batch has rooted. Care should be taken not to allow the shoots to acquire hyperhydricity because such shoots do not root well and have to be carried to the phase 2 of rooting.

Hardening and transplantation of chickpea has been a serious problem for the tissue culture grown plants. To overcome this problem different laboratories have adapted variety of strategies. Polisetty et al., (1996) reported that placing the plants in liquid can increase the hardening frequency. Some workers resorted to inefficient in vitro grafting by utilizing the root system from the pre-germinated seedlings (Krishnamurthy et al., 2000). However, such methods are not only time consuming and technique specific and the success rates varied among various laboratories. The rooting method described above not only increased the rooting frequency but also promoted a distinct increase in the number of hardened plants. The comprehensive protocol for hardening and establishment of the in vitro grown chickpea plants is described in the above sections is reproducible and efficient. The main problems with the establishment of chickpea plants were lack of growth of rooting system and humidity acclimatization. Owing to the efficiency of static hydroponics system that ensures the growth of root system can be considered as the best method for hardening. The other method of hardening and transplantation through three different stages was also efficient and reliable, however, simplicity, rate and in total the efficiency of hydroponics systemis superior. In addition, the 3-stage method employs humidity establishment by covering and their gradual opening in the later stages, which is very case sensitive. Even minor variations of humidity management resulted in either

wilting or drying of the plants. This problem was negated in the hydroponics system. However, a careful examination of growth of the stem and root system, leaf morphology should be done while hardening the rooted plants in static hydroponics. Potting medium was one of the important factors that affect chickpea hardening and establishment. Chickpea generally prefers black soil. However, growth of root system was greatly inhibited by this potting medium in all the three stages described in the above sections. Sand was used at higher proportions to provide better aeration and relatively less amount of black soil was added to maintain the water holding capacity. Other factors like temperature, light intensity, photoperiod, irrigation and were also standardized. Optimal conditions for hardened chickpea were also standardized and the walk-in type Conviron™ growth chamber ideally provided these conditions. Establishment in the green house or any other harsh environment can be detrimental to the hardening of chickpea. One limitation of the chickpea establishment studies is that the requirement of walk-in type growth chamber, which makes hardening process somewhat restricted to few institutes that can afford the facility. Static hydroponics system is an efficient system that can harden in vitro produced chickpea plants in the normal culture room conditions without compromizing the integrity and vitality of the plant. In vitro derived chickpea plants showed an early flowering and maturity. In some instances the plants flowered in culture tubes while they were rooting. This suggests that mild conditions of temperature, light intensity and humidity provided in the culture room might activate respective genetic elements for flowering and maturity. This rate of maturity was found to be slower in case of field-sown seedlings. Removing the flowers as and when they appeared considerably decreased the rate of maturity. This treatment promoted a better growth of

stem and leaves. Secondary branches were also promoted by this treatment. This sort of promotion of vegetative growth gains significance while maintaining and processing of invaluable transgenic plants.

5.1.3 Histological studies of multiple shoot development from AM4 explant:

Development of multiple shoots from AM4 explant was found to be adventitious phenotypically. However, their adventitious nature was confirmed after the histological observations carried out at different stages of growth of the explant. Broadly the AM4 explant development occurs in two stages, 1. up to axillary bud removal and 2. up to the stage of removal of multiple shoot buds originating from regenerating area. Presence of axillary bud exerts pressure of apical dominance on the tissue of cotyledonary node region. Hence, as expected removal of axillary bud negates the apical dominance and different regions in the uniform non-meristematic area received stimulus from the applied cytokinin resulting in the development of meristemoid regions. These regions on further growth developed into multiple shoot buds that originated adventitiously. Removal of the regenerating multiple shoot buds at the end of stage 2, results in increased meristemoid development and also provides multiple wounding areas for successful infection of the tissue with *Agrobacterium* for genetic transformation. Basing on these results it can be suggested that removal of multiple shoot buds on day-5 or day-6 and co-cultivation for 72 hours could give better frequency of genetic transformation.

5.2 Genetic transformation:

Transforming the AM4 explants with the binary constructs containing *BtCrylAb* and *SBTI* genes, produced chickpea transgenics. In accordance with the conventional

molecular analysis of the transgenic plants, the plants were initially screened for GUS histochemical assay and about 60% of the plants showed positive reaction. *uid*A gene activity was clearly demonstrated in vascular tissue. The terminal young leaflets showed a clear blue color activity of the incorporated *uid*A gene. However, the later stages of the molecular analysis showed some variable results in terms of number of positives in PCR confirmation and Southern analysis.

Polymerase chain reaction is one of the most efficient system for analysis of the putative transformants. The selective amplification of a fragment specific to the incorporated gene invariably confirms the presence of the respective gene in the genome of the transgenic plant. However, due to some variations unknown the PCR results showed some variable results in the putative transformants. The plants with *BtCrylAb* gene showed a 50% transformation frequency with respect to the *npt*II gene in the PCR confirmation analysis. Five plants out of 10 showed amplification of the 700 bp fragment that was specific to the *npt*II gene. Similarly five out of 9 tested plants with *SBTI* showed amplification of the respective fragment. With respect to 1.2 kb fragment amplification from the *uid*A gene, there was slightly increased frequency. 8 out of 10 *BtCrylAb* plants showed amplification and 5 out of 9 *SBTI* plants showed the respective amplified fragment. It can be suggested here that some impurities incumbent in the reaction mixture might be interfering with the amplification. Hence, frequency of confirmed transformation with respect to the marker genes can be fixed in the range of 50 to 70% of the putative transformants. Much lower frequency of transformation was observed with respect to the agronomically important and insect resistance genes *BtCrylAb* and *SBTI*. Only four out of 10 tested samples from the plants transformed with *BtCrylAb* showed

amplification of the respective 908 bp fragment. Comparatively high GC content of the primer and the gene could be the reason for this lower frequency compared to the marker genes. Only one sample from the plants transformed with *SBTI* gene out of 9 tested plants showed the amplification of respective 497 bp fragment suggesting some incumbent problems with the primer composition.

Southern analysis has been the most authentic technique to confirm the putative transformants ever since the genetic transformation research began. This technique is widely used to analyze the incorporation and copy number of the integrated gene in the genome of the transgenic plant. PCR amplified fragments of *npt*II and *BtCryIAb* gene were used to probe the integrated gene in the transgenic plants. Chickpea was found to be highly recalcitrant in the tissue culture system due to which the transformation frequency reported to date has been very low at around 2% (Kar et al., 1997; Krishnamurthy et al., 2000). Biolistic-mediated transformation was done into zygotic embryos and the transient expression of nptII and uidA genes was studied (Husnain et al., 1997). However, modification of the axillary meristem explant by way of removing the axillary bud and negating the apical dominance of some shoot buds regenerating initially, gave much better results in terms of transformation frequency. T_0 generation of the putative transformants was tested for incorporated genes and around 70% of the tested plants showed incorporation of the transgene in their genomes. Initial experiments when the conditions for optimal restriction of the genomic DNA were not standardized, showed the signal for the transgenes at a non-specific regions of the blots and standardization of the conditions such as quantities of enzyme, BSA and water was done and the actual results were obtained. A total of 7 plants out of 10 tested plants showed integration of the *npt*II

with a variable copy number. CB1 showed four copies of integrated *npt*II genes. Samples CB2, CB3, CB7 and CB11 showed two copies each and samples CB4 and CB5 showed single copy insertions. With respect to *BtCrylAb* gene integration the genomic DNA of putative transformants was digested with *Eco*RI enzyme that releases 3 kb fragment of the *BtCrylAb* gene. A total of 8 plants showed integration of *BtCrylAb* gene out of CB1, CB2 and CB11 showed very faint bands. However, CB3, CB4, CB5, CB8 and CB9 showed very prominent bands. This system using *Agrobacterium* as the mediator for the transferring the necessary genes into the plant genome resulted in relatively low frequency of plants with single copy insertions.

5.3 Conclusions:

In conclusion the regeneration system is very efficient in terms of adventitious multiple shoot regeneration and the protocol gains significance owing to its person and lab independent reproducibility. This regeneration system resulted in a comparatively high frequency of genetic transformation and recovery of valuable transgenic plants. The transgenic plants with the incorporated *BtCrylAb* and *SBTI* genes will be very useful in forthcoming future and can considerably add to the field of biotechnological improvement of chickpea. The diagrammatic representation of the protocol for efficient regeneration and transformation of chickpea is as follows (see following page).

Explant Preparation

-Germinate seeds on SIM [MS+4 μM TDZ+2 μM kinetin] for 1 wk

-Prepare AME explant by removing axillary bud from the cotyledonary node

↓ *Agrobacterium* infection

Shoot bud induction

Step 1: Culture AME explant on SIM medium for 2 wk

Step 2: Sub-culture explants with shoots buds to MS basal medium for 5 d

↓

Shoot elongation

Step 1: Transfer shoots to SEM1 [MS+5 μM 2-iP+2 μM kinetin] for 10 d

Step 2: Transfer unelongated shoots to SEM2 [MS+2 μM GA_3] for 2 to 3 wk

↓

Rooting of shoots

Phase 1: Transfer shoots toliquid RIM [MS+9.4 mM KNO_3+2% sucrose+5 μM IBA] on filter paper bridge for 2 wk [60-70% shoots rooted]

Phase 2: Pulse treatment of shoots with 100 μM IBA and culture on filter paper bridge in liquid MS for 2 wk [10-20% shoots rooted]

Phase 3: Transfer unrooted shoots to static hydroponic system containing 1/4 Arnon's nutrient solution+3 μM IBA for 2-3 wk [10-15% shoots rooted]

↓

Transplantation

Step 1:Transfer rooted shoots to 8 cm (dia) pots containing 2-4 mm sand. Cover the plants with polypropylene bags and gradually open the covers over 7-10 d period.

OR

Suspend the rooted shoots in Magenta jars containing 1/4 Arnon's nutrient solution.

Step 2: Transfer the hardened plants to 20 cm (dia) pots containing sand:black soil (3:2)+Cell Rich (5%) +rice straw compost (5%).

Schematic representation of the protocol for in vitro regeneration of whole plants from axillary meristem explant (AME) of chickpea [Jayanand & Sharma, 2003]

Explant Preparation

-Germinate seeds on SIM [MS+4 μM TDZ+2 μM kinetin] for 1 wk

-Prepare AME explant by removing axillary bud from the cotyledonary node

Agrobacterium infection

Shoot bud induction

Step 1: Culture AME explant on SIM medium for 2 wk

Step 2: Sub-culture explants with shoots buds to MS basal medium for 5 d

Shoot elongation

Step 1: Transfer shoots to SEM1 [MS+5 μM 2-iP+2 μM kinetin] for 10 d

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Phase 3: Transfer unrooted shoots to static hydroponic system containing 1/4 Arnon's nutrient solution+3 μM IBA for 2-3 wk [10-15% shoots rooted]

Transplantation

Step 1:Transfer rooted shoots to 8 cm (dia) pots containing 2-4 mm sand. Cover the plants with polypropylene bags and gradually open the covers over 7-10 d period.

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Suspend the rooted shoots in Magenta jars containing 1/4 Arnon's nutrient solution.

Step 2: Transfer the hardened plants to 20 cm (dia) pots containing sand:black soil (3:2)+Cell Rich (5%) +rice straw compost (5%).

Schematic representation of the protocol for in vitro regeneration of whole plants from axillary meristem explant (AME) of chickpea [Jayanand & Sharma, 2003]

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APPENDIX

1. MURASHIGE AND SKOOG (1962) MEDIUM

SALT	CONC (mg/L)	STOCK (per L)	USE (per L)
Major salts (X50)			
NH_4NO_3	1650	33.0 g/200ml	10 ml
KNO_3	1900	38.0 g/400 ml	20 ml
KH_2PO_4	170	3.4 g/ 200 ml	10 ml
$CaCl_2$	440	8.8 g/200 ml	10 ml
$MgSO_4.7H_2O$	370	7.4 g/200 ml	10 ml
Minor Salts (X100)			
H_3BO_3	6.20	6.20 mg/L	5 ml should be
KI	0.83	83.0 mg/L	taken from the stock
$MnSO_4.4H2O$	22.3	2230 mg/L	solution prepared by
$ZnSO_4.7H2O$	8.6	860 mg/L	mixing all the
$Na_2MoO_4.2H_2O$	0.25	25 mg/L	constituents
$CuSO_4.5H_2O$	0.025	2.5 mg/L	
$CoCl_2.6H_2O$	0.025	2.5 mg/L	
IRON (X 100)			
$Na_2EDTA.2H_2O$	37.3	3.73 g/L	} 10 ml
$FeSO_4.7H_2O$	27.8	2.78 g/L	
OR			
$FeNa_2EDTA$	40	2.0 g/500 ml	10 ml
Organics (X100)			
Glycine	2.0	200 mg/L	}
Nicotinic acid	0.5	50 mg/L	} 10 ml
Thiamine HCl	0.1	100 mg/L	}
Pyridoxine HCl	0.5	50 mg/L	
m-Inositol	100	5.0 g/500 ml	10 ml

2. Fixative used for fixation of tissue culture samples:

Acetic acid and 95% Ethanol were mixed at 1:3 proportions.

3. Hematoxylin-Eosin stain: 50 g of ammonium or potassium alum was dissolved in 1 litre of water without heating. Then 1 g hematoxylin was dissolved in this solution. Further, 0.2 g of sodium iodate, 1 g of citric acid and 50 g chloral hydrate were added. The components were shaken until all of them are in solution. The color of the stain was reddish violet. For Eosin stock solution, 1 g of Eosin (water soluble) was dissolved in 20 ml of distilled water and made up to 100 ml with 95% ethanol.

3. DNA extraction buffer (DELLAPORTA method)

100 mM Tris pH8

50 mM EDTA pH8

500 mM NaCl

10 mM Mercaptoethanol

4. Primary wash buffer (1 litre) (for Southern analysis)

Name of the compound	Working concentration
Urea	2 M
SDS	0.5%
NaP (pH 7.0)	50mM
NaCl	150 mM
$MgCl_2$	1mM
Blocking Reagent	0.2%

5. Secondary wash buffer (20X stock)(for Southern analysis)

Tris base 121 g. 1M

NaCl 112 g. 2M

Adjust pH to 10.0. Make upto 1 litre with water. This can be kept for up to 4 months. 1:20 i.e., 2 ml/L of 1M $MgCl_2$

Table 3.1

Primer compositions of all the four genes used for genetic transformation, their respective annealing temperatures, and size of the respective fragments amplified.

Name of the gene	Sequence of the primers		Annealing temperature (°C)	Size of the fragment amplified
*npt*II	Forward	GAG GCT ATT CGG CTA TGA CTG	58.5	0.7 kb
	Reverse	ATC GGG AGC GGC GAT ACC GTA		
*uid*A	Forward	GGT GGG AAA GCG CGT TAC AAG	57.5	1.2 kb
	Reverse	GTT TAC GCG TTG CTT CCG CCA		
BtCryIAb	Forward	TCA CCC AGT TCC TCC TCA GCG AGT T	63.1	0.908 kb
	Reverse	GGC GTT GCC CAT CGT GCC GTA GAG C		
SBTI	Forward	AAC AGC ATT TGG TGG AAT AAG	58.0	0.497 kb
	Reverse	AAA GGC CAT GAT TTT TCT TGG		

Table 4.1

Induction of somatic embryos on MS containing combinations of 2,4,5-T (2.0 and 5.0 μM) with TDZ, BAP, kinetin or zeatin. The results were recorded at the end of 4 weeks and represent means of three replications.

Medium name	Concentration of Plant Growth Regulators (μM)					No. of explants cultured	No. of explants responding	% Explants with embryo-genesis	Mean embryos per responding explant
	2,4,5-T	TDZ	BAP	kinetin	zeatin				
JEM1	2.0	0.5	-	-	-	36	6	17	3.33 ± 0.58
JEM2	2.0	1.0	-	-	-	36	8	22	4.67 ± 1.53
JEM3	2.0	-	0.5	-	-	36	8	22	2.33 ± 1.53
JEM4	2.0	-	1.0	-	-	36	6	17	1.33 ± 0.58
JEM5	2.0	-	-	-	0.5	36	10	28	4.67 ± 2.08
JEM6	2.0	-	-	-	2.0	36	13	36	3.33 ± 1.53
JEM7	2.0	-	-	-	5.0	36	14	39	4.00 ± 2.00
JEM8	2.0	-	-	0.5	-	36	16	44	6.67 ± 1.53
JEM9	2.0	-	-	2.0	-	36	17	47	7.67 ± 1.53
JEM10	2.0	-	-	5.0	-	36	17	47	6.33 ± 1.15
JEM11	5.0	0.5	-	-	-	36	5	14	3.33 ± 0.58
JEM12	5.0	1.0	-	-	-	36	6	17	2.33 ± 1.53
JEM13	5.0	-	0.5	-	-	36	7	19	2.33 ± 1.53
JEM14	5.0	-	1.0	-	-	36	6	17	2.33 ± 1.53
JEM15	5.0	-	-	-	0.5	36	12	33	6.67 ± 0.58
JEM16	5.0	-	-	-	2.0	36	13	36	5.33 ± 0.58
JEM17	5.0	-	-	-	5.0	36	15	42	4.67 ± 0.58
JEM18	5.0	-	-	0.5	-	36	14	39	9.33 ± 1.53
JEM19	5.0	-	-	2.0	-	36	18	50	8.67 ± 2.52
JEM20	5.0	-	-	5.0	-	36	19	53	7.33 ± 1.53

Table 4.2

Induction of embryos on MS containing combinations of 2,4,5-T (10.0 and 15.0 μM) with TDZ, BAP, kinetin or zeatin. The results were recorded at the end of 4 weeks and represent means of three replications.

Medium name	Concentration of Plant Growth Regulators (μM)					No. of explants cultured	No. of explants responding	% explants with embryo-genesis	Mean embryos per responding explant
	2,4,5-T	TDZ	BAP	kinetin	zeatin				
JEM21	10.0	0.5	-	-	-	36	4	11	4.67 ± 0.58
JEM22	10.0	1.0	-	-	-	36	5	14	2.67 ± 1.53
JEM23	10.0	-	0.5	-	-	36	7	19	2.67 ± 0.58
JEM24	10.0	-	1.0	-	-	36	6	17	3.67 ± 0.58
JEM25	10.0	-	-	-	0.5	36	9	25	6.33 ± 1.15
JEM26	10.0	-	-	-	2.0	36	13	36	4.33 ± 0.58
JEM27	10.0	-	-	-	5.0	36	15	42	6.67 ± 2.08
JEM28	10.0	-	-	0.5	-	36	15	42	13.67 ± 1.15
JEM29	10.0	-	-	2.0	-	36	20	56	19.00 ± 2.00
JEM30	10.0	-	-	5.0	-	36	21	58	16.67 ± 1.15
JEM31	15.0	0.5	-	-	-	36	4	11	1.67 ± 1.15
JEM32	15.0	1.0	-	-	-	36	5	14	2.33 ± 1.53
JEM33	15.0	-	0.5	-	-	36	7	19	2.67 ± 0.58
JEM34	15.0	-	1.0	-	-	36	6	17	1.33 ± 1.53
JEM35	15.0	-	-	-	0.5	36	9	25	3.67 ± 1.15
JEM36	15.0	-	-	-	2.0	36	12	33	3.67 ± 2.08
JEM37	15.0	-	-	-	5.0	36	14	39	3.33 ± 1.53
JEM38	15.0	-	-	0.5	-	36	17	47	9.67 ± 1.15
JEM39	15.0	-	-	2.0	-	36	20	56	11.67 ± 1.53
JEM40	15.0	-	-	5.0	-	36	21	58	7.67 ± 1.15

Table 4.3

Induction of embryos on MS containing combinations of 2,4-D (5.0 and 10.0 μM) with TDZ, BAP, kinetin or zeatin. The results were recorded at the end of 4 weeks and represent means of three replications.

Medium name	Concentration of Plant Growth Regulators (μM)					No. of explants cultured	No. of explants responding	% explants with embryo-genesis	Mean embryos per responding explant
	2,4-D	TDZ	BAP	kinetin	zeatin				
JDM1	5.0	0.5	-	-	-	36	5	14	1.33 ± 0.58
JDM2	5.0	1.0	-	-	-	36	7	19	2.00 ± 1.00
JDM3	5.0	-	0.5	-	-	36	6	17	1.00 ± 1.00
JDM4	5.0	-	1.0	-	-	36	8	22	0.67 ± 0.58
JDM5	5.0	-	-	-	0.5	36	9	25	2.33 ± 1.15
JDM6	5.0	-	-	-	2.0	36	12	33	1.33 ± 0.58
JDM7	5.0	-	-	-	5.0	36	15	42	2.00 ± 1.00
JDM8	5.0	-	-	0.5	-	36	14	39	3.00 ± 1.00
JDM9	5.0	-	-	2.0	-	36	19	53	4.00 ± 1.00
JDM10	5.0	-	-	5.0	-	36	20	56	3.00 ± 1.00
JDM11	10.0	0.5	-	-	-	36	6	17	1.33 ± 0.58
JDM12	10.0	1.0	-	-	-	36	8	22	1.00 ± 1.00
JDM13	10.0	-	0.5	-	-	36	8	22	1.00 ± 1.00
JDM14	10.0	-	1.0	-	-	36	6	17	1.00 ± 1.00
JDM15	10.0	-	-	-	0.5	36	9	25	3.33 ± 0.58
JDM16	10.0	-	-	-	2.0	36	13	36	2.67 ± 0.58
JDM17	10.0	-	-	-	5.0	36	14	39	2.33 ± 0.58
JDM18	10.0	-	-	0.5	-	36	16	44	5.00 ± 1.00
JDM19	10.0	-	-	2.0	-	36	17	47	4.33 ± 1.53
JDM20	10.0	-	-	5.0	-	36	18	50	3.00 ± 1.00

Table 4.4

Induction of embryos on MS containing combinations of 2,4-D (15.0 and 20.0 μM) with TDZ, BAP, kinetin or zeatin. The results were recorded at the end of 4 weeks and represent means of three replications.

Medium name	Concentration of Plant Growth Regulators (μM)					No. of explants cultured	No. of explants responding	% explants with embryo-genesis	Mean embryos per responding explant
	2,4-D	TDZ	BAP	kinetin	zeatin				
JDM21	15.0	0.5	-	-	-	36	6	17	2.67 ± 0.58
JDM22	15.0	1.0	-	-	-	36	8	22	1.00 ± 1.00
JDM23	15.0	-	0.5	-	-	36	8	22	1.33 ± 0.58
JDM24	15.0	-	1.0	-	-	36	6	17	1.67 ± 0.58
JDM25	15.0	-	-	-	0.5	36	9	25	2.67 ± 0.58
JDM26	15.0	-	-	-	2.0	36	12	33	2.33 ± 0.58
JDM27	15.0	-	-	-	5.0	36	14	39	3.33 ± 1.53
JDM28	15.0	-	-	0.5	-	36	16	44	7.33 ± 0.58
JDM29	15.0	-	-	2.0	-	36	17	47	6.00 ± 1.00
JDM30	15.0	-	-	5.0	-	36	19	53	8.67 ±0.58
JDM31	20.0	0.5	-	-	-	36	5	14	1.00 ± 1.00
JDM32	20.0	1.0	-	-	-	36	5	14	1.00 ± 1.00
JDM33	20.0	-	0.5	-	-	36	7	19	1.67 ± 0.58
JDM34	20.0	-	1.0	-	-	36	6	17	0.33 ± 0.58
JDM35	20.0	-	-	-	0.5	36	9	25	2.00 ± 1.00
JDM36	20.0	-	-	-	2.0	36	12	33	1.33 ± 1.53
JDM37	20.0	-	-	-	5.0	36	15	42	1.67 ± 1.15
JDM38	20.0	-	-	0.5	-	36	14	39	4.67 ± 0.58
JDM39	20.0	-	-	2.0	-	36	20	56	9.33 ± 1.53
JDM40	20.0	-	-	5.0	-	36	22	61	4.00 ± 1.00

Table 4.5

Induction of embryos from various explants. The explants were cultured on JEM29 medium that contained MS with 10 μM 2,4,5-T and 2 μM kinetin. The results are the mean of three replicates.

Explant		No. of explants cultured	No. of explants responded	% explants showing embryo-genesis	Average no. of embryos per explant	Time taken for embryo induction (weeks)
Parts of mature embryo axis	Plumule	40	35	87.5	20.0±1.7	5
	Radicle	40	11	27.5	3.7±2.1	6
	Side arms	40	32	80	14.7±0.6	4
	Middle portion	40	38	95	15.3±2.1	5
Leaflets		40	31	77.5	23.7±3.1	5
Stem segments		40	27	67.5	11.3±0.6	8
Root segments		40	23	57.5	10.7±1.5	9

Table 4.6
Different combinations of plant growth regulators used to achieve maturation of induced somatic embryoids and responses observed

Medium name	Plant growth regulators (μM)						Result
	ABA	TDZ	zeatin	kinetin	GA_3	BAP	
JCM1	5	-	-	-	-	-	1% embryoids showed conversion into torpedo stage; failed to grow further
JCM2	10	-	-	-	-	-	2% of the embryoids showed conversion into torpedo stage; failed to grow further
JCM3	15	-	-	-	-	-	Embryos survived for a long time with little or no conversion
JCM4	20	-	-	-	-	-	Embryos survived for a long time with little or no conversion
JCM5	-	2	-	-	-	-	Embryoids converted into soft callus mass
JCM6	-	5	-	-	-	-	Embryoids converted into soft callus mass
JCM7	-	10	-	-	-	-	Embryoids converted into soft callus mass
JCM8	-	-	5	-	-	-	Embryoids showed signs of conversion but did not turn into torpedo stage
JCM9	-	-	10	-	-	-	Embryoids grew into green globuloid structures and failed to convert further
JCM10	-	-	15	-	-	-	Embryoids enlarged in size turned into green and failed to grow further
JCM11	-	-	-	5	-	-	Embryoids showed signs of conversion with groves but failed to grow further
JCM12	-	-	-	10	-	-	Embryoids showed signs of conversion with groves but failed to grow further
JCM13	-	-	-	15	-	-	Embryoids showed signs of conversion with groves but failed to grow further
JCM14	-	-	-	-	5	-	Embryoids slightly enlarged and showed no signs of conversion
JCM15	-	-	-	-	10	-	1% embryoids showed conversion into torpedo stage with no signs of further growth
JCM16	-	-	-	-	15	-	2% embryoids showed conversion into torpedo stage with no signs of further growth
JCM17	-	-	-	-	20	-	1% embryoids showed conversion into torpedo stage with no signs of further growth
JCM18	-	-	-	-	-	2	Embryoids converted into soft callus mass
JCM19	-	-	-	-	-	5	Embryoids converted into soft callus mass
JCM20	-	-	-	-	-	10	Embryoids converted into soft callus mass

Table 4.7

Induction of multiple shoots from mature embryo axis explants on media containing BAP as the principal growth regulator. A total of 36 explants per treatment were cultured and there was 100% response in terms of number of explants responding. The results were recorded at the time of 2nd and 4th weeks. All the results are the mean of three replicates.

Media	Growth regulators (μM)		No. of shoots per explant (after 2 week)	No. of shoots per explant (after 4 week)
	BAP	kinetin		
JBC 1	5	2	7.2 ± 0.6	11.3 ± 0.6
JBC 2	10	2	13.8 ± 2.1	20.3 ± 1.2
JBC 3	20	2	9.7 ± 1.3	17.7 ± 1.2
JBC 4	30	2	10.7 ± 1.5	13.0 ± 1.0
JBC 5	40	2	8.3 ± 1.4	11.3 ± 1.5
JBC 6	50	2	6.0 ± 2.0	9.3 ± 0.6
JBC 7	100	2	3.7 ± 1.5	5.1 ± 2.0
JBC 8	5	5	8.0 ± 1.0	9.7 ± 1.2
JBC 9	10	5	11.3 ± 1.6	17.0 ± 1.0
JBC10	20	5	9.3 ± 0.6	13.7 ± 1.6
JBC 11	30	5	9.0 ± 1.1	11.0 ± 2.0
JBC 12	40	5	8.3 ± 1.5	10.3 ± 1.6
JBC 13	50	5	6.3 ± 1.5	9.0 ± 2.6
JBC 14	100	5	3.7 ± 2.5	5.3 ± 1.5

Table 4.8

Effect of TDZ, 2-iP and kinetin on shoot regeneration from the explants derived from axillary meristems of chickpea. The results were recorded at the end of 2nd and 4th weeks and the values are means of three replicates.

Media	Growth regulators (μM)			No. of explants cultured	No. of shoots per responding explant (after 2 weeks)	No. of shoots per responding explant (after 4 weeks)
	TDZ	2-iP	Kinetin			
JCR 1	2	-	-	32	7.3 ± 0.6	11.3 ± 0.6
JCR 2	4	-	-	32	14.7 ± 2.1	26.3 ± 1.2
JCR 3	6	-	-	32	13.7 ± 2.3	25.7 ± 1.2
JCR 4	8	-	-	32	13.7 ± 1.5	22.0 ± 1.0
JCR 5	10	-	-	32	12.3 ± 1.2	19.3 ± 1.5
JCR 6	20	-	-	32	11.0 ± 2.0	19.3 ± 0.6
JCR 7	30	-	-	32	6.7 ± 1.5	15.0 ± 2.0
JCR 8	40	-	-	32	9.0 ± 1.0	11.7 ± 1.5
JCR 9	50	-	-	32	5.3 ± 0.6	9.0 ± 1.0
JCR 10	100	-	-	32	3.3 ± 0.6	4.7 ± 0.6
JCR 11	4	5	2	32	13.0 ± 2.0	23.0 ± 2.0
JCR 12	4	5	4	32	15.3 ± 1.5	20.3 ± 0.6
JCR 13	4	10	2	32	23.3 ± 1.5	43.0 ± 2.6
JCR 14	4	10	4	32	14.7 ± 2.5	25.3 ± 2.5

Table 4.9

Effect of pH of the culture medium on multiple shoot regeneration form the axillary meristem explants of chickpea. Results were recorded at the end of 3 weeks and the values are mean from three replicates.

Media	pH	No. of explants cultured	No. of explants with shoots	No. of shoots per explant	Percent explants with shoots (%)
JPH 1	4.0	40	37	20.3 ± 1.3	92.5
JPH 2	4.5	40	38	217 ± 0.6	95.0
JPH 3	5.0	40	40	28.3 ± 1.5	100
JPH 4	5.5	40	40	29.7 ± 0.5	100
JPH 5	6.0	40	39	23.7 ± 0.6	97.5
JPH 6	6.5	40	37	23.7 ± 1.2	92.5
JPH 7	7.0	40	33	15.7 ± 1.5	82.5
JPH 8	7.5	40	35	11.3 ± 0.5	87.5
JPH 9	8.0	40	27	6.3 ± 1.2	67.5

Table 4.10

Effect of inclusion of cotyledon tissues along with the regenerating axillary meristem on shoot forming capacity of the axillary meristem explants. Results were recorded from 1 to 5 weeks to show the promotion of regenerating ability and rate of multiple shoot induction by the included cotyledon.

Size of the cotyledon included	No. of shoots per responding explants (weeks)[1]				
	1	2	3	4	5
Zero	0	5.3 ± 1.5	10.3 ± 2.1	14.3 ± 1.2	19.7 ± 1.2
Half	5.7 ± 2.1	10.3 ± 1.2	19.7 ± 3.2	33.0 ± 1.7	45.3 ± 2.1
Full	6.3 ± 1.2	15.3 ± 0.6	27.3 ± 1.5	36.3 ± 1.2	53.3 ± 6.1

Table 4.11

Effect of age of the explant donor seedlings on regeneration capacity of different explants derived from axillary meristems. Regenerating multiple shoots were counted in the third week and the values are means of three replicates.

Seedling age (d)	No. of shoots per explants[1]			
	AM1	AM2	AM3	AM4
2	17.0 ± 2.0	17.3 ± 2.1	4.0 ± 1.0	17.3 ± 1.5
4	15.7 ± 1.5	18.3 ± 1.5	6.7 ± 1.5	22.3 ± 2.5
6	10.7 ± 1.5	23.3 ± 2.1	3.7 ± 0.6	27.7 ± 3.8
8	10.3 ± 2.1	24.0 ± 2.6	7.3 ± 1.5	22.0 ± 3.0
10	7.7 ± 3.1	17.7 ± 1.5	3.7 ± 0.6	19.7 ± 3.1
12	10.0 ± 1.0	12.7 ± 2.1	2.7 ± 0.6	15.7 ± 1.5
14	6.7 ± 2.5	13.3 ± 1.5	1.3 ± 0.6	11.7 ± 1.2
16	6.3 ± 1.5	10.3 ± 1.5	1.0 ± 1.0	9.7 ± 1.5

[1] Mean ± Standard error of three replications

Table 4.12

Induction of multiple shoot buds from various seedling explants derived from axillary meristems. The number of multiple shoots were counted in the third week prior to their transfer to the shoot elongation medium.

Explant	No. of Explants cultured	No. of Explants responding (%)	No. of shoots per explant[1]
Embryo axis	40	38 (95.0)	13.7 ± 1.5
Shoot tip	40	36 (90.0)	10.3 ± 2.1
Axillary bud	40	40 (100)	17.3 ± 2.5
AM1	40	33 (82.5)	23.7 ± 0.6
AM2	40	37 (92.5)	30.3 ± 2.9
AM3	40	23 (57.5)	7.7 ± 2.3
AM4	40	40 (100)	31.0 ± 1.7

[1] Mean ± Standard error of three replications

Table 4.13

Effect of media compositions on elongation of the regenerated shoots. Results were recorded from three replicate experiments involving shoots induced on JCR13 medium (see Table 4.8)[a]

Culture media	Plant growth regulators (μM)				Elongation I[b]	Elongation II
	2iP	BAP	Kinetin	GA3		
CEL 1	2	-	2	-	1.2	2.3
CEL 2	5	-	2	-	3.1	4.2
CEL 3	-	2	2	-	1.1	2.5
CEL 4	-	5	2	-	1.7	3.1
CEL 5	-	-	2	-	0.3	0.5
CEL 6	-	-	5	-	0.5	0.6
CEL 7	-	-	-	2	1.2	7.5
CEL 8	-	-	-	5	1.1	4.8

[a]Each replicate experiments consisted of a total of 40 explants with induced shoot buds at 18 to 23 per explant and the number of shoots elongating per explant was the average of all the 40 explants. Data compiled from three replicated experiments.

[b]Elongation I: number of shoots elongated per explant by the end of second week; Elongation II: number of shoots elongated on the next two or three subcultures.

Table 4.14

Effect of media constituents on rooting of in vitro formed and elongated shoots of chickpea.

Rooting media	Medium constituents [a]			No of shoots with roots [b]		% shoots rooted
	Sucrose (%)	IBA (μM)	NAA (μM)	Phase 1	Phase 2	
CPR 1 (1/2 MS)	2.0	-	-	4.1	-	10.25
CPR 2 (MS)	3.0	-	-	3.3	-	8.25
CPR 3	0.0	5.0	-	0.1	0.0	0.25
CPR 4	1.0	5.0	-	17.0	3.2	50.50
CPR 5	1.5	5.0	-	30.5	8.2	96.75
CPR 6	2.0	5.0	-	25.9	10.1	90.00
CPR 7	2.5	5.0	-	19.9	5.7	64.00
CPR 8	3.0	5.0	-	16.8	7.0	59.50
CPR 9	2.0	5.0	-	27.2	12.4	99.00
CPR 10	2.0	10.0	-	17.1	3.5	51.50
CPR 11	2.0	-	5.0	3.9	-	9.75
CPR 12	2.0	-	10.0	6.4	-	16.00

[a]All the media were used as liquid containing filter paper bridge. CPR3 to CPR8 contained sucrose at various concentrations with IBA as the rooting hormone added at 5 μM concentration. CPR1, CPR2, CPR11 and CPR12 did not contain Phase 2 as there was no pulse treatment with IBA.

[b]Results were recorded at the end of 3 weeks for Phase 1 and 5 weeks for Phase 2. Each combination tested under three replication. Total number of shoots per replicate was 40.

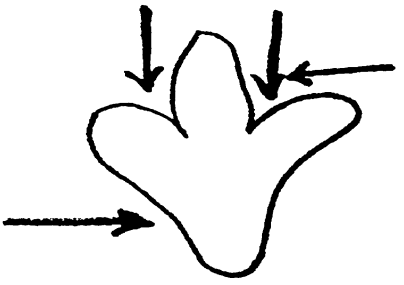
Table 4.15

Lethal dose testing and effect of TDZ on lethal effect of kanamycin. Number of days of explant survival was counted until the explant bleached or showed no signs of growth.

kanamycin (mg/L)	No. of days of explant survival			
	Zero TDZ	2 μM TDZ	4 μM TDZ	10 μM TDZ
5	>30	>40	>40	>40
10	25	30	>35	>35
15	15	20	25	25
20	5	8	10	10
25	2	5	7	8
30	0	2	2	3

Figure 3.1

Diagrammatic representation of the explants derived from mature embryo axis, plumule, radicle, side arms and middle portion. (Arrows shows sites of surgery)



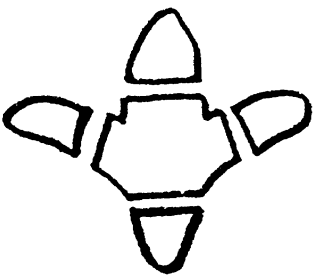


Figure 3.2

Preparation of axillary meristem explants AM1, AM2, AM3 and AM4. Final stage is the stage of regeneration of multiple shoots after one week of culture on shoot induction medium. Large arrows show progression of preparation of explants. Medium arrows show sites of surgery and small arrows show sites of multiple shoot regeneration.

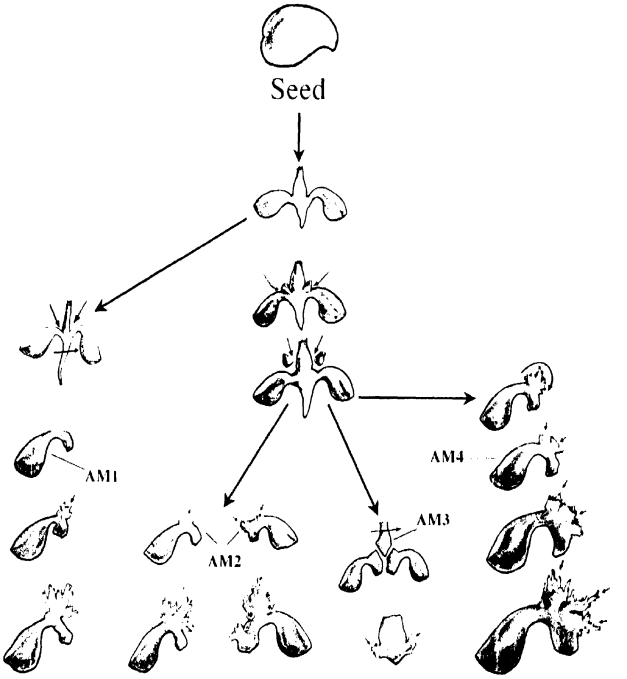
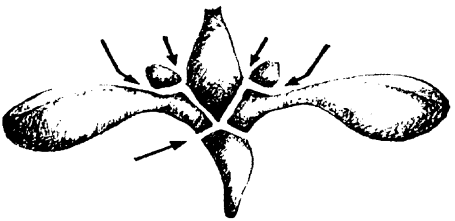

Seed
AM1
AM2
AM3
AM4

Figure 3.3 A – C

Diagrammatic representation for preparation of axillary meristem explant (AM2) showing the sites of surgery. A. 6-day old seedling showing prominent axillary bud, B. Processing of the axillary meristem explant (arrows shows the sites of surgery), C. Axillary meristem explant (AM2) showing the axillary meristem region with intact cotyledon.

A



B

C

Figure 3.4

Restriction map of the plasmid pRT99:GUS-Int used for biolistic-mediated gene transfer.

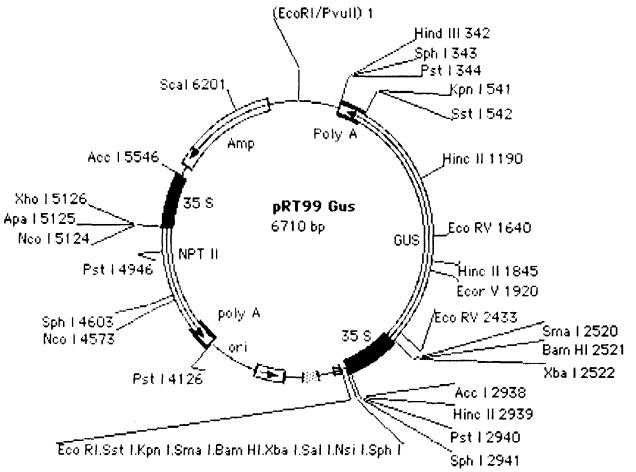

(EcoRI/PvuII) 1
Hind III 342
Sph I 343
Pst I 344
Kpn I 541
Sst I 542
Poly A
Hinc II 1190
Eco RV 1640
GUS
Hinc II 1845
Ecor V 1920
Eco RV 2433
Sma I 2520
Bam HI 2521
Xba I 2522
35 S
Acc I 2938
Hinc II 2939
Pst I 2940
Sph I 2941
Eco RI.Sst I.Kpn I.Sma I.Bam HI.Xba I.Sal I.Nsi I.Sph I
Pst I 4126
ori
poly A
Sph I 4603
Nco I 4573
Pst I 4946
NPT II
Xho I 5126
Apa I 5125
Nco I 5124
35 S
Acc I 5546
Amp
ScaI 6201
pRT99 Gus
6710 bp

Figure 3.5

Restriction map of the plasmid pHS723:Bt used for *Agrobacterium*-method of transformation.

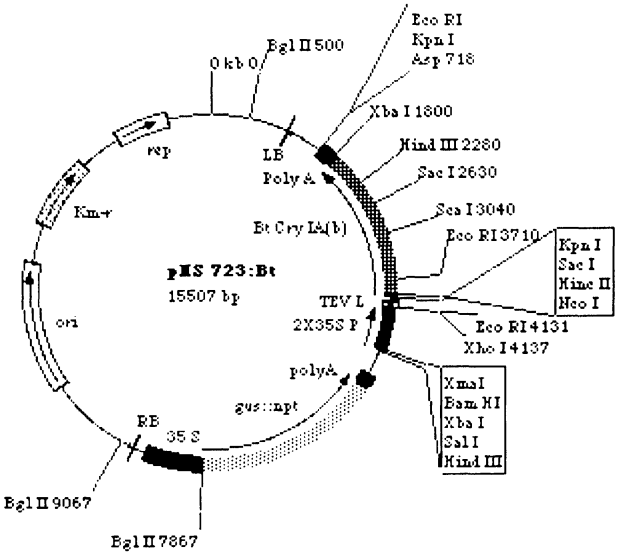

Eco RI
Kpn I
Asp 718
Bgl II 500
0 kb 0
Xba I 1800
Hind III 2280
Sac I 2630
Sca I 3040
Eco RI 3710
Kpn I
Sac I
Hinc II
Nco I
Eco RI 4131
Xho I 4137
XmaI
Bam HI
Xba I
Sal I
Hind III
LB
Poly A
rep
Km-r
ori
Bt Cry IA(b)
pMS 723:Bt
15507 bp
TEV L
2X35S P
polyA
gus::npt
RB
35 S
Bgl II 9067
Bgl II 7867

Figure 3.6

Restriction map of the plasmid pHS737:SBTI used for *Agrobacterium*-method of transformation.

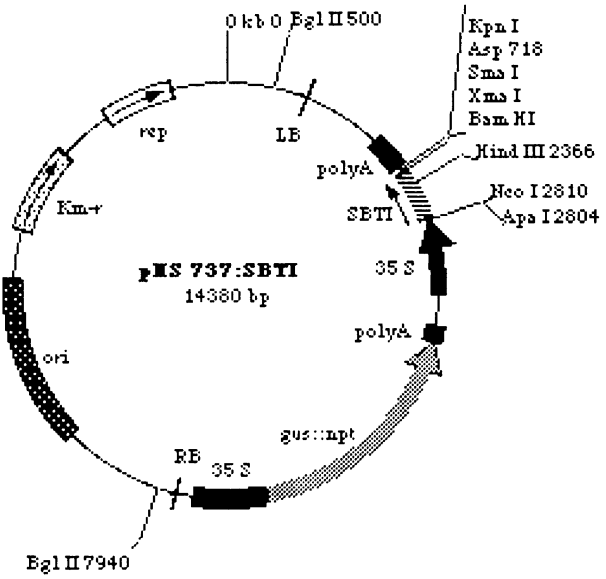

0 kb 0
Bgl II 500
Kpn I
Asp 718
Sma I
Xma I
Bam HI
rep
LB
polyA
Hind III 2366
Km-r
SBTI
Nco I 2810
Apa I 2804
pMS 737:SBTI
14380 bp
35 S
polyA
ori
gus::npt
RB
35 S
Bgl II 7940

Figure 4.1

Induction of somatic embryos from mature embryo axis and leaflet explants after 6 weeks of culture on the medium containing 2,4,5-T as principal growth regulator. A, B and C. Induction of embryos from mature embryo axis explant, D. Induction of somatic embryos from leaflet explant.

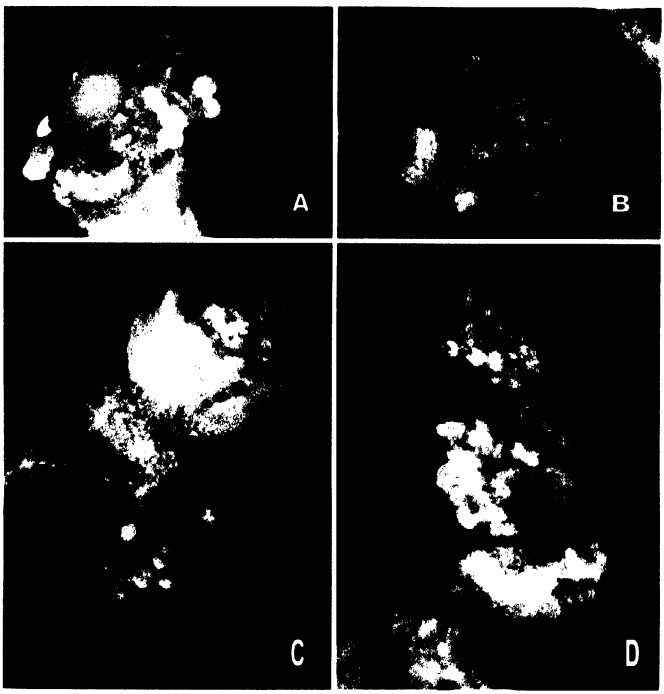

A
B
C
D

Figure 4.2

Induction of somatic embryos from mature embryo axis explant by using 2,4-D as principal growth regulator. A. Top view of the embryo axis showing multiple globular embryos, B. Lateral view of embryo axis showing multiple globular embryos formed from plumule region.

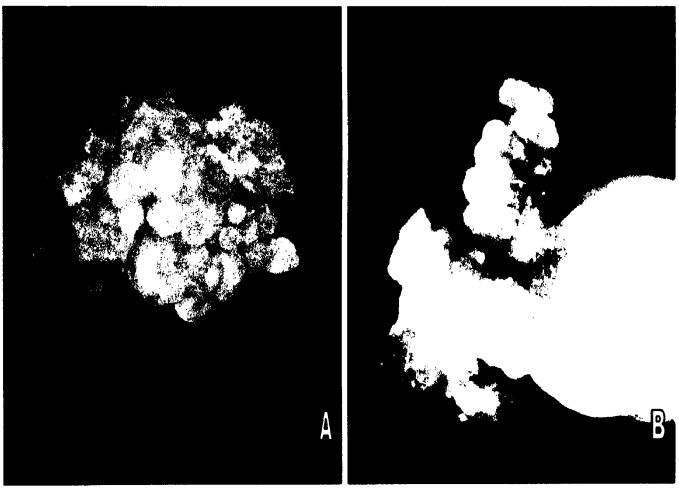

A
B

Figure 4.3

Effect of concentration of BAP on multiple shoot regeneration from mature embryo axis explant after 15 days of culture on shoot induction medium. A. Multiple shoot regeneration at 10 μM BAP concentration, B. Multiple shoot regeneration with 30 μM BAP, C. Multiple shoot regeneration with 40 μM BAP, D. Multiple shoot regeneration with 50 μM BAP, D. Multiple shoot regeneration with 100 μM BAP.

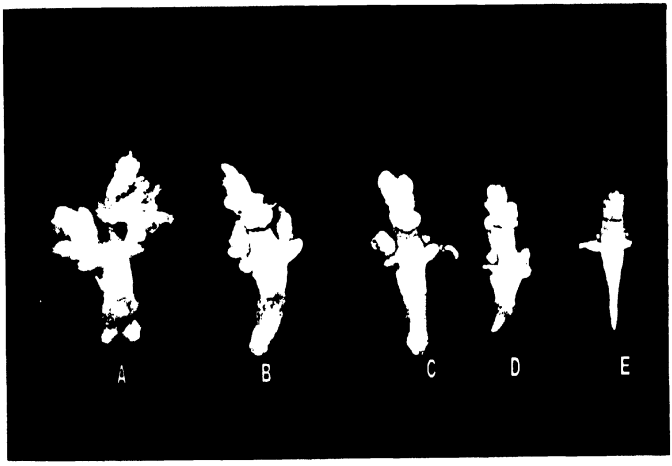

A
B
C
D
E

Figure 4.4

Multiple shoot regeneration from different explants at the third week of culture on shoot induction medium. A. Shoot tip showing multiple shoots emerging from meristematic region, B. Axillary bud showing multiple shoots originating from tip and basal regions, C. Mature embryo axis showing multiple shoots emerging from shoot tip as well as axillary bud regions, D. AM1 explant showing multiple shoots from axillary region, E. AM2 explant showing swollen basal region and multiple shoots emerging synchronously as cluster, F. AM4 explant showing synchronous multiple shoots from different places of axillary meristem region.

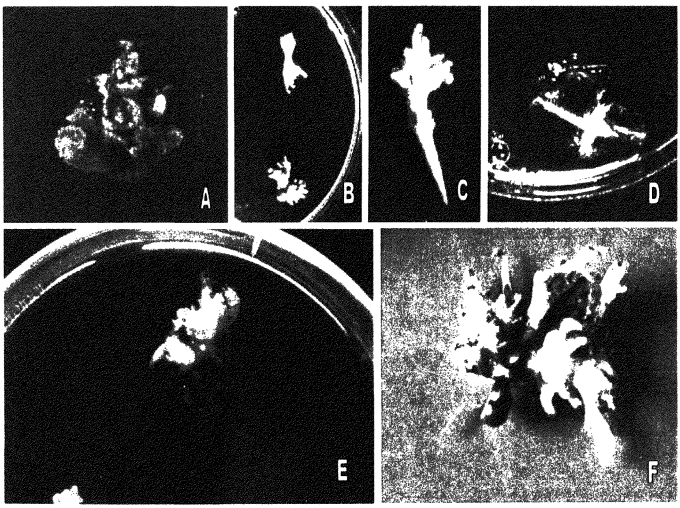

A
B
C
D
E
F

Figure 4.5

Stages of multiple shoot regeneration from AM4 explant after culture on the shoot induction medium. A. Explant showing the area of axillary bud removal (arrow shows the site of surgery), B. Multiple shoot buds originating from the area left by axillary bud removal (arrow shows the shoots buds emerging from axillary meristem area), C. Explant showing swollen area after removal of regenerating shoot buds (this stage of explant was named as AM4 explant) after 7 days of culture on shoot induction medium, D. Regeneration of multiple shoot buds from the cut portions of the regenerating tissue after 6 days of culture on MS, E. Exuberant multiple shoot growth from the regenerating tissue after 10 to 12 days of culture on MS, F. Multiple shoots originating from different parts of the regenerating area after 12 to 14 days of culture on MS.

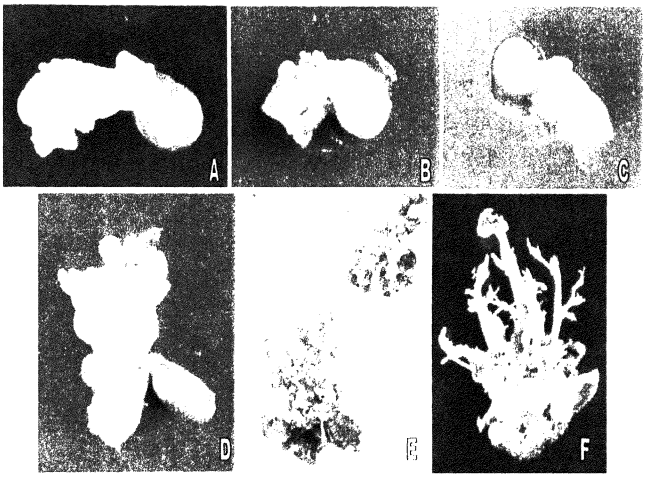

A
B
C
D
E
F

Figure 4.6

Various stages of elongation of regenerating shoot buds on shoot elongation medium; A. Elongation of young shoot on shoot elongation medium 1 (SEM1) after 1 week of culture, B. Elongation of shoot on shoot elongation medium 2 (SEM2) after 2 weeks of culture, C. Elongation of shoots on SEM2 after 3 weeks.

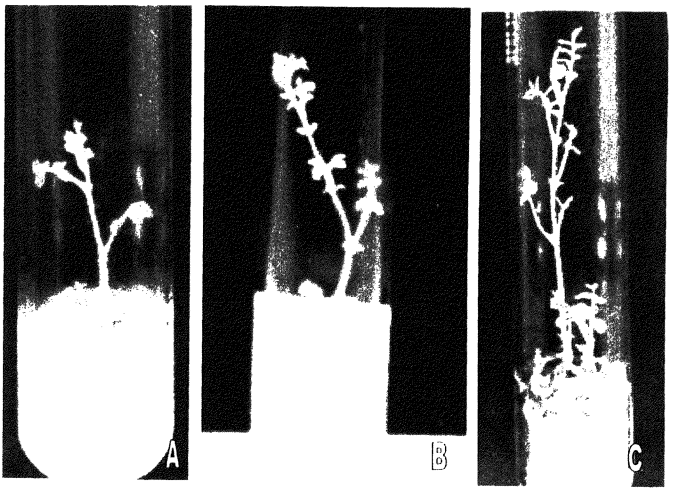

A
B
C

Figure 4.7

Rooting of elongated shoots on root induction medium containing 5 µM IBA; A. Rooting on filter paper bridge immersed in liquid root induction medium, B. Rooting on solid root induction medium containing 5 µM IBA.

A
B

Figure 4.8

Stage 1 hardening of tissue culture grown chickpea after transferring to 8 cm pot containing 2-4 mm sand.

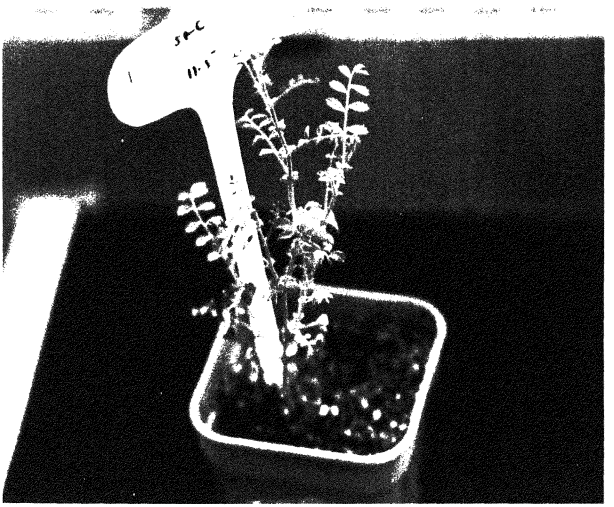


Figure 4.9

Alternative methods of hardening of rooted plantlets of chickpea prior to transplantation; A. Hardening process by embedding the root system in the coarse sand with the cotton plug open. ¼ Arnon's solution was used for irrigation, B. Hardening process in static hydroponics system with root system immersed in the liquid medium. The liquid medium comprised of ¼ Arnon's solution, C. A plant from B showing profuse growth of root system in the static hydroponics system after 15 days. This plant is ready for transplantation into pots.

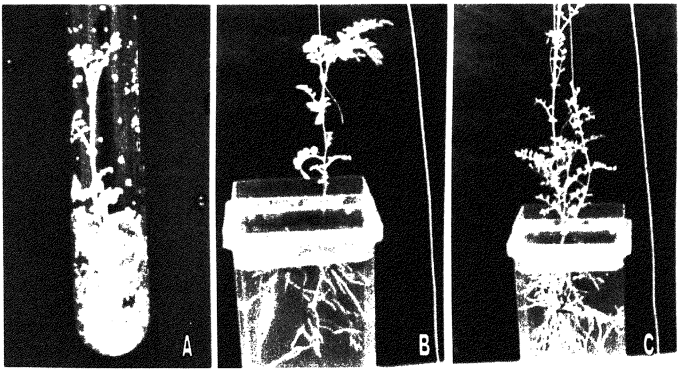

A
B
C

Figure 4.10

Hardened and transplanted chickpea plants growing in the glasshouse; A. Plant growing without any post-hardening treatment, B. Plant with multiple branches after post-hardening treatment of terminal bud amputation and juvenile flower bud removing, C. Group of plants being maintained in 20 cm pots after hardening.


A
B
C

Figure 4.11

A complete scheme of regeneration and recovery of whole plants through tissue culture method using axillary meristem explant (AM2) obtained from *in vitro* grown seedlings of chickpea. A. Axillary meristem explant (AM2) on the first day of culture on shoot induction medium, B. Shoot buds regenerating from the region left by the removal of axillary bud after 7 days, C. Cluster of multiple shoot buds originating from region left by the removal of axillary bud after 12 days, D. A closer view of the multiple shoots regenerating from the axillary meristem explant after 15 days of culture on shoot induction medium, E. Elongation of the shoot buds after 7 days of culture on shoot elongation medium, F. Rooting of the elongated shoot bud on the filter paper bridge immersed in liquid rooting medium after 8 days of culture, G. Picture showing *ex vitro* static hydroponics system for hardening of the rooted shoot after 15 days, H. An alternative method for hardening of the plantlet obtained through tissue culture in which the root system was immersed in sand with the cotton plug of the culture tube kept open, I. Hardened and transplanted plant showing normal morphology.

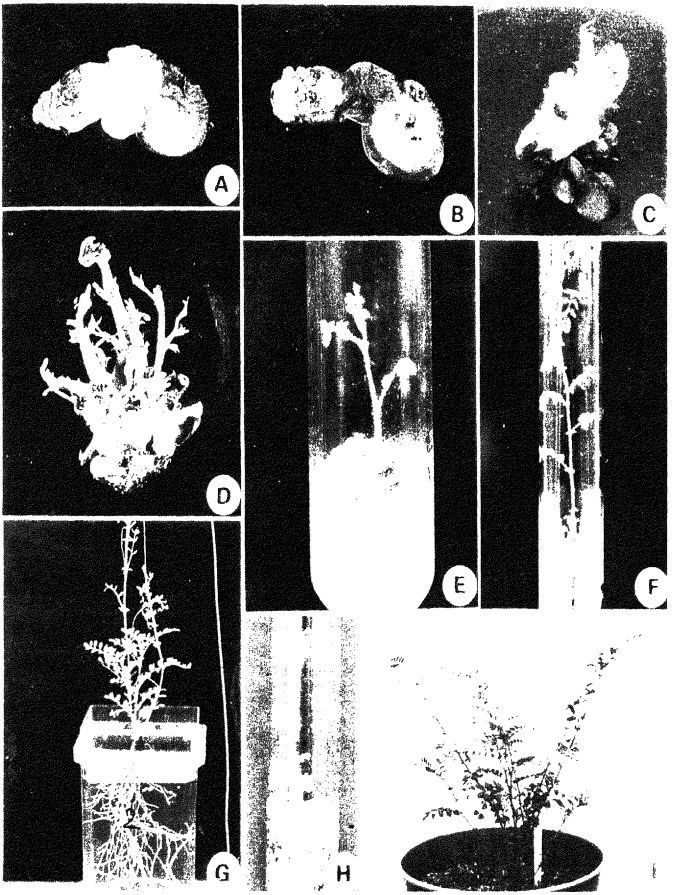

A
B
C
D
E
F
G
H

Figure 4.12

Histological sections showing status of axillary meristem. A. Longitudinal section of the growing axillary bud just before its removal from 6-day-old seedling showing actively dividing meristematic cells and uniform non-meristematic tissue in the basal region. B. LS of explant after removal of axillary bud in the process of preparing axillary meristem explant.


A


B

Figure 4.13

Histological studies of development of multiple meristemoids from axillary meristem region of AM4 explant after the removal of axillary bud. A. Longitudinal section of the axillary meristem area on day-1 after axillary bud removal, B. Development of meristemoids at the basal portion of axillary bud on day-2 (the small bud appearing is a shoot bud emerging from the basal portion of the removed axillary bud), C. Appearance of meristemoids in the axillary meristem area on day-3, D. Increased number of meristemoids in the axillary meristem area on day-4, E. Increase in the number and growth of meristemoids on day-5, F. Exponential increase in the number and growth of meristemoids on day-6, G. Longitudinal section of the multiple shoot buds as seen on day-6, H. Meristematic activity of dividing cells at various places in the axillary meristem area in different direction on day-7.

A
B
C
D
E
F

Figure 4.14

Closer observation of development and growth of meristemoids in the axillary meristem area of AM4 explant. A. Closer view of the meristemoids on day-4 after the removal of axillary bud, B. Growth and division of meristematic cells in the meristemoid region on day-5, C. Growth and division of meristematic cells around meristemoid region on day-6, D. Growth and division of meristematic cells of meristemoid region on day-7, E. Closer view (40X) of meristemoid region and cell division activity, F. Hectic meristematic activity of the dividing and growing cells.


A
B
C
D
E
F
G
H

Figure 4.15

Restriction analysis of the plasmids used for transformation. A. The plasmid pRT99:GUS-Int (6.9 kb) was used in biolistic method of transformation. Lane 1 shows λDNA digested with *BstE* II enzyme as marker. Lanes 2 and 3 have duplicate plasmid un-restricted sample and lane 4 shows plasmid after restriction with *EcoR*I showing four fragments. B. Two un-restricted plasmid constructs that were used in *Agrobacterium*-mediated transformation. Lane 1 shows λDNA digested with *Hind* III enzyme as marker. Lanes 2 and 3 had pHS737:SBTI (14.3 kb) plasmid while lanes 4 and 5 had pHS723:Bt (15.5 kb) plasmid preparations.

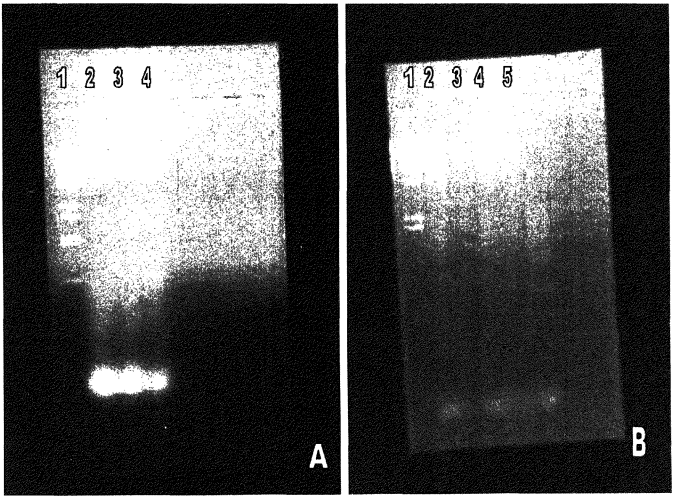

1 2 3 4
A
1 2 3 4 5
B

Figure 4.16

GUS histochemical assay of the leaflets from putatively transformed plants of chickpea. A. A closer view of the leaflet showing GUS activity in the veins, B, C. GUS activity as seen in the petiole and veins of leaflets.

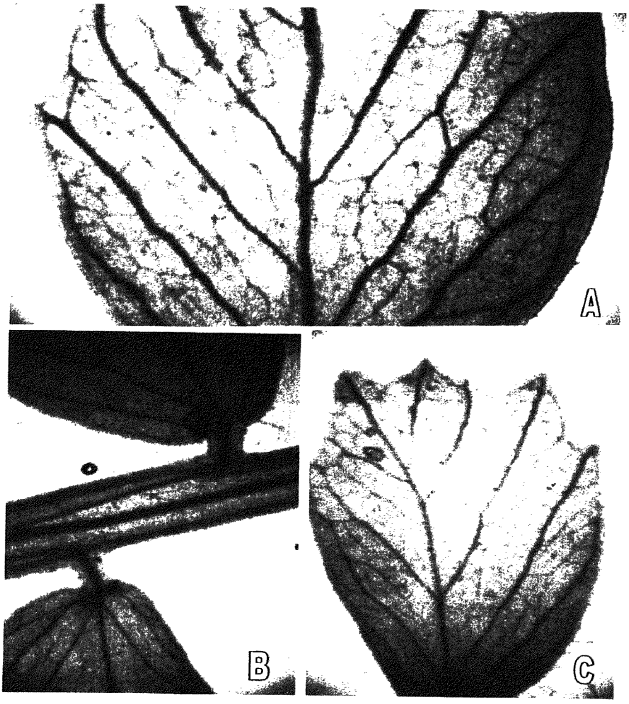

A
B
C

Figure 4.17

PCR amplification of 700 bp fragment of *npt* II gene from the genomic DNAs of T_0 generation plants transformed with Bt cryIAb and SBTI genes via *Agrobacterium*-mediated transformation. A. *npt*II-PCR of plants transformed with pHS723:Bt via *Agrobacterium*-mediated transformation. Lanes 1 to 10 had transformed samples and show the amplification of *npt*II gene. Lane 11 negative control and 12 to 17 were positive controls from plasmid pHS723:Bt used for transformation. Lane 18 is - DNA and λ DNA-*BstE* II marker was added in the lane 19. B. *npt*II-PCR of plants transformed with pHS737:SBTI vector via *Agrobacterium*-mediated transformation. Lanes 1 to 9 had transformed samples CS5 to CS9. Lane 10 was negative control and 11 to 15 were positive controls of pHS737:SBTI vector that was used in the transformation. λ DNA-*BstE* II marker was added in the lane 16.

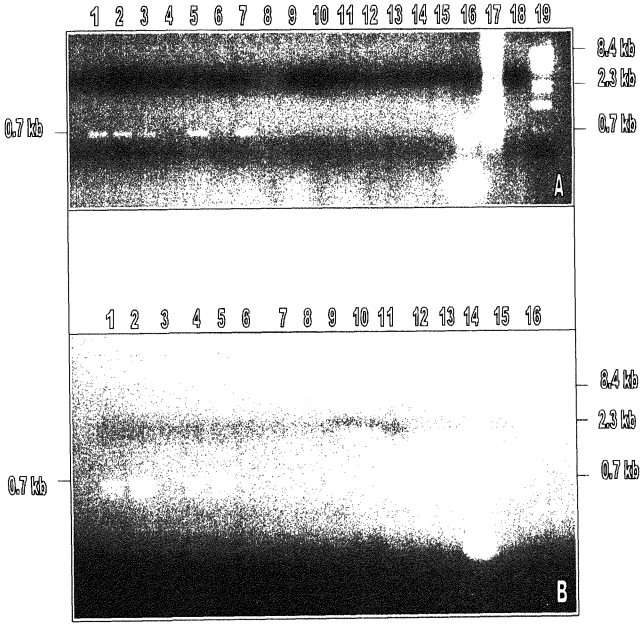

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
8.4 kb
2.3 kb
0.7 kb
0.7 kb
A
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
8.4 kb
2.3 kb
0.7 kb
0.7 kb
B

Figure 4.18

PCR amplification of 1.2 kb fragment of *uid*A gene from genomic DNA samples of T_0 generation putative transgenic plants transformed with pHS723:Bt and pHS737:SBTI vectors via *Agrobacterium*-mediated transformation. A. GUS-PCR analysis of plants transformed with pHS723:Bt vector. Lanes 1 to 10 were added with putative transformant samples, while lane 11 was positive control and lane 12 negative control. Sample added in lane 13 was -DNA. λ DNA-*BstE* II marker was added in the lane 14. B. GUS-PCR analysis of plants transformed with pHS737:SBTI. Lanes 1 to 9 were putative transformant samples, while lane 10 and 11 were positive controls of plasmid pHS737:SBTI and lane 12 was negative control. Sample added in lane 13 was -DNA. λ DNA-*BstE* II marker was added in the lane 14.

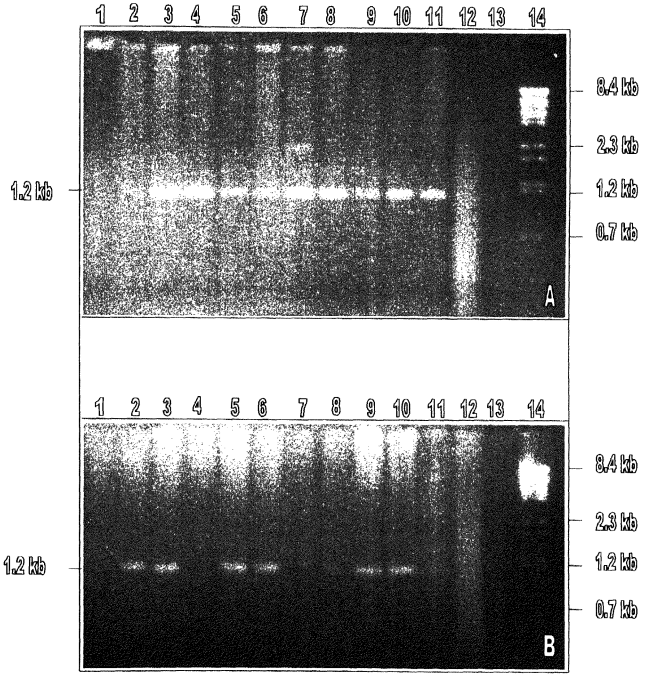

1 2 3 4 5 6 7 8 9 10 11 12 13 14
1.2 kb
8.4 kb
2.3 kb
1.2 kb
0.7 kb
A
1 2 3 4 5 6 7 8 9 10 11 12 13 14
1.2 kb
8.4 kb
2.3 kb
1.2 kb
0.7 kb
B

Figure 4.19

PCR amplification of SBTI and B*tcryIAb* genes from genomic DNAs of putative transformants at T_0 generation transformed via *Agrobacterium*-mediated transformation. A. PCR analysis of plants transformed with pHS737:SBTI vector. Lane 1 through lane 9 are putative transformant samples showing amplification of 497 bp fragment. Negative control samples were added in the lanes 10, 11 and 12. pHS737:SBTI plasmid samples were positive controls in the lanes 13, 14, 15 and 16. Lane 17 was -DNA and λ DNA-*BstE* II marker was added in lane 18. B. PCR analysis of plants transformed with pHS723:Bt vector. Lane 1 through lane 11 were added with putative transformants showing amplification of 908 bp fragment. Lane 12, 13, 14 and 15 had the negative control. Lanes 16, 17 and 18 were positive controls of pHS723:Bt plasmid vector. Lane 19 was -DNA and lane 20 was added with λ DNA-*BstE* II marker.

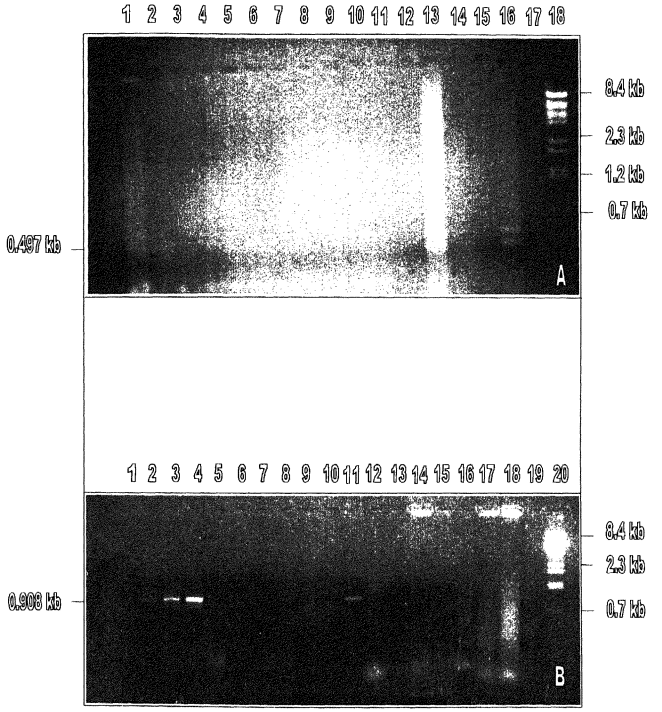

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
8.4 kb
2.3 kb
1.2 kb
0.7 kb
0.497 kb
A
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
8.4 kb
2.3 kb
0.7 kb
0.903 kb
B

Figure 4.20

DNA profile of genomic DNA isolated from putative transformants of chickpea. A. Purified DNA profile of genomic DNA of B*tcryIAb* plants. DNA was prepared in duplicate samples. Lanes 1 to 10 shows first set and lanes 11 to 20 shows the second set. B. Effect of amount of BSA, water and enzyme in the reaction mixture to digest the genomic DNA sample of chickpea. Unless otherwise stated, the total volume of reaction mixture was maintained at 25 µl with 10 µl of genomic DNA, 2 µl (10 units) of enzyme, 2.5 µl of reaction buffer and the rest being water. In case of volume variations arising due to the changing enzyme and water concentrations, the reaction buffer volume was maintained accordingly with its working concentration always kept at 1X.

Lane 1: Unrestricted genomic DNA (5 µg. was added to the well)

Lane 2: 1 µg. genomic DNA digested with 10 units of enzyme

Lane 3: 5 µg. genomic DNA digested with 10 units of enzyme

Lane 4: 5 µg. genomic DNA digested with 15 units of enzyme and double the quantity of water

Lane 5: 5 µg. genomic DNA digested with 20 units of enzyme with double the quantity of water

Lane 6: 5 µg. genomic DNA digested with 10 units of enzyme and 0.1% BSA

Lane 7: 5 µg. genomic DNA digested with 10 units of enzyme, 0.1% of BSA and double the quantity of water

Lane 8: 5 µg. genomic DNA digested with 10 units of enzyme, 0.3% of BSA

Lane 9: 5 µg. genomic DNA digested with 10 units of enzyme, 0.3% of BSA and double the quantity of water

Lane 10: 5 µg. genomic DNA digested with 10 units of enzyme, 0.5% of BSA

Lane 11: 5 µg. genomic DNA digested with 10 units of enzyme, 0.5% of BSA and double the quantity of water

Lane 12: 5 µg. genomic DNA digested with 10 units of enzyme, 1.0% of BSA

Lane 13: 5 µg. genomic DNA digested with 20 units of enzyme

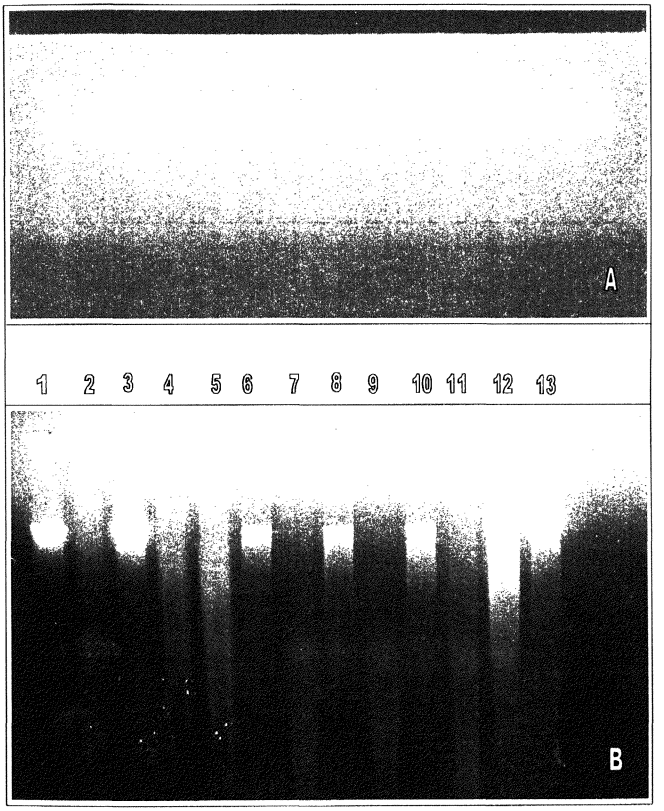

A
1 2 3 4 5 6 7 8 9 10 11 12 13
B

Figure 4.21

Southern blots of the T_0 generation putative transformants of chickpea transformed with *BtcryIAb* and SBTI genes via *Agrobacterium*-mediated transformation. These blots show the signal accumulated on the higher molecular weight region due to the limited restriction of genomic DNA. This problem was encountered when the factors affecting restriction of genomic DNA, such as quantity of enzyme, water and BSA were not standardized. A. Southern blot with probe for *cryIAb* gene. Lanes 1 to 10 has putatively transformed samples of CB1 to CB10. B. Southern blot with probe for *npt*II gene. Lanes 1 to 10 were added with putative transformants transformed with *BtcryIAb* gene. Lane 11 has negative control, while lane 12 has the positive control. Restricted genomic DNA samples from putative transgenic transformed with SBTI gene were added in the lanes 13 to 21.

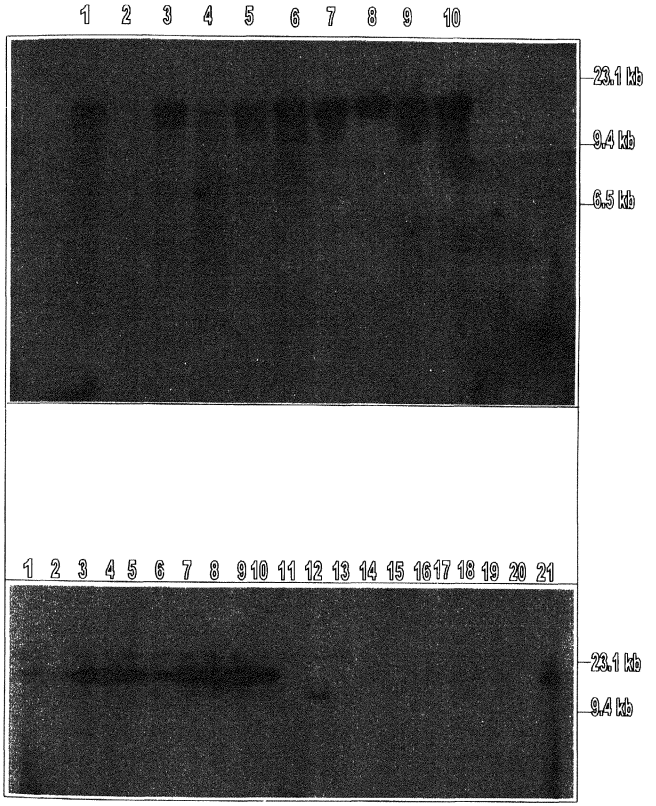

1 2 3 4 5 6 7 8 9 10
23.1 kb
9.4 kb
6.5 kb
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
23.1 kb
9.4 kb

Figure 4.22

Southern analysis of the T_0 generation of *BtCryIAb* putative transgenic plants of chickpea transformed via *Agrobacterium*-mediated transformation. Genomic DNA was restricted with *Eco*RI enzyme. Analysis of copy number with respect to the *npt*II gene. Lanes 1 to 11 were added with transformant samples CB1 to CB11. Lane 1: CB1, Lane 2: CB2, Lane 3: CB3, Lane 4: CB4, Lane 5: CB5, Lane 6: CB6, Lane 7: CB7, Lane 8: CB8, Lane 9: CB9, Lane 10: CB10 and Lane 11: CB11. Lane 12 has the negative control and lane 13 has the positive control (Restricted plasmid pHS723:Bt that was used in genetic transformation).

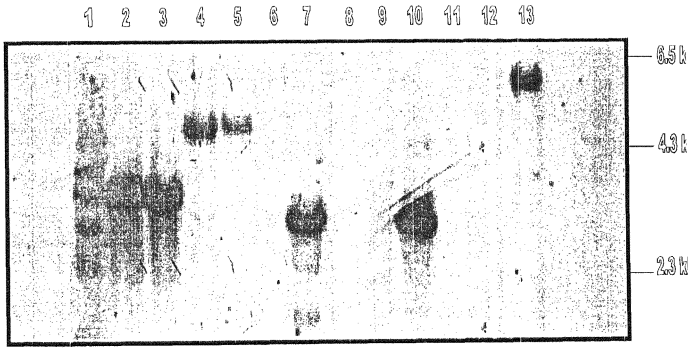
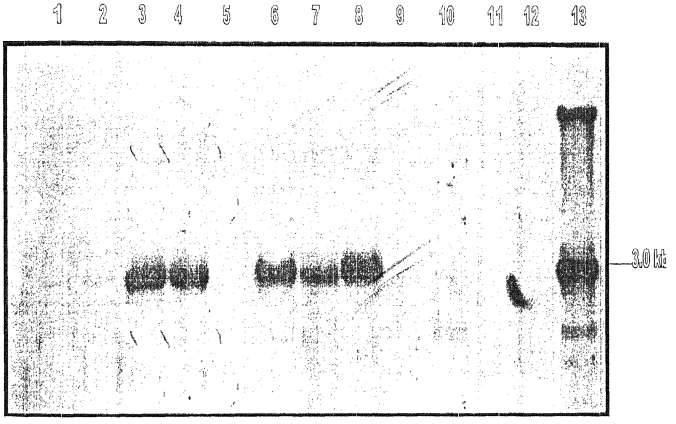

1 2 3 4 5 6 7 8 9 10 11 12 13
6.5 k
4.3 k
2.3 k

Figure 4.23

Southern analysis of theT_0 generation of *BtCryIAb* plants. Genomic DNA was restricted with *Eco*RI enzyme that has two restriction sites within the *BtCryIAb* gene. This double cut releases the 3 kb fragment of *BtCryIAb* gene. Analysis of the transgenics for the *BtCryIAb* gene. Lanes 1 to 11 were added with transformant samples, Lane 1: CB1, Lane 2: CB2, Lane 3: CB3, Lane 4: CB4, Lane 5: CB6, Lane 6: CB5, Lane 7: CB7, Lane 8: CB8, Lane 9: CB9, Lane 10: CB10 and Lane 11: CB11. Lane 12 has the negative control and lane 13 has the positive control Restricted plasmid pHS723:Bt with *Eco*RI enzyme).


1 2 3 4 5 6 7 8 9 10 11 12 13
3.0 kb