

**STUDIES ON *Agrobacterium*-MEDIATED INSECT RESISTANCE
GENE TRANSFER IN CABBAGE (*Brassica oleracea* L. var.
capitata) AND MOLECULAR ANALYSIS OF
REGENERATED PLANTLETS**

Thesis

by

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*Submitted in partial fulfilment of the requirements
for the degree of*

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in

BIOTECHNOLOGY



**COLLEGE OF HORTICULTURE
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CERTIFICATE-I

This is to certify that the thesis entitled, “**Studies on *Agrobacterium*-mediated insect resistance gene transfer in Cabbage (*Brassica oleracea* L. var. *capitata*) and molecular analysis of regenerated plantlets**”, submitted in partial fulfilment of the requirements for the award of degree of **DOCTOR OF PHILOSOPHY in BIOTECHNOLOGY** to Dr. Yashwant Singh Parmar University of Horticulture and Forestry, Nauni, Solan (H.P.) is a record of bonafide research work carried out by **Ms. Geetika Gambhir (H-2009-01-D)** under my guidance and supervision. No part of this thesis has been submitted for any other degree or diploma.

The assistance and help received during the course of investigations has been fully acknowledged.

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CERTIFICATE-II

This is to certify that the thesis entitled, “**Studies on *Agrobacterium*-mediated insect resistance gene transfer in Cabbage (*Brassica oleracea* L. var. *capitata*) and molecular analysis of regenerated plantlets**”, submitted by **Ms. Geetika Gambhir (H-2009-01-D)** to Dr. Yashwant Singh Parmar University of Horticulture and Forestry, Nauni, Solan (H.P.) in partial fulfilment of the requirements for the award of degree of **DOCTOR OF PHILOSOPHY** in **BIOTECHNOLOGY** has been approved by the Student’s Advisory Committee after an oral examination of the same in collaboration with the external examiner.

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This is to certify that all the mistakes and errors pointed out by the external examiner have been incorporated in the thesis entitled, “**Studies on *Agrobacterium*-mediated insect resistance gene transfer in Cabbage (*Brassica oleracea* L. var. *capitata*) and molecular analysis of regenerated plantlets**” submitted to Dr Y.S. Parmar University of Horticulture and Forestry, Nauni-Solan (H.P.) by **Ms. Geetika Gambhir (H-2009-01-D)** in partial fulfilment of the requirements for the award of degree of **DOCTOR OF PHILOSOPHY** in **BIOTECHNOLOGY**

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Needless to say errors and omissions are mine.

Place:

Date :

Geetika Gambhir

LIST OF ABBREVIATIONS

%	per cent
β-ME	β-Mercaptoethanol
μl	Microlitre
μM	Micromolar
°C	Degree Celsius
35S	1,2,3,5-yl urea) CaMV 35S promoter
ABA	Abscissic acid
AgNO ₃	Silver nitrate
BA	6-Benzyl adenine
BAP	6-Benzyl aminopurine
bp	Base pair bromide)
<i>Bt</i> toxin	<i>Bacillus thuringiensis</i> δ-endotoxin
CaMV	Cauliflower mosaic virus
CD	Critical difference
Cm	Centimeter
CpTI	Cowpea trypsin inhibitor
CRD	Completely randomized design
<i>cry</i>	Insecticidal crystal protein (toxin) gene
ct	Cycle threshold
CTAB	(N-cetyl N,N,N-trimethyl ammonium
cv.	Cultivar
DNA	Deoxy ribonucleic acid
EDTA	Ethylenediamine tetra acetic acid
ELISA	Enzyme linked immunosorbent assay
Fm	Fermi molar
g/l	Gram per litre
GA ₃	Gibberellic acid
<i>gus</i>	β-glucuronidase
ha	Hectare
HCl	Hydrochloric acid
HgCl ₂	Mercuric chloride
hpt	Hygromycin phosphotransferase
hr	Hour
IAA	Indole 3-acetic acid
IBA	Indole 3-butyric acid
Kn	Kinetin

LB	Left-border of T-DNA
lbs/inch ²	Pounds per square inch
mg/l	Milligram per litre
min	Minute
ml	Milli litre
mM	Milli molar
mm	millimeter
MS	Murashige and Skoog medium (1962)
N	Normal
NAA	α -Naphthalene acetic acid
NaOH	Sodium hydroxide
nm	Nanometer
Nos Pro	Nopaline synthase promoter
Nos Ter	Nopaline synthase terminator
<i>npt-II</i>	Neomycin phosphotransferase-II
NTC	non-transformed control
OD	Optical density
Ori	Origin of replication
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PGR	Plant growth regulators
RB	Right border of T-DNA
RNA	Ribonucleic acid
rpm	Rotations per minute
RT	Real Time
RT	Reverse Transcriptase
S.E.	Standard error
spp.	Species
TAE	Tris Acetate EDTA
T-DNA	Transfer DNA
TDZ	Thiadiazuron (N-phenyl-N-thidiazol-
UV	Ultra violet
v/v	Volume by volume
var	variety
vir	Virulence region
w/v	Weight by volume
YMB	Yeast manitol broth

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Chapter-1

INTRODUCTION

Brassica vegetables are an economically important and highly diversified group of crops belonging to the family *Brassicaceae*. *Brassica oleracea* is one of the major species of this group which includes many distinct vegetable and fodder varieties, such as cabbage, broccoli, Brussels sprouts, cauliflower, collards, Savoy cabbage, kohlrabi, rutabaga, and turnip. They are consumed worldwide as food of high nutritional value. *Brassica* vegetables contain little fat and are a source of vitamins, minerals, fiber, important proteins, and phytochemicals (ascorbic acid, lutein, carotene, tocopherol and phenolics) that could be beneficial in the prevention of tumors (Singh *et al.*, 2006). Regeneration of these plants from green explants culture is very important for genetic improvement, recent advances in cell and molecular biology have facilitated the transfer of foreign gene into plant, which is the first step for the genetic improvement of crops using the new biotechnological approaches. *Agrobacterium*-mediated transformation has been the most widely used in these types of work and it has been known for many years that some species including *Brassica* species are susceptible to infection by *Agrobacterium tumefaciens* strains. Thus, the applications of this technology in vegetable crops can enhance yield, nutrition quality, improved resistance to diseases, pests and herbicides. The preferred target area of research in gene transfer also includes tolerance to various abiotic stresses, plant productivity, usage of male sterility for production of hybrids and increased storage life. Among all vegetable crops, potato, tomato, brinjal, peppers, cucumber, cauliflower, cabbage and carrot have received the maximum attention particularly in the areas relating to insect pests, disease and herbicide resistance and quality improvement (Ram and Dasgupta, 2004).

Cabbage (*Brassica oleracea* L. var. *capitata*) is an important vegetable crop of the family *Brassicaceae* and is used as a leafy green vegetable. It is an herbaceous, biennial, dicotyledonous flowering plant distinguished by a short stem upon which is crowded a mass of leaves, usually green but in some varieties red or purplish, which while immature form a characteristic compact, globular cluster. The plant is also called head cabbage or heading cabbage and in Scotland a bowkail, from its rounded shape. Cabbage leaves often have a delicate, powdery, waxy coating called bloom.

The cultivated cabbage is derived from a leafy plant called the wild mustard plant, native to Eastern Mediterranean region as well as Asia Minor region, where it is common along the seacoast, also called sea cabbage and wild cabbage (Decoteau, 2000). This vegetable for its medicinal properties, declaring that “It is the cabbage that surpasses all other vegetables”. This crop is grown in more than 90 countries throughout the world which include countries like China, Japan, Korea, India and Poland.

The Food and Agriculture Organization of the United Nations (FAO) reports that world production of cabbage and other brassica's (these plants being combined by the FAO for reporting purposes) for calendar year 2011 was 68,840,531 metric tonnes and cultivated on 2,373,818 ha land. This was primarily grown in China (43 percent) and India (11 percent with production of 79,49,000 metric tonnes on 369,000 ha land).

Cabbage grows best in relatively cool and moist weather. It can tolerate frost to a great extent. Cabbage exhibits vegetative growth at temperatures of 15-20°C but temperature below 0°C hinders growth. Plants perform best when grown in well-drained soil in a location that receives full sun. Different varieties prefer different soil types, ranging from lighter sand to heavier clay, but all prefer fertile ground with a pH 6.0-6.8 (Salunkhe and Kadam, 1998). Plants are generally started in protected locations early in the growing season before being transplanted outside, although some are seeded directly into the ground from which they will be harvested. Growers normally place plants 12 to 24 inches (30 to 61cm) apart. Some varieties of cabbage have been developed for ornamental use these are generally called "flowering cabbage". They do not produce heads and feature purple or green outer leaves surrounding an inner grouping of smaller leaves in white, red, or pink.

Cabbage is consumed as salad, boiled vegetables, cooked in curries, pickling as well as dehydrated vegetables. As it is excellent source of vitamin C and also contains significant amount of glutamine, an amino acid that has anti-inflammatory properties. Infact, Cabbage can be included in dieting programs, as it is a low calorie food. It is a cruciferous vegetable, and has been shown to reduce the risk of some cancers. This is possibly due to the glucosinolates found in cole crops, which serve as metabolic detoxicants, or due to the sulphoraphane content, also responsible for metabolic anti-carcinogenic activities. It is also a source of indole-3-carbinol, a chemical which boosts DNA repair in cells and appears to block the growth of cancer cells (Salunkhe and Kadam, 1998; Lakshmi 2009). Purple

cabbage also contains anthocyanins, which in other vegetables have been proven to have anti-carcinogenic properties. It is also rich in minerals such as phosphorus, potassium, calcium, iron, protein and vitamin C. In ayurvedic medicine, cabbage leaves are prescribed for cough, fever, skin diseases, peptic ulcers, urinary discharges and haemorrhoids. The seeds are diuretic laxative, stomachic, and antihelminthic. Fresh cabbage juice is reported to have antibacterial activity and has been shown to inhibit the growth of various strains of lactic acid bacteria (Brown and Hutchinson, 1949).

The cultivation of this vegetable is severely challenged by infestation of insect-pests, including the major insect cabbage looper (*Trichoplusiani hubner*), cabbage worm (*Pieris rapae* L.), diamondback moth (*Plutella xylostella* L.), cabbage aphids (*Brevicoryne brassicae* L.) and onion thrips (*Thrips tabaci* Lindeman) (Salunkhe and Kadam, 1998) which results in great loss of the yield and damage to the quality of cabbage production (Shelton *et al.*, 1982; Gould *et al.*, 1984; Theunissen *et al.*, 1995). To check the losses caused by these insect-pests, a number of pesticides are being used on this crop. Unfortunately, major insect pests are developing resistance to most of the applied chemicals insecticides.

In 2009, 14 million farmers planted 134 million ha of transgenic crops in 25 countries (James, 2009). The adoption of transgenic crops has saved pesticide costs and reduced environmental impact from pesticides (Qaim and Zilberman, 2003; Kleter *et al.*, 2007). The conventional insect control method heavily relies on the intensive and extensive use of chemical pesticides. Chemical insecticides cause severe environmental pollution and residual effect and also bring about adverse effects on people and beneficial insects. Moreover, Diamondback Moth (DBM) has evolved resistance to chemical insecticides (Hama, 1992; Shelton *et al.*, 1993). More recently, genetic engineering has provided a promising method for breeders to obtain insect resistant plants (Qaim and Zilberman, 2003) that is why present studies proposed to introduce *cryIAa* gene in cabbage which is a natural insecticide.

Bt transgenic crops express insecticidal crystal (*cry*) proteins from *Bacillus thuringiensis* (*Bt*). Various *cry* proteins are highly toxic to lepidopterans, coleopterans or dipterans, but do not harm human beings or the environment (Crickmore *et al.*, 1998; Bravo and Soberon, 2008). *Bt* toxins are expressed constitutively throughout the *Bt* plant. *Bt* toxins are only active in insects when ingested orally, as their mode of action is in the midgut. An important feature of the midgut is that the pH of the luminal fluid is about 12, which is

essential for dissolving the crystalline *Bt* protoxin, usually soluble only above pH 9.5. When insects come into contact with and ingest the crystalline inclusions, these inclusions as well as the toxins present in these inclusions (i.e. Cry(stal) protoxins; *cry*) first need to be proteolytically processed before binding to receptors is possible (Pietrantonio and Gill, 1996). Hence, the specificity of *Bt* strains may be caused by the presence or absence of specific proteases in an insect midgut. Transgenic *Bt* plants produce toxins instead of protoxins, as only the part of the gene that codes for the active toxin is inserted in the plant genome. As a result, after ingestion, the δ -endotoxins may bind directly to specific receptors on the midgut membrane, without the involvement of insect proteolytic enzymes. In the case of *Bt* plants, specificity may be based on the presence of specific receptors. Binding of the toxins to the receptors is a prerequisite for their insecticidal activity, although binding alone is not sufficient for toxicity (De Maagd *et al.*, 2001). After binding, it is thought that the toxin molecules insert into the gut membrane and form pores. These pores cause leakage and swelling of the cells due to an influx of positive ions (K^+) and then water. The swelling continues until the cells lyse, allowing the alkaline gut juices to leak into the insect's blood, raising the blood pH, which causes paralysis and insect death. One of the primary advantages of using *Bt* genes for insect control in transgenic plants is the specific insecticidal action toward certain insect orders (e.g. *Bt cryI* is specifically toxic to the order Lepidoptera), and therefore beneficial insects, birds, and mammals (including humans) are not harmed.

Being an economically important crop, genetic improvement programme can be a reliable way to tackle the cultivation problems of the cabbage plants. Genetic transformation using direct or indirect methods is an effective means, consuming less time compared to other methods of genetic improvement such as through the production of haploid or double-haploid plants and conventional breeding. *Agrobacterium*-mediated transformation system is the most widely applied method for cabbage genetic transformation (Cardoza and Stewart Jr., 2004). Attempts have been made to increase yield and quality of cabbage via genetic transformation techniques (Yu and Cao, 2004; Paul *et al.*, 2005; Lei *et al.*, 2006; Deng-Xia *et al.*, 2011). In *Agrobacterium*-mediated transformation technique, the availability of an effective binary vector system is a precondition. A binary vector that offers a high copy number in *Escherichia coli* and *Agrobacterium*, small in size, with suitable bacterial and plant selectable marker genes and includes one of the most widely used reporter genes is most applicable (Slater *et al.*, 2003).

The application of genetic engineering in cabbage cultivation is of great significance to generate new improved and desirable traits like insect and diseases resistant varieties. Several systems of genetic transformation have been used but *Agrobacterium tumefaciens* are most common. *Agrobacterium tumefaciens* has been used routinely to obtain transgenic plants and this technology is now available for many crops (Gasser and Fraley, 1989; Day and Lichtenstein, 1992; Lindsey, 1992; Jain, 1993; Dunwell, 2000; Srivastava, 1997, 1998, 2001a, 2001b 2002, 2003, 2012a, 2012b, 2013; Ranjekar *et al.*, 2003; Cardoza and Stewart Jr., 2004; Hua *et al.*, 2005; Xing *et al.*, 2008; McPherson and MacRae, 2009; Ahmad *et al.*, 2012, Awasthi and Srivastava, 2013).

In the present investigation of *Agrobacterium*-mediated insect resistance gene (*cryIAa*) transfer in cabbage (cv. Pride of India) has been carried out. The permission to use genetically engineered *Agrobacterium tumefaciens* strain containing insect resistance gene (*cryIAa*) was obtained from Institutional Biosafety Committee (IBSC) of Dr Y.S. Parmar University of Horticulture and Forestry, Solan.

The present studies were proposed to achieve the following objectives:

- i) To standardize a protocol of *Agrobacterium*-mediated *cryIA(a)* gene transfer in cabbage
- ii) Molecular characterization of putative transgenic plantlets of cabbage.

Chapter-2

REVIEW OF LITERATURE

Regeneration of plants from explant(s) culture is very important for its genetic improvement. Recent advances in cell and molecular biology have facilitated the transfer of foreign gene(s) into plant, which is the first step for the genetic improvement of crops. These techniques have enabled the production, characterization and selection of transgenic plant cultivars in a short period. With the development of gene transfer techniques applicable to many plant species, Genetic transformation is enabling researchers to introduce foreign genes, with known function from the model plants, into crop plants. It is also increasingly used to introduce homologous sequences from the crops, back into the crops, to either over express or silence native genes. A better understanding of gene function in crops will facilitate the transfer of knowledge of plant systems from model species and support targeted selection of key traits in plant breeding programmes (Srivastava *et al.*, 2013).

The expression of foreign gene(s) introduced into plants was first achieved in the early 1980s. In the 30 years following these initial successes, a revolution in plant genetic engineering has been witnessed, with the transformation of well over 100 different plant species now a routine procedure. The ability to manipulate the plant genome has come through intensive research into vector system based on the soil bacterium *Agrobacterium tumefaciens* and alternative strategies involving direct DNA gene transfer. *Agrobacterium*-mediated gene transfer has been extensively utilized for transfer of foreign DNA (gene) into number of plant species. Most of the transgenic plants produced to date were created through the use of *Agrobacterium* system (Gasser and Fraley, 1989; Day and Lichtenstein, 1992; Lindsey, 1992; Jain, 1993;; Singh and Sansavini, 1998; Dunwell, 2000, Srivastava, 1997, 1998, 2001a, 2001b, 2002, 2003, 2012, 2013; Ranjekar *et al.*, 2003; Cardoza and Steward Jr., 2004). The success of this approach resulted from improvement in tissue culture and genetic engineering methods. *Agrobacterium* is a causative agent of crown gall disease and the utility of this bacterium as a gene transfer system first recognized by Chilton *et al.* (1977), who demonstrated that the crown gall actually produced as result of the transfer and integration of genes from the *Agrobacterium* (Ti-plasmid) into the genome of plant cells.

The first transgenic plants expressing engineered foreign genes were tobacco plants produced by the use of *Agrobacterium tumefaciens* as a vector (Horsch *et al.*, 1984 and De Block *et al.*, 1984). An efficient method for introducing cloned gene(s) into plant cells and plant was given by Fraley *et al.*, 1983; Herrera Estrella *et al.*, 1983 and Zambrysky *et al.*, 1983. Genetic transformation of plants to confer resistance to insect pests offers an ecofriendly method of crop protection. Considerable progress has been made in developing transgenic crops with resistance to the target insect-pests over the past two decades (Vaeck *et al.*, 1987 and Chakrabarty *et al.*, 2002). Impressive results have been obtained with the expression of *Bt (cry)* genes, cowpea trypsin inhibitor gene, serine proteinase inhibitor gene and cysteine proteinase inhibitor gene (Gatehouse *et al.*, 1980; Wolfson and Murdock, 1987; Houseman *et al.*, 1989; Johnson *et al.*, 1989; Liang *et al.*, 1991; Bai *et al.*, 1992; Ding *et al.*, 1998; Jin *et al.*, 2000; Cho *et al.*, 2001; Awasthi, 2003; Chakrabarty *et al.*, 2002; Zhang *et al.*, 2004; Paul *et al.*, 2005; Lingling *et al.*, 2005; Zhao *et al.*, 2006b; Hua *et al.*, 2009 and Deng-Xia *et al.*, 2011; Srivastava, 2013). Such transgenic plants have shown considerable promise in reducing insect damage, both under laboratory and field conditions and thus, reducing the need to use pesticides for crop pest management. To protect the crop plants from insect pest attack, a massive application of pesticides not only leaves harmful residues in the food, but also causes adverse effects on non-target organisms and the environment.

A brief resume of work done on various aspects of plant regeneration and genetic transformation in cabbage and related *Brassica* species has been presented here under the following headings:

2.1 Plant regeneration studies in cabbage and related *Brassica* species

2.1.1 Direct organogenesis studies in cabbage and related *Brassica* species

2.1.2 Indirect organogenesis studies in cabbage and related *Brassica* species

2.1.3 Somatic embryogenesis studies in cabbage and related *Brassica* species

2.2 Genetic transformation studies in cabbage and related *Brassica* species

2.2.1 *Agrobacterium*-mediated reporter/marker gene transfer in cabbage and related *Brassica* species

2.2.2 Direct gene transfer technique-mediated reporter/marker gene transfer in cabbage and related *Brassica* species

2.2.3 *Agrobacterium*-mediated desirable gene transfer in cabbage and related *Brassica* species

2.2.4 Direct gene transfer technique-mediated desirable gene transfer in cabbage and related *Brassica* species

2.3 Factors affecting transformation frequency in different plant species.

2.1 Plant regeneration studies in cabbage and related *Brassica* species

Regeneration of whole plants from cultured tissues or cells is a prerequisite for successful applications of *in vitro* techniques of gene transfer, mass propagation and somaclonal variation studies (Pavlovic *et al.*, 2012). There are two major systems of plant regeneration, viz. organogenesis (Direct and Indirect) and somatic embryogenesis.

2.1.1 Direct organogenesis studies in cabbage and related *Brassica* species.

Shoot regeneration from the explants without callus formation has an important role in *Agrobacterium*-mediated gene transfer experiments where the objective is to recover the donor genotype intact apart from the inserted gene.

The tissue culture work in cabbage was initiated by Bajaj and Nietsh (1975) and Primo-Millo and Harada (1975). They had successfully regenerated red cabbage plants using various explants viz. roots, stem, cotyledon and leaves by culturing on a standard medium having different concentrations of various cytokinins and auxins.

Dai *et al.* (1994) studied high frequency regeneration by using hypocotyl and cotyledon as an explants and cultured them on MS medium *in vitro*. Hundred per cent differentiation was observed in MS medium containing 2.0mg/l BA.

Petrova and Antonova (1996) reported factors affecting plantlet regeneration from seedling explants of *Brassica oleracea* L. var. *capitata*. They excised cotyledon and hypocotyl explants from 6 to 7 days old seedlings and cultured them on MS medium supplemented with 1-5mg/l BAP and 0.1–1.0mg/l NAA. MS medium supplemented with 5mg/l BAP and 1mg/l NAA induced the highest regeneration frequency. Hypocotyls were more responsive than cotyledons.

Zhang *et al.* (1997) did optimization growth regulators in medium for efficient plant regeneration of Chinese cabbage. Cotyledons of four days old seedlings of 4 varieties of

Chinese cabbage were cultured on MS medium containing different combinations of NAA and BA, and 4.0mg/l AgNO₃. The ratio of NAA: BA was crucial for regeneration frequency.

In vitro shoot induction and multiplication of nodal explants of cabbage on MS medium supplemented with 18.5µM kinetin, 17.7µM BAP or 0.45µM thidiazuron were briefly considered by Souza *et al.* (1998). Cultures were maintained under 16 hours light/8 hours dark. After 30 days no significant differences were found for multiple shoot induction, but the kinetin treatment showed the best shoot length.

Zhang *et al.* (1998) examined medium conditions for reliable shoots regeneration from cotyledonary explants of Chinese cabbage (*Brassica campestris* L. subsp. *pekinensis*). Maximum shoot regeneration was obtained in the presence of 5.0mg/l BA and 0.5mg/l NAA. Shoot induction was further improved by addition of AgNO₃ as well as higher concentrations (1.2-1.6 %) of agar in the regeneration medium; when 123 genotypes were tested a large variation in regeneration frequency was observed, ranging from 0 to 95 %.

Pua *et al.* (1999) studied effect of ethylene inhibitors and exogenous putrescine on shoot regeneration capacity of cultured hypocotyl explants from 18 cultivars of cabbage (*Brassica oleracea* L.var. *capitata*), cauliflower (*Brassica oleracea* L. var. *botrytis*) and broccoli (*Brassica oleracea* L. var. *italica*). All cultivar were poorly regenerated on MS medium supplemented with 0.5-1.0mg/l NAA in combination with 1.0-4.0mg/l BA. Shoot regeneration of some cultivar was enhanced by addition of 10µM AgNO₃ to the medium. The presence of 10µM aminoethoxyvinyl glycine (AVG) or 10µM exogenous putrescence was also beneficial to regeneration, whereas combination chemical treatment with vacuum infiltration had a little effect. The results supported the hypothesis that ethylene produced by explants often inhibit shoot regeneration *in vitro*.

Effect of physical factors on shoot regeneration in cabbages was studied by Souza *et al.* (1999). Shoot cuttings from *in vitro* cultured cabbage plantlets were cultured on modified MS solid or liquid medium. In a second experiment, cuttings were cultured on solid MS medium in flasks with metal vitafilm or plastic lids. Shoot regeneration was not affected by medium or lid type.

Cao *et al.* (2000) developed an efficient and fast method for plant regeneration from petiole explants with cotyledons in Chinese cabbage (*Brassica campestris* L. subsp. *chinensis* [*B. chinensis*]). A half-strength MS medium supplemented with 2.0mg/l BA, 0.45mg/l NAA and 7.5mg/l AgNO₃ was optimum for obtaining the highest frequency of shoot regeneration. Shoot formation was observed directly after 6 days of inoculation.

Xie *et al.* (2000) analysed the effects of seedling age and supplemented culture media on bud induction from cotyledons in *Brassica campestris* L. subsp. *chinensis* [*B. chinensis*]). Results indicated that explants from five to nine days old seedlings were the most efficient for inducing adventitious buds. Benzyladenine (BA) concentration was the key factor in adventitious bud differentiation; BA concentration lower than 2.0mg/l reduced the frequency of adventitious bud induction, whereas a combination of 6mg/l BA and 0.05mg/l NAA improved adventitious buds induction. Culture medium containing 12.0g/l agar, 5.0mg/l AgNO₃ and 2.0-3.0mg/l gibberellic acid (GA₃) increased the frequency of differentiation.

A protocol for production of common cabbage through organogenesis was developed by Donato *et al.* (2001). Young leaves were inoculated in a basic medium supplemented with 0.5, 1.0, 2.0 and 5.0mg/l NAA in combination with kinetin or BAP at 0.0, 0.01, 0.05, 0.1 and 1.0mg/l. Kinetin and benzyladenine were not effective for shoot development. However, addition of 1.0mg/l NAA was efficient for direct organogenesis of shoots.

Kang *et al.* (2001) developed a simple and efficient system to regenerate plants from cotyledon and hypocotyl tissues of Chinese cabbage (*Brassica campestris* L. subsp. *pekinensis* [*B. pekinensis*] cv. Seoul). Among the various combinations of naphthalene acetic acid (NAA) and 6-benzyladenine (BA) tested, the best shoot induction medium for cotyledons, with 2.67 shoots per explant, contained 2.0mg/l NAA, 1.0mg/l BA and 16.7mg/l AgNO₃. The shoot induction medium with 1.0mg/l NAA, 5.0mg/l BA and 16.7mg/l AgNO₃ was best for shoot induction from hypocotyl explants, with 1.87 shoots per explant.

Kondratenko (2001) analysed the effect of plant extracts and new synthetic substrates of phytohormones on plant regeneration from protoplasts of cabbage (*Brassica oleracea* L. var. *capitata*) and developed a protocol for the isolation and culture of protoplasts obtained from fully expanded young leaves (2-3cm wide) of cabbage cultivars Dithmarscher Fruner, Lyunskaya Ranyaya, Kharkovskaya Zimnaya, Ukrainskaya Osen and Lesya. The combined application of cabbage stump extract and auxin regulators resulted in successful plant

regeneration at 3.0–3.5 months after protoplast isolation, whereas the application of 2,4-D inhibited shoot regeneration.

Cheng *et al.* (2001a) developed an *in vitro* method for high frequency shoot regeneration and continuous production of rapid cycling *Brassica oleracea* in large numbers. The high regeneration capacity was tissue dependent. High frequency shoot regeneration was obtained with N⁶-benzylAdenine or thidiazuron supplemented media. Rooting of regenerated shoots of *B. oleracea* occurred readily on α -naphthalene acetic acid supplemented media. Rooted plantlets were successfully established in soil and developed normal fertile flower and viable seeds.

In vitro experiment on Chinese cabbage to determine the effect of silver nitrate, pH, agar concentration and dark period on shoot induction from cotyledon and hypocotyl explants was studied by Kang *et al.* (2002). The initial dark treatments decreased shoot induction for both explants. Both pH and agar concentrations in the culture media did not affect shoot induction from hypocotyl explants. For the cotyledon explants, the best shoot induction was obtained from the culture media supplemented with 20mg/l NAA, 1.0mg/l benzylAdenine, and 4mg/l silver nitrate. The best shoot induction for hypocotyl explants was obtained from the culture media supplemented with 1.0mg/l NAA, 50mg/l benzylAdenine and 8.0mg/l silver nitrate.

Zhang *et al.* (2002a) identified genotypic factors influencing shoot regeneration from cotyledonary explants of Chinese cabbage (*Brassica campestris* L. subsp. *chinensis*). A large variation in shoot regeneration frequency and mean shooting number per cotyledon was observed ranging from 0 to 97.5 %, among 123 cultivars of *B. pekinensis* and 20 cultivars of *B. chinensis*. Seven *B. pekinensis* and five *B. chinensis* cultivars showed regeneration frequencies of more than 80 %. Shoot regeneration frequency was not related to origin, cultivar types and days to maturity.

Zhang *et al.* (2002b) analysed the effect of hormone combinations, AgNO₃ and agar concentration on shoot and plant regeneration from cotyledonary explants of Chinese cabbage (cultivars Beijing New 2, Beijing 80, Youhuang, CR Xinhuang, Xinza0 89-8 and Xiabao). Maximum shoot regeneration was obtained in MS medium containing 5.0mg/l BA, 0.5mg/l NAA, 2.0mg/l AgNO₃ and solidified with 1.2-1.6 % agar.

High efficient shoot regeneration systems were established using cotyledonary explants of two Chinese cabbage (*Brassica campestris* L.) cultivars by Lu *et al.* (2003). Effects of two kinds of cytokinins used in this experiment on shoot regeneration were compared. The regeneration frequency of HY and LB were 92.8 and 82.4 % respectively in the medium of MS+ 2.0mg/l BA+ 0.5mg/l NAA+ 5.0mg/l AgNO₃, and the regeneration frequency of HY in medium of MS+ 0.25mg/l TDZ+ 0.5mg/l NAA+ 5.0mg/l AgNO₃ was up to 98.8 %. As a kind of cytokinins, TDZ was more active than BA. In the medium supplemented with TDZ, it was more efficient and rapid for shoot regeneration; there were more buds on the explants than in the media with BA. AgNO₃ was indispensable for the shoot regeneration.

Eight parental lines of Chinese cabbage were cultured *in vitro*. The effect of growth regulator combinations, different genotypes, seedling age, cotyledon sampling place and explants on shoot and plant regeneration from cotyledon explants were studied by Zhao *et al.*, (2004). Maximum shoot regeneration was obtained in MS medium supplemented with 6-benzylAdenine, naphthalene acetic acid, and AgNO₃ at 2.0, 0.5 and 0.05mg/litre, respectively. The best medium for rooting was MS medium supplemented with naphthalene acetic acid at 0.2mg/litre. The survival rate of transplanted plantlets was as high as 90 % in spring.

Wang *et al.* (2005) reported shoot regeneration potential of 14 genotypes of Chinese cabbage (*Brassica campestris* L. subsp. *pekinensis* [*B. pekinensis*]). The regeneration frequency significantly varied among the different cultivars. The effect of 6-BA (benzylAdenine) and TDZ (thidiazuron) on shoot regeneration was studied using the genotypes that exhibited superior shoot regeneration potential. Shoot regeneration was optimum in the MS (Murashige and Skoog's) medium with 5.0mg/l 6-BA + 0.5mg/l NAA + 2.0mg/l AgNO₃ and MS medium with 0.1mg/l TDZ + 5.0mg/l 6-BA + 0.5mg/l NAA + 2.0mg/l AgNO₃ + 0.25mg/l ABA (abscisic acid), but highest frequency of shoot regeneration (90.6 %) was obtained with the latter medium.

Fan *et al.* (2005) used cotyledon as explants for shoot regeneration in two high quality Chinese cabbage, Aichi Hakusai and Shinriso Hakusai, from Japan. The effect of hormone combinations, AgNO₃ concentration and seedlings age on shoot regeneration was studied. Compared with 6-BA, TDZ was more effective in inducing shoot regeneration. Shoot regeneration from cotyledons of the two cultivars reached 75.0 and 55.0 %, respectively, in

MS medium containing NAA at 0.5mg/l and TDZ at 1.0mg/l. Addition of AgNO₃ at 5.0mg/l to this medium further increased the frequency of shoot regeneration to 96.7 and 80.0 %, respectively. Seedlings age of 5 to 8 days was optimum for shoot regeneration.

Yu *et al.* (2005b) developed an efficient and fast method for plant regeneration of three varieties of Chinese cabbage. An efficient system was also established using cotyledon with petiole as explants. MS medium supplemented with 2.0mg/l BA, 0.5mg/l NAA, 4.0mg/l AgNO₃ and 0.3mg/l ABA was optimum for Chinese cabbage.

Zhu *et al.* (2006) inoculated hypocotyl explants of Chinese cabbage cv. Guilong No.5 with morphological lower parts inserted on the medium (A), horizontal to the medium (B) or placed upwards (C). Changes in soluble protein content and enzyme activities (peroxidase, POD; superoxide dismutase, SOD; and catalase, CAT) during shoot regeneration were evaluated. Under treatment A, adventitious shoots formed at eighth to ten days of culture, shoot regeneration % age reached 87.8 %, and 15.1 shoots were regenerated per explant. Treatment A showed higher regeneration ability than B and C. Soluble protein content, and POD and SOD activities increased, while CAT activity decreased before shoot primordium appeared.

Chen and Hou (2008) analysed effects of genotype, explant and hormone on shoot regeneration of four varieties including Shulü, Suzhouqing, Liangbaiye and Aikang 5 of non-heading Chinese cabbage, to select the easily regenerating variety, and set up better regeneration system for transgenic work. The regeneration frequency of Shulü was the highest among different cultivars. The regeneration frequency of petiole with cotyledon was the highest among different explants types. Addition of 0.5mg/l thidiazuron (TDZ) into the germination medium could make the seedlings stronger which was beneficial to regeneration, leading to a regeneration rate of over 60.00 % for all mediums. The regeneration rate of petiole with cotyledon was the highest in the medium MN+ 0.5mg/l TDZ+ 0.5mg/l NAA+ 7.5mg/l AgNO₃ and the rate reached 80.00 %. The result indicated that TDZ played an obviously persistent role in inducing organogenesis.

Pavlović *et al.* (2010) studied *in vitro* shoot regeneration ability of *Brassica oleracea* varieties (red cabbage, broccoli, Savoy cabbage and cauliflower). Cotyledon, hypocotyl and root explants of 7 days old seedlings were incubated on Murashige and Skoog's (MS) medium supplemented with 1.0mg/l 6-benzylAdenine (BA) or 6-furfurylaminopurine (KIN)

in combination with 0, 0.1, and 0.2 mg/l indole-3-butyric acid (IBA). Hypocotyls showed the best explants in almost all varieties tested with a minimum regeneration potential of 75 % and producing 3.5-7.4 shoots per explant. The BA-supplemented media were optimal for both shoot regeneration and multiplication. Shoots rooted maximally (100 %) on plant growth regulator-free MS medium containing 2 or 4 % sucrose. Increased sucrose content improved plant acclimatization in the greenhouse.

Liu *et al.* (2011b) observed a high regeneration system of heading Chinese cabbage. The effects of different combinations of hormones (6-BA, NAA, TDZ), cutting treatments and inoculation methods on regeneration rate of adventitious bud were studied in four heading Chinese cabbage with the first leaf as explants when the second leaf just came out. The results showed that the best regeneration rate and regeneration coefficient of '06J28' reached as high as 87.63 % and 2.62 respectively in MS+ 4.0mg/l 6-BA+ 0.14mg/l NAA; The regeneration rate and regeneration coefficient of '06J28' were 80.73 % and 1.89 respectively in the MS medium supplemented with 0.3mg/l TDZ and 0.14mg/l NAA, and TDZ showed similar effect with 6-BA. The best cutting and inserting mode was that: The young leaf was cut off at the cross of petiole and hypocotyl, the whole leaf with petiole was kept as explants, which was vertically inserted in medium with petiole end. Comparing the four genotypes, the highest regeneration rate reached 87.63 % in '06J28', while the highest regeneration coefficient reached 3.76 in '92S105'. They established a high regeneration system of heading Chinese cabbage and set up a basis for transgenic improvement further.

Cristea *et al.* (2012) studied the effect of silver nitrate (AgNO_3) in modulating the morphogenetic reaction of white cabbage anthers cultivated *in vitro*. Silver ions, in the form of nitrate play an important role in promoting the somatic embryogenesis and organogenesis, which led its wide spread use in different plant tissue culture *in vitro*. Therefore, they focused on testing different concentrations of AgNO_3 (5, 10, 15, 25, 30, 50 and 60 μM) added to a pre-tested medium formula– Murashige and Skoog (MS), 1962 basal medium supplemented with benzylaminopurine (BAP)- 8.9 μM and naphthylacetic acid (NAA) 2.7 μM . The biological material was represented by unopened flower buds containing anthers with microspores at late uninucleate to binucleate stage. In the culture condition investigated in this study, the data obtained promotes the utilization of AgNO_3 in a concentration of 50 μM for the successful induction and sustaining of regenerative processes of white cabbage anthers cultivated *in vitro*.

Pavlovic *et al.* (2012) tested fourteen genotypes of cabbage (*Brassica oleracea* L. var. *capitata*), were tested for their ability to regenerate shoots *in vitro*. Five of them are early, while nine are late genotypes. Lateral buds from plants grown in the open field were used as explants. In all genotypes, lateral buds showed the high %age of shoot formation, ranged from 80 to 100 %. They were incubated on Murashige and Skoog's (MS) media supplemented with 1.0 and 2.0mg/l of benzylAdenine (BA) or 1.0 mg/l 6-furfurylaminopurine (KIN) in combination with 0, 0.5 and 1.0mg/l indole-3-butyric acid (IBA). The BA supplemented media were optimal for both growth and multiplication of shoots. In both groups of genotypes, the highest index of multiplication (IM) was achieved on medium supplemented with 2.0mg/l BA and 1.0mg/l IBA, in R9 early genotype (IM 8.53) and K1 late genotype (IM10.06). R5 early and in K29 and K75 late genotypes had no multiplication on medium with 1.0 mg/l KIN (IM 1.00). Also, in all genotypes the lowest index of multiplication was observed on media supplemented with KIN (without or in combination with IBA).

2.1.2 Indirect organogenesis studies in cabbage and related *Brassica* species

In vitro regeneration of plants from callus cultures is often an essential final step for shoot multiplication and can be used to generate somaclonal variation for selection of desirable traits. It can also provide an additional tool for cellular genetic manipulation in crop improvement programs.

Mascarenhans *et al.* (1978) induced callus and roots on 5mm explants of different explants of cabbage seedlings on MS media containing kinetin, coconut, milk, inositol and an auxin.

Lillo and Shahin (1986) showed that pre-treatment on a medium containing 4.5fM 2,4-D; 2.2fM BA and 0.5fM NAA encouraged later shoot formation from both protoplasts derived callus and root segments of cabbage. A medium containing BA and GA₃ with sucrose as osmoticum was suitable for shoot regeneration. Roots were produced on hormone free medium and plants were grown to maturity in soil. Plants were regenerated from protoplasts in 6-7 weeks, reducing the chances of uncontrolled genetic changes in culture. Root segments produced multiple shoots suggesting that the method may be suitable for micropropagation.

Koda *et al.* (1988) analysed the effects of phytohormones and gelling agents on plant regeneration from protoplasts of red cabbage. The number of calluses developed after 4 weeks of culture of isolated protoplasts on MS medium containing 0.8 % agar and 0.5 or 1mg/l kinetin was unaffected by the concentration of growth regulators. Shoot regeneration was best with (5-20mg/l) cytokinin concentrations.

Regenerants of tomato and cabbage from tissue culture were obtained by Bel'-Skaya *et al.* (1990). Suitable conditions were established for callus formation and plantlet regeneration in 3 tomato and 6 cabbage varieties. Regenerants of both crops were obtained from explants of different origin. Conditions were determined for the micropropagation and rooting of 6 self-compatible lines of cabbage.

Plants were regenerated from hypocotyl callus of intergeneric and interspecific hybrids between radish, cabbage and Chinese cabbage by Hagazi and Matsubara (1991). Hypocotyl segments were cultured on MS medium containing combinations of different phytohormones: 2,4-D + BA or Kn ; or NAA + BA/ Kn. For shoot regeneration, callus obtained from the hypocotyl was sub-cultured at month long interval on a callus initiation or MS media with or without cytokinins. As many as 70 % of the calli from interspecific hybrids between cabbage and Chinese cabbage differentiated into shoot on the initial medium.

Wang *et al.* (1991) isolated protoplasts of the cytoplasmic male sterile (CMS) line of cabbage from leaves and hypocotyl of seedlings grown *in vitro*. Callus obtained was made to differentiate and complete plantlets were regenerated about 3 weeks after transfer to auxin free medium for root regeneration.

Eisner *et al.* (1992) established methods for *in vitro* regeneration of various varieties of cabbage and broccoli in conditions used for genetic transformations. Best results were obtained with five to seven days old hypocotyls. Acetone in the medium had a beneficial effect, stimulating the production of callus and shoots, increasing morphogenetic activity in the explants and accelerating the rate of regeneration. Therefore, for transformation some conditions need to be optimized before hand.

Havel *et al.* (1994) experimented with cabbage hypocotyls by culturing on MS medium containing benzolinon and showed that as a result of its effect on callus formation,

bud induction, shoot and root regeneration and peroxidase activity, it can be included that benzolinon has an auxin like activity but it is not as effective as NAA in tissue culture.

The plantlet regeneration from cotyledon protoplast culture of non-heading Chinese cabbage was observed by Hou *et al.* (2000). Protoplasts obtained from 14 to 18 days old seedlings of Chinese cabbage (*Brassica campestris* L. subsp. *chinensis* [*B. chinensis*]) divided and grew in the presence of KM8P1 medium supplemented with 2,4-D (0.5mg/litre), 6-BA [benzylAdenine] (0.25mg/litre), NAA (0.5mg/litre), glucose (9.0g/100 ml), sucrose (1.0g/100ml) and galactose (0.03g/100ml). Regenerated plants were formed from callus after bud induction and root initiation.

Hou *et al.* (2000) cultured protoplasts isolated from hypocotyls from five days old seedlings of *Brassica campestris* L. subsp. *chinensis* on KM8P1 medium with 0.5mg/l 2,4-D, 0.25mg/l BA and 1.0mg/l NAA. Microcalli formed after 21 days of culture, and these were then multiplied on MS medium with 1mg/l 2,4-D. Shoots were initiated on MS medium containing 10.0mg/l BA and 0.3mg/l NAA and then transferred to a hormone-free medium to develop roots.

Mesophyll protoplasts of vegetable brassicas were regenerated by a simple protocol which was developed by Kirti *et al.* (2001). Protoplasts from the *in vitro* grown leaf material of cabbage cv. Golden Acre, cauliflower cv. Pusa Snowball and broccoli cv. KTS1 were cultured in Kao's medium with 0.25mg/l 2,4-D, 1.0mg/l NAA, 0.5mg/l BAP and 7.2 % glucose, initially in the dark for 3 days and subsequently exposed to light. Osmoticum reduction in protoplast cultures on the seventh, tenth and thirteenth day with a medium of lower osmoticum consisting of the basal medium used for plating (with 3.4 % sucrose instead of glucose and supplemented with 0.1mg/l 2,4-D and 0.5mg/l BAP) resulted in rapid colony growth. Microcalli were planted on K3 medium with 0.1mg/l 2,4-D, 1.0mg/l BAP and 3.4 % sucrose and gelled with 0.5 % agarose (pH 5.8). Transfer of calli to another medium with 2.0mg/l zeatin regenerated shoots from cauliflower protoplast-derived calli, whereas a medium with kinetin and zeatin both at 2.0mg/l supported shoot regeneration in cabbage and broccoli.

A protocol for rapid and efficient plant regeneration from protoplasts of red cabbage by a novel nurse culture method was developed by Chen *et al.* (2004). When the protoplasts of red cabbage were cultured on modified MS medium containing various combinations of

BA, NAA and 2,4-D, they did not continue dividing due to browning. However, they successfully divided and formed micro-calli at a high efficiency when they were mixed and co-cultured with those of tuber mustard at a 1:1 ratio. Red cabbage-like plantlets were regenerated from these protoplast derived callus at a frequency ranging from 33 to 56 % in all the experiments, where three cultivars of red cabbage were tested.

Wang and Wang (2007) used MS medium supplement with 6-BA, NAA and 2,4-D to induce calli, adventitious buds and adventitious roots from under hypocotyl explants of cabbage. The experiments showed that 1mg/l 6-BA and 0.3mg/l NAA was the best for inducing calli from under hypocotyls. It's easy for inducing adventitious buds on MS medium supplement with 6-BA(1.0mg/l)+ NAA(0.2mg/l) and NAA(0.5mg/l) as well as inducing adventitious roots when hormone were 6-BA(1.0mg/l)+ NAA(0.2mg/l), 6-BA(1.0mg/l)+ NAA(0.3mg/l) and 6-BA(4.0mg/l)+ NAA(0.1mg/l), respectively. The experiments also showed that genotype has significant influence on adventitious bud differentiation.

Farzinebrahimi *et al.* (2012) regenerated *Brassica oleracea* L. var. *italica* by using leaf and shoot tip explants on Murashige and Skoog (MS) basal medium supplemented with various concentrations of (BAP) and (IBA), alone and in combinations to achieve plant regeneration. The highest mean numbers of shoot was observed on combinations of 1.0mg/l and 1.5mg/l of BAP and IBA, 9.49 and 8.69, respectively. The highest number of shoot produced (11.55) per shoot tip explant was recorded on 2.5mg/l BAP. In addition, highest %age of roots (100 %) and highest mean number of roots produced per leaf explant (8.47) occurred on medium with combination of 1.5mg/l IBA and BAP by shoot tip explant (4.51).

Sharma *et al.* (2013) studied morphogenetic potential of cotyledon and hypocotyl explants of cabbage (*Brassica oleracea* L. var. *capitata*) to develop a reliable plant regeneration protocol. Nine days old aseptically grown seedlings of cabbage were used as source of explants for plant regeneration. Out of 36 combinations of growth regulators used in MS medium, high frequency shoot regeneration from cotyledon and hypocotyl explants was obtained through callus on MS medium supplemented with 1.5mg/l BAP and 0.50mg/l NAA and 2mg/l BAP and 0.25mg/l IAA.

2.1.3 Somatic embryogenesis studies in cabbage and related *Brassica* species

Induction of somatic embryogenesis requires a single hormonal signal to induce a bipolar structure capable of forming a complete plantlet. In contrast, organogenesis requires

two different hormonal signals and thus two different culture media—one to induce a shoot bud, and another to induce a root primordia (Phillips *et al.* 1995). Therefore, somatic embryogenesis is more attractive than organogenesis as plant regeneration.

The somatic embryogenesis work on cabbage was started quite some time back by Terada *et al.* (1987). They developed an efficient procedure for obtaining somatic hybrids between *Brassica oleracea* L. and *Brassica campestris* L. Hypocotyl protoplasts of *Brassica oleracea* L. were fused with mesophyll protoplasts from three different varieties of *Brassica campestris* L. by the polyethylene glycoldimethylsulfoxide method. The selection of somatic hybrids utilized the inactivation of *B. oleracea* protoplasts by iodoacetamide (IOA) and the low regeneration ability of *Brassica campestris* L. The efficiency of recovery of somatic hybrids depended upon the IOA concentration, and when 15 mM IOA was used, 90 % of the regenerated plants were found to be hybrid. All the hybrids had intermediate leaf morphology and possessed LAP isozymes of both parental species. The chromosome analysis revealed a considerable variation in chromosome number of somatic hybrids, showing the occurrence of multiple fusion and chromosome loss during the culture. Some of the hybrids flowered and set seeds.

Cao *et al.* (1990) used microspores from 2-8mm long buds of 14 rapid cycling hybrid genotypes reached a size of 30-40 μ m after 2 days of incubation in liquid medium and exhibited the first nuclear divisions from day 3 onwards. Globular embryos arose after 12 days of culture and were transferred to M₈ solid medium. Plantlets developed after several subcultures on M₈ and T₀ media. Embryogenesis occurred only when 7mm long buds were used, coinciding with the presence of binucleate or trinucleate spheric microspores. Mean embryo yield/bud ranged from 0.16 for AIB33.7.4s X Krautprinz 26 to 8.64 in AIB33.7.4s X Menza 31. No embryos were formed in hybrids with AIB33.8.1s as the ♀ parent, nor in AIB33.7.4s X Strukton 17 and AIB33.6s X Casquedor 24. Embryogenesis was also influenced by temperature pretreatment of donor plants. Microspore culture was considered more reliable than anther culture, with a broader genotypic response.

Cotyledonary explants of Chinese cabbage were cultured on MS medium supplemented with various concentration of 2,4-D, reported by Choi *et al.* (1996). Upto 20 % of the cotyledonary explants produced somatic embryos with or without intervening callus production upon transfer to MS basal medium most of the somatic embryos developed into plantlets.

In vitro somatic embryogenesis in cabbage was reported by Donato *et al.* (2000). Leaf explants were cultured in MS medium supplemented with various combinations of 2,4-D/BAP and 2,4-D/kinetin. During the induction phase of calluses (phase I), development of calluses with embryogenic characteristics was observed from media supplemented with 5.0mg/l 2,4-D or 1.0mg/l 2,4-D + 0.1mg/l BAP, or 1.0mg/l 2,4-D + 1.0mg/l BAP, or 2.0mg/l 2,4-D + 0.05mg/l BAP, these were chosen for sub-culture in regeneration media (phase II). In this phase, culture media were supplemented with reduced concentrations of 2,4-D, BAP and GA₃ or without growth regulators. The calluses that were sub-cultured in the regeneration media R₃ (0.1mg/l BAP) and R₈ (0.001mg/l 2,4-D) and M₃₀ (0.1mg/l 2,4-D + 1.0mg/l BAP), respectively, developed structures similar to embryos.

Jiang *et al.* (2005) Isolated microspores from 20 varieties of cabbages were cultured in NLN-13 with activity charcoal liquid medium, 18 varieties produced microspore-derived embryos and plant regeneration. The production of microspore-derived embryos varied remarkably between these varieties. Embryo yield per bud ranged from 0.026 to 26.89. Sucrose concentrations and hormone concentrations in NLN medium were compared. The results show NLN with sucrose (130mg/l) was better in the production of microspore-derived embryos. Average embryo yield was higher in 33°C high temperature treatment for 24 hours. Microspore-derived embryos on B5 medium with 6-BA 0.1mg/l and NAA (0.1mg/l) and sucrose 30mg/l and agar 11mg/l were good to regenerated plants.

Górecka and Krzyzanowska (2007) elaborated the method of plant regeneration from cabbage (*Brassica oleracea* L. var. *capitata*), anther-derived embryos. They observed that this method should be efficient enough to secure plant material for creating new F₁ hybrids of cabbage. The factors taken into consideration were varieties and regeneration media. Out of all embryos, 20.8 % grew or produced shoots and 43.4 % died during the first passage. In the second passage a more intensive shoot production was observed. On B5-2 medium, containing sucrose (20g/l) and kinetin (20mg/l) most embryos (18.3 %) produced shoots but on MS medium containing sucrose (20g/l), BA (1.0mg/l) and NAA (0.001mg/l), most produced callus (51.0 %). The shoots rooted on B5 medium supplemented with 1mg/l IAA. From among varieties considered in this study, most embryos of Kamienna Glowka produced shoots during first passage (16.2 %) and least died (38.5 %). The best medium for the regeneration was B5-2. The genotype had an effect on plant regeneration from androgenetic embryos but not as pronounced as in the process of androgenesis. After 3 passages it was

possible to obtain from each survived embryo, enough plants for the subsequent stages of cabbage breeding.

Effects of chilling, activated charcoal and AgNO₃ on plant regeneration were studied by Gao *et al.* (2009) and the chromosome ploidy of regenerated plants was identified by using Microspore-derived cotyledonary embryos of non heading Chinese cabbage. The results showed that cold treatment (4°C) for 5d increased the rate of embryo germination and the number of shoots. Adding activated charcoal (1.0g/l) in the medium showed no distinct difference for embryo germination, but the phenomenon of vitrification had been relieved. Adding 5.0 or 7.0mg/l AgNO₃ in the medium efficiently improved the embryo germination. The ploidy analysis results showed that non-heading Chinese cabbage had a high level (50 %-100 %) of spontaneous chromosome doubling rate; the ploidy variation of different genotypes had diversity. The rate of chimera was high in some genotypes with the highest value of 42.86 %.

Yang *et al.* (2010a) used cotyledon, hypocotyl or root explants of 7 days old broccoli seedlings. Explants were cultured on MS agar or liquid medium supplemented with 1.0mg/l 2,4-D. The frequency of direct somatic embryo formation was 100 % when root explants were cultured in liquid medium.

Improve induction of embryogenesis in white cabbage (*Brassica oleracea* L. var. *capitata*) microspore cultures was done by Yuan *et al.* (2012). The effect of NLN-13 liquid medium pH on isolated microspore embryogenesis was investigated in five white cabbage genotypes. Relatively high pH (6.2 or 6.4) was more effective on microspore embryogenesis in most of the white cabbage genotypes than the pH of 5.8, especially for inducing microspore-derived embryos in recalcitrant genotype ‘Zhonggan No. 8’. 2—(N-Morpholino) ethanesulfonic acid (MES) and the arabinogalactan-protein from gum arabic were tested on four out of five genotypes to see if they could increase embryo yield in microspore cultures. Adding MES or gum arabic alone was effective for these four genotypes, but the frequency of embryos derived from microspores was still low. However, the combination of 10mg/l gum arabic and 3mM MES in NLN-13 at pH 6.4 significantly enhanced microspore embryogenesis efficiency (with embryo production of 4.57–222.97 embryos per bud), especially with recalcitrant genotype “Zhonggan No. 8” for which it was increased by about 35-fold.

Wang *et al.* (2012) investigated the effect of AG and AGPs on microspore embryogenesis and plant regeneration by using 3 late-bolting hybrids of Chinese cabbage (*Brassica campestris* L.subsp. *pekinensis* (Lour) Olsson) and 3 hybrids of Pakchoi (*Brassica campestris* L.subsp.*chinensis*) Makino var.*communis* Tsen et Lee) as material. The results showed that adding 10mg/l AG, 10mg/l AGPs or 10mg/l AG+ 10mg/l AGPs to the medium of NLN-13 could improve the ability of microspore embryogenesis and support plant regeneration.

2.2 Genetic transformation in cabbage and related *Brassica* species

Genetic transformation systems have been described for many plant species. It can bring improvement and is used as a tool for functional genomics in plants (Ahmad *et al.*, 2012). The technology involves the delivery of defined foreign genes into plant cells, obtaining integration of the genes into plant genomes and observing expression of the genes in the regenerated plants. This successful application of plant transformation technology is due to advances in rDNA technology and the development of techniques which allow plant regeneration from cells or tissues (Srivastava, 2012a). Genes are introduced either with the help of *Agrobacterium tumefaciens* or through direct delivery of DNA (Davey *et al.*, 1989). It plays a vital role in *Brassicaceae* family where the conventional breeding programmes require a long time to screen for desired traits. A few examples, but are not limited, are discussed here:

2.2.1 *Agrobacterium*-mediated reporter/marker gene transfer in cabbage and related *Brassica* species

Genetic transformation is a potential tool for the improvement of crop plants. Recombinant DNA techniques for introducing foreign genes into plants involve the use of dominant selectable markers that are expressed at the cell and whole-plant levels (Mathew and Litz, 1990). Selection and recovery of transformed cells or tissues and elimination of *Agrobacterium* from the cultures require the use of selective agents and/or antibiotics which is a critical feature in obtaining transgenic plants. Genes encoding antibiotic resistance and herbicide tolerance are widely employed as selective markers to identify the rare transformed cells (Vetten de *et al.*, 2003; Miki & McHugh, 2004).

Genetic transformation studies in cabbage had started sometime back by Shahin and Yashar (1986). They transformed cabbage by introducing *npt-II* gene via disarmed *A.*

tumefaciens using punctured cotyledon method followed by selection of cells with 60mg/l kanamycin. The foreign gene was expressed in all transgenic plants recovered from callus resistant to kanamycin.

Christey and Earle (1988) reported high plant regeneration efficiency of *Brassica oleracea* L. and *B. campestris* L. by using peduncle explants. The procedure was also considered for the transformation of *Brassica oleracea* L. through the co-cultivation with *Agrobacterium tumefaciens*. The transformants were obtained using *A. tumefaciens* strain LBA4404 carrying gene specific to kanamycin resistance and β -glucuronidase activity.

Yu and Shao (1988) produced plants from hypocotyl segments of an early maturing F₁ hybrid of cabbage by using Ti-plasmid derived vector. Hypocotyl segments were grown *in vitro* and infected with *Agrobacterium* strain C58C1. The transformants had shown the expression of *nos* and *npt-II* genes.

David and Tempe (1988) inoculated cauliflower explants with different *Agrobacterium rhizogenes* strains. Numerous hairy roots were induced on cauliflower hypocotyls by agropine-type strains. Fewer roots were obtained with mannopine-type strains. *In vitro* cultures were established both from normal and hairy roots. Plant regeneration occurred spontaneously from normal and transformed roots. Regenerated plants contained the same opines (if present) as root cultures. Some mannopine-positive regenerants displayed a modified phenotype. Relationships between phenotype, opine content, T-DNA content and/or expression were discussed.

Hosoki *et al.* (1989) reported genetic transformation and regeneration of *Brassica oleracea* L. var. *acephala* by *Agrobacterium rhizogenes* mediation using leaf tissue. Hairy root produced with adventitious buds when transferred to media supplemented with zeatin and NAA. The transformation rate was 44 % as determined by the presence of mannopine, one of the opines produced by transformed tissue.

He (1990) transformed hypocotyl segments of cabbage with T-DNA segment (root inducing) by co-cultivation of *Agrobacterium rhizogenes*. After 4 months they were transferred to solid MS medium without plant growth regulators. Transformed roots re-differentiated into callus clumps and then again redifferentiated into shoot buds which were rooted to get plantlets.

He *et al.* (1991) used a strain of *A. rhizogenes*, harbouring the binary vector pBin19 and induced roots *in vitro* on hypocotyl segments of cabbage. Among these roots, transformed roots containing the kanamycin resistant gene were identified by their ability to grow on a medium having kanamycin.

Berthomieu and Jouanin (1992) transformed rapid cycling cabbage (*Brassica oleracea* L. var. *capitata*) leaf petiole with *A. rhizogenes*. Two strains A4K and A4H, harbouring the *npt-II* (Kanamycin resistance gene) and *hpt* (hygromycin resistance gene), respectively, were used to obtain transgenic cabbage plants. Axenic root clones resistant to kanamycin or hygromycin B were established, most of which did not exhibit hairy root phenotype.

Beclin *et al.* (1993) worked on the use of *aux-2* gene from *Agrobacterium rhizogenes* as a conditional negative marker in transgenic cabbage. The *aux-2* gene was introduced into a rapid cycling cabbage genotype. Expression of this gene in the transgenic progeny grown *in vitro* led to an altered root phenotype.

Berthomieu *et al.* (1994) reported transformation of rapid cycling cabbage and showed molecular evidence for regeneration of chimeras. One transgenic plant was obtained for an average of 25 explants inoculated. Southern blot analysis showed that most of the plants, proved to be chimeras.

Christey *et al.* (1997) reported a procedure for the production of transgenic *Brassicac*s via Ri-mediated transformation. Transgenic hairy root lines were selected for 12 vegetable *brassica* cultivars and lines representing six varieties: broccoli, Brussels sprout, cabbage, cauliflower, rapid cycling (all *Brassica oleracea* L.) and Chinese cabbage (*Brassica campestris* L.). Leaf explants or petioles of intact cotyledons were co-cultivated with *Agrobacterium* strain A4T harbouring various binary vectors. The T-DNA region of all binary vectors contained a neomycin phosphotransferase-II (*npt-II*) gene for kanamycin resistance, in addition to other genes. Hairy root lines grow prolifically on hormone-free medium containing kanamycin. Transgenic shoots were regenerated from all cultivars either spontaneously or after transfer of hairy roots to a hormone containing medium. Southern analysis confirmed that the plants were transgenic.

Bhalla and Smith (1998b) developed an efficient and simpler method for genetic transformation and regeneration of cauliflower, *Brassica oleracea* L. var. *botrytis*. Explants

from 4 days old seedlings were inoculated and co-cultivated with *Agrobacterium tumefaciens* strain LBA4404 harbouring a binary vector with the *npt-II* gene under the regulatory control of nopaline synthase promoter and terminator sequences, permitting transformed shoots to be selected on kanamycin containing medium. After three months rooted transformed plantlets were successfully transferred and grown under glasshouse conditions. Higher numbers of transformed plants were obtained from cotyledon than hypocotyl explants indicating cotyledons of cauliflower are more amenable to genetic transformation. Integration and expression of the introduced transgene were analysed by DNA gel blot and PCR analysis and NPT-II expression assay.

Cai *et al.* (1999) studied the factors influencing bud regeneration and genetic transformation of *Brassica campestris* L. subsp. *pekinensis*. The seeds of self incompatible line 42 were germinated, and the cotyledons from seedlings of different ages were cultured on MS medium. Adventitious buds from the cultured cotyledons were detached and cultured on MS media containing various phytohormones, such as IAA, iPA (isopentenyl adenosine), gibberellins, abscisic acid, NAA, CPPU [N-(2-chloro-4-pyridyl)-N-phenylurea] and nitrates. Results showed that the frequency of adventitious buds was significantly affected by the age of cotyledons and the concentration of cytokinins on the medium. The highest adventitious bud frequency of 30–60 % was recorded in cotyledon of 8-10 days old. Among the phytohormone treatments, the highest adventitious bud frequency of 38.5 – 43.1 % was noted on the treatments with 0.1mg/l NAA and 1-2mg/l CPPU. In culture for genetic transformation, when cefotaxime, carbenicillin and kanamycin were added separately or in combination, kanamycin at 5-10mg/l gave the best results.

Pius and Achar (2000) developed an efficient protocol for *Agrobacterium* – mediated transformation of four commercial cultivars of *Brassica oleracea* L. var. *capitata* by *A. tumefaciens* strain LBA 4404 with neomycin phosphotransferase gene (*npt II*) and CaMV 35S – peroxidase gene cassette using cotyledonary explants. The frequency of transgenic plants was calculated on the basis of GUS (β -glucuronidase) activity detected by histochemical X-gluc test. Southern blot analysis revealed that integration of marker genes occurred in single and multiple loci in the genome.

Zhang *et al.*(2000) produced transgenic Chinese cabbage plants by inoculating cotyledons with *Agrobacterium tumefaciens* EHA101 carrying a binary vector pIG 121 Hm,

which contains kanamycin-resistance, hygromycin resistant genes and a GUS reporter genes. Highest infection was obtained for co-cultivation of 15 minutes and was then kept for 3 days in co-cultivation medium. When cotyledons were inoculated with *A. rhizogenes* strain produced transformed roots and kanamycin resistant callus. Transformants were identified and sub-cultured on MS medium with 3mg/l kanamycin. Shoots were differentiated after 40 days in culture. A histochemical GUS assay, PCR and Southern blot analysis confirmed that transformation had occurred. Genetic analysis of progeny showed that the transgenes were inherited in a Mendelian fashion.

Cogan *et al.* (2001) scored production of transgenic roots for eight *Brassica oleracea* L. cultivars from broccoli (Marathon F1, Trixie F1, Corvet F1 and Green Duke F1), cabbage (Hawke F1), cauliflower (white rock) and Kale (Kaliaan) following inoculation with an *A. rhizogenes* cell lines (strain LBA 9402 pRi 1855) carrying a binary plasmid bearing the green fluorescence protein (*gfp*) gene in the T-DNA. Significant differences in the number of explants producing transgenic roots were observed between cultivars ranging from 1.5 % for Marathon F1 to 57.8 % for the green Duke F1. The double haploid lines produced through anther culture showed considerable variation for transgenic root production with some lines showing increased efficiency compared to the parental F1 cultivars. No apparent segregation distortion for transgenic root production was observed in the double haploid lines following anther culture.

A number of factors that are known to influence genetic transformation were evaluated to optimize *Agrobacterium*-mediated transformation of hypocotyl explants of cauliflower variety Pusa Snowball K-1 by Chakrabarty *et al.* (2002). The binary vector p35SGUSINT mobilized into *Agrobacterium* strain GV2260 was used for transformation and transient GUS expression was used as the basis for identifying the most appropriate conditions for transformation. Explant age, preculture period, bacterial strain and density were found to be critical determinants of transformation efficiency.

Tsukazaki *et al.* (2002) established a transformation system using doubled haploid lines of cabbage. Hypocotyl explants that had been precultured for 3 days on Murashige and Skoog medium containing 50mM acetosyringone were inoculated and cocultured with *Agrobacterium tumefaciens* strain LBA4404 (pIG 121 Hm) for 3 days. Kanamycin tolerant

shoots were regenerated onto shoot induction medium 3 months after *Agrobacterium* inoculation. The transformation efficiency was about 3 % under optimum conditions.

Sharma and Srivastava (2003) standardized a technique of plant regeneration and genetic transformation in cabbage (*Brassica oleracea* L. var. *capitata*) by *Agrobacterium tumefaciens* strain LBA 4404 containing *npt-II* and *gus* genes using hypocotyl explants. The putative transformed cells/tissues were identified by their growth on the selective medium containing kanamycin and *gus* positive calli were obtained.

Singh and Srivastava (2003) reported genetic transformation studies in cabbage (*Brassica oleracea* var. *capitata* L. cv. Pride of India) using *Agrobacterium tumefaciens* strain LBA 4404 containing β -glucuronidase (*gus*) gene in binary vector pBI 121 along with a kanamycin resistance (*npt-II*) gene. Effect of preincubation time and co-cultivation time on frequency of transformation was studied, and it was found that preincubation time and co-cultivation time of 48 hours was optimum. Most of the putative transgenic shoots/plantlets obtained on selective media were GUS positive.

Gribova *et al.* (2005) developed the selection scheme for obtaining significantly more efficient hybrids of cabbage (*Brassica oleracea* L. var. *capitata*). Transgenic fertile analogues of sterile parental types of 4 lines (Ges 2, Meg 2, Drv 2 and Zmu 7) of cabbage were obtained by *Agrobacterium*-mediated transformation, which were *bar* gene hemizygotic.

Yu *et al.* (2005a) have introduced *npt-II* gene into Chinese cabbage (*Brassica campestris* L. var. *chinensis* cv. Shanghaiqing and cv. Youqing) by *Agrobacterium*-mediated transformation using the binary plasmid vector pB135S-AMF. The time required for pre-culturing and co-cultivation was 3 and 2 days, respectively. The efficiency of inoculation with LBA4404/pB135S-AMF was significantly higher than with AHA105/pB135S-AMF. Molecular analysis of kanamycin resistant seedlings showed that the frequency of Southern blotting was 82.2 % and most of the transformations contained multi-copies of the gene. The results indicate a stable transformation.

Sharma *et al.* (2012b) did plant regeneration and *Agrobacterium*-mediated gene transfer in cauliflower (*Brassica oleracea* L. var. *botrytis* cv. Pusa Snowball K1). They obtained higher frequency of shoot regeneration in cotyledon (68 %) and hypocotyl (84 %) explants on MS medium supplemented with 2.0mg/l BAP + 0.5mg/l IAA. Additional of

0.05mg/l IBA in nutrient medium promoted regeneration of roots. *Agrobacterium tumefaciens*, strain LBA 4404 was used for genetic transformation studies. Transformed cells were found to be able to grow on selective medium. Successful genetic transformation were confirmed by β -glucuronidase enzyme assay.

2.2.2 Direct gene transfer technique-mediated reporter/marker genes transfer in cabbage and related *Brassica* species

Direct gene transfer studies in *Brassica* species was initiated by Eimert and Siegemund (1992). There are only few reports on direct gene transfer technique-mediated reporter/marker genes in *Brassica* species which are discussed below.

A novel method in the field of genetic engineering of higher plants was presented by Neuhaus *et al.* (1987): microinjection into multicellular structures which had a high competence for plant regeneration through embryogenesis. Microspore-derived embryoids of *Brassica napus* L. were individually selected and microinjected with *npt-II* gene constructions. High frequency regeneration of haploid plants through embryogenesis was achieved within 8 weeks. Transformation efficiencies between 27 % and 51 % were determined by DNA dot blot analysis of primary regenerants. Stable integration of fulllength microinjected genes into high molecular weight DNA was proven by Southern analysis of genomic DNA isolated from regenerated plants. Transformed plants were tested for expression of the *npt-II* gene by enzyme assay. The chimeric nature of the primary regenerants was demonstrated after their *in vitro* segregation through secondary embryogenesis into pure transformants.

Eimert and Siegemund (1992) used three methods of genetic transformation in cauliflower i.e. i) leaf disc transformation, ii) PEG-mediated transformation, iii) electroporation of protoplasts with a frequency of 0.000002, 0.0004 and 0.000035, respectively. The *npt-II* gene was used as a selectable marker. Eimert *et al.* (1992) microinjected cauliflower seedlings with different *Agrobacterium tumefaciens* strain and expression and integration of *npt-II* gene was demonstrated.

Cho *et al.* (1994) carried out transformation of Chinese cabbage (*Brassica campestris* L. var. *pekinensis*) by particle bombardment with tungsten particle size of 0.7–1.1 mm at a target of 3cm. Transgenic plantlets were regenerated from leaf discs on minimal MS medium with 1.0mg/l NAA and 5.0mg/l BAP.

Tuan and Garg (2001) studies gene transformation in *Brassica* sp. using particle bombardment. Cotyledon and hypocotyl of different species of *Brassica* had been used as target explants. Transient expression of *uidA* gene had been obtained when either been constructed with CaMV35S or Actin promoters. The highest expression was recorded between 10 to 15 hours after bombardment. Plasmids pBI121, pBI221 and pDM803 were used to carry *uidA* gene. Further transformation events should be carried out to obtain highest transformation efficiency.

Mesophyll protoplasts of *Brassica oleracea* L. var. *botrytis* were successfully transformed using polyethylene glycol (PEG) by Radchuk *et al.* (2002). The success of plant transformation depended on both gene transfer and plant regeneration. Parameters, such as PEG and vector concentrations and heat shock conditions were tested in experiments on transient expression of the β -glucuronidase (EC 3.2.1.31) gene and the most suitable conditions for DNA uptake were determined. Two antibiotic resistance marker genes for neomycin phosphotransferase (EC 2.7.1.95) and hygromycin phosphotransferase (EC 2.7.1.104), and three vector plasmids with different lengths were used to obtain stable transformants.

Yan *et al.* (2004) studied the effect of the developmental stage of floral bud, and sucrose and surfactant concentrations on the transformation of Chinese cabbage (landrace variety Shanghaiqing) by microinjection of *Agrobacterium* into flower buds.

2.2.3 *Agrobacterium*-mediated desirable gene transfer in cabbage and related *Brassica* species

A number of novel resistance genes of different origin were discovered and used for plant transformation. First transgenic insect resistant plant contained *cry* (*Bt*) gene isolated from bacterium *Bacillus thuringiensis*. In the middle of 1990's transgenic maize and, some time later on, cotton having *cry* genes were developed and produced commercially.

Brassica species are seriously damaged by lepidopteran which result in yield loss as this is an extremely diverse species which includes several important vegetable nutritionally as well as economically. These important crops are severely affected by many insect pests like cabbage looper (*Trichoplusia hubner* L.), cabbage worm (*Pieris rapae* L.), diamondback moth (*Plutelia xylostella* L.), cabbage aphids (*Brevicoryne brassicae* L.) and

onion thrips (*Thrips tabaci* L.) (Salunkhe and Kadam, 1998). Few examples of cabbage and related *Brassica* species containing gene for insect resistance has been discussed here:

Berthomieu and Jouanin (1992) transformed rapid cycling cabbage (*Brassica oleracea* L. var. *capitata*) leaf petiole with *A. rhizogenes*. Two strains A4K and A4H, harbouring the *npt-II* (kanamycin resistance gene) and *hpt* (hygromycin resistance gene), respectively, were used to obtain transgenic cabbage plants. Axenic root clones resistant to kanamycin or hygromycin B were established, most of which do not exhibit hairy root phenotype.

Bai *et al.* (1992) reported genetic transformation of cabbage using an insecticidal crystal gene [*cryIA(c)*] of *Bacillus thuringiensis* fused to a modified CaMV35S promoter constructed in a binary vector which was transformed into *A. tumefaciens* with a disarmed Ti plasmid. Cotyledon segments of more than 10 Chinese cultivars of *Brassica oleracea* L. var. *capitata* were used in transformation experiments. Transgenic plantlets selected on 30mg/l kanamycin and hybridized with the probe *cryIA(c)* gene. Bioassay of two transgenic plants showed that they were significantly tolerant to *Plutella xylostella* larvae attack.

Metz *et al.* (1995) transformed cabbage seedlings using *Agrobacterium tumefaciens* strain AB1, harbouring binary vector carrying the *npt-II* gene under control of CaMV 35S promoter and *Bacillus thuringiensis* [*cryIA(c)*] gene. Several hundred transformants with resistance to kanamycin and *Plutella xylostella* that contained the *npt-II* and *cryIA(c)* genes were obtained.

Jun *et al.* (1995) developed protocol for the regeneration and genetic transformation of Chinese cabbage (*Brassica campestris* L. subsp. *pekinensis* cv. 'Spring Flavour') by *Agrobacterium tumefaciens* strain LBA 4404 carrying a disarmed binary vector pTOK/BKS-1 using cotyledonary petiole explants. The T-DNA region of this binary vector containing the nopaline synthase/neomycin phosphotransferase-II (*npt-II*) chimeric gene for kanamycin resistance and the cauliflower mosaic virus 35S/coat protein gene of tobacco mosaic virus L. (TMV-L) chimeric gene. Polymerase chain reaction and Southern blot hybridization analyses confirmed the introduction of the T-DNA into the Chinese cabbage genome. Further, western blot analysis using polyclonal TMV antiserum showed most of the regenerants (5 out of 6) expressed TMV coat protein gene.

Cai *et al.* (1997) used cotyledon of Chinese cabbage for co-cultivation with *Agrobacterium tumefaciens* strain LBA4404 carrying *cpTi* and *npt-II* genes after preculturing of 24 hours on shoot induction medium containing 20mg/l AgNO₃, 0.1mg/l NAA and 10mg/l kinetin. Transformants (transgenic shoots) were selected on selective medium containing 10mg/litre kanamycin.

Liu *et al.* (1998) reported transgenic plants with herbicide resistance using microspore derived embryos of Chinese cabbage. Microspore derived embryos of Chinese cabbage were co-cultivated with *Agrobacterium tumefaciens* strain C58 harboring *bar* gene that confers resistance to Basta, a phosphinothricin based herbicide. Six green plants were regenerated from 1006 treated embryoids on selective medium containing 5.0mg/l PPT. Southern blotting revealed that the *bar* gene had been integrated into the genome of the plants and Basta resistance was observed.

Lim *et al.* (1998) reported genetic transformation of herbicide resistance gene (*bar*) in Chinese cabbage (*Brassica campestris* L. subsp. *penkinensis*). Genetic transformation was achieved using hypocotyl explants which were precultured on the regeneration medium (containing of MS salts along with 1.0mg/l NAA and 3.0mg/l BAP) and then co-cultivated for two days with *Agrobacterium tumefaciens* containing *npt-II* and *bar* genes. Shoots regenerated directly from the explants on the selection medium containing 10mg/l kanamycin in a few weeks. Putative transgenic plants were analysed by PCR using *bar* gene primers. 10 out of 14 regenerated plants were transgenic.

The *Bacillus thuringiensis cry IB* gene driven by the 35S CaMV promoter and NOS terminator was placed in the pDE1001 binary vector alone and together with an analogous cassette for the *cryIAb* gene (Gonzalez *et al.*, 1998). Kanamycin resistant cabbage (*Brassica oleracea* L. cv. Hercules 31) plants were obtained after co-cultivation of hypocotyls with *Agrobacterium tumefaciens* harbouring the pDK-B2 and pDK-B5 constructions. T-DNA insertion into the plant genome was analysed by Southern blotting. A field trial to evaluate the resistance of the transgenic clones obtained to diamondback moth (*Plutella xylostella*) showed no differences in damage level when compared with non-transformed plants.

Cai *et al.* (1999) have introduced *Bt* gene into the 4 cabbage lines using *Agrobacterium* strain LBA 4404 (pGBI 4A2B). GUS and PCR tests showed successful incorporation of *Bt* gene. Bioassay of transgenic plants showed 74.9 % mortality of *Pieris*

rapae after 4 days of feeding but there was no significant effect on the mortality of *Plutella xylostella*.

Cao *et al.* (1999) reported genetic transformation of *Brassica oleracea* L. var. *italica* by *Agrobacterium tumefaciens*-mediated transformation. A synthetic *Bacillus thuringiensis* (Bt) *cryIC* gene was introduced into *B. oleracea* var. *italica* by co-cultivation of hypocotyl and petiole explants. 21 *cryIC* transgenic plants were obtained from 400 hypocotyl and petiole explants. These studies indicated that such transgenic plants will be useful in studies of resistance management strategies involving multiple transgenes.

Henzi *et al.* (1999) produced an improved protocol for development for *Agrobacterium rhizogenes*-mediated transformation of broccoli. This procedure uses compounds that enhance the virulence of *A. rhizogenes* and a *Brassica campestris* L. feeder cell layer. Leaf explants or intact cotyledons of three broccoli cultivars: Green Beauty, Shogun and Green Belt, were co-cultivated with *A. rhizogenes* strain A4T harbouring the binary vector pLN35. The T-DNA of this binary vector contains genes encoding antisense 1-aminocyclopropane-1- carboxylic acid (ACC) oxidase (35S-ACC-5-7 %) and neomycin phosphotransferase II (NOS-NPTII-NOS). Two cultivars were successfully transformed, Shogun and Green Beauty, with a transformation efficiency of 35 % and 17 %, respectively. Fertile plants were regenerated from kanamycin-resistant hairy roots by transfer to hormone-containing media. Integration of the T-DNA was confirmed by the polymerase chain reaction (PCR) and Southern analyses. Analysis of ethylene production by fully open flowers of three transgenic lines of Shogun demonstrated the feasibility of down-regulating ethylene biosynthesis using an antisense ACC oxidase gene. One transgenic line, Sh:2, showed a 91 % reduction in ethylene production after 96 hours in comparison to the non-transgenic control.

Zhang *et al.* (1999) have introduced arrowhead proteinase inhibitor (API) gene into small Chinese cabbage (*Brassica campestris* L. var. *chinensis*) via *Agrobacterium tumefaciens*-mediated transformation using cotyledon explants with petioles. *Agrobacterium* strain LBA 4404 carrying the pBI-API contained a neomycin phosphotransferase II gene for kanamycin resistance. Following co-cultivation, the explants were transferred to fresh shoot induction medium with 10–15mg/l kanamycin and 300–500mg/l carbenicillin. The transformation rates were above 0.2–2.0 %. In the presence of AgNO₃ in the medium, the transformation rates were upto 5 %. Integration of *api* gene was confirmed by PCR and Southern hybridization analysis.

Zhang *et al.* (2000) produced transgenic Chinese cabbage plants by inoculating cotyledons with *Agrobacterium tumefaciens* EHA101 carrying a binary vector pIG 121 Hm, which contains kanamycin-resistance, hygromycin resistant genes and a GUS reporter genes. Highest infection obtained for infection of co-cultivation for 15 min and then kept for 3 days in co-cultivation medium. When cotyledons were inoculated with *A. rhizogenes* strain produced transformed roots and kanamycin resistant callus. Transformants were identified and subcultured on MS medium with 3.0mg/l kanamycin. Shoots were differentiated after 40 days in culture. A histochemical GUS assay, PCR and Southern blot analysis confirmed that transformation had occurred. Genetic analysis of progeny showed that the transgenes were inherited in a Mendelian fashion.

Yu *et al.* (2000) used a plasmid vector containing the *barnase* gene and Kanamycin gene derived from plasmid pBI 121 for transforming stem tip callus of young seedlings of Chinese cabbage. Potential transformed plantlets were selected on MS medium containing 50mg/l kanamycin. PCR analysis was used to detect transformed plantlets.

Jin *et al.* (2000) developed a transgenic head cabbage (*Brassica oleracea* L. var. *capitata*) resistant to diamondback moth larvae through *Agrobacterium tumefaciens*-mediated transformation with *Bt* genes using modified procedures. All cabbage plants transformed with a synthetic *Bt* gene, *cryIAb3* were resistant to larvae of diamondback moth, whereas all plants transgenic for *cryIAb3*, a wild type *Bt* gene, were susceptible. The *cryIAb3* expression was put under the transcriptional control of the soyabean wound inducible VSpB promoter, and transgenic cabbage plants were obtained. Insect bioassay showed that such plants were all resistant to diamondback moth even without reduction for the expression of *Bt*.

Cho *et al.* (2001) transferred a synthetic *Bacillus thuringiensis* gene *cryIC* to three Korean cultivars of Chinese cabbage (*Brassica compestris* L. subsp. *pekinensis*) via *Agrobacterium tumefaciens*-mediated transformation of hypocotyl explants. Hygromycin resistance served as an efficient selective marker. The transformation efficiency ranged from 5 to 9 %. Transformation was confirmed by Southern blot analysis, PCR, Northern hybridization and progeny tests. The transgenic plants and their progeny were resistant to diamondback moth. Both susceptible diamondback moth and a diamondback moth population resistant to *cryIA* were controlled by the *cry IC*-transgenic plants.

Zhu *et al.* (2001) using cotyledonary petioles of Chinese cabbage [*Brassica campestris* L. subsp. *Pekinensis* (*B. pekinensis*) cv. Fushan Dabaotou], factors effecting plant regeneration and *Agrobacterium tumefaciens*-mediated genetic transformation were investigated. The turnip mosaic virus (TMV) coat protein (CP) gene was introduced into Chinese cabbage. Preliminary molecular analysis indicated that 5 of the 32 putative transgenic plants showed that the TuMV-CP gene was integrated into the chromosome of the plants. Northern blot analysis and ELISA test indicated that the TuMV gene was expressed at both transcriptional and translational levels. The TuMV-CP gene segregated in a 3:1 ratio in the T1 generation. Resistance assay revealed that plants of the T1 generation showed high levels of resistance to TuMV infection.

Cheng *et al.* (2001b) studied the basis for introducing LMV-CP into the Chinese cabbage (*B. campestris* L. subsp. *pekinensis*/*B. pekinensis*) genome and its highly effective and permanent expression using *Agrobacterium tumefaciens*-mediated transformation.

Chakrabarty *et al.* (2002) used the optimized protocol, the synthetic *cryIA(b)* gene was mobilized into cauliflower. Molecular analyses of transgenics established the integration and expression of the transgene. Insect bioassays indicated the effectiveness of the transgene against infestation by diamondback moth (*Plutella xylostella*) larvae.

Yang *et al.* (2002) successfully transferred modified *CpTI* (Cowpea trypsin inhibitor) gene (SCK) into Chinese cabbage (*Brassica campestris* L. subsp. *pekinensis*) GP-11 and Znongbai 4 by *Agrobacterium tumefaciens*-mediated transformation. Kanamycin resistant plants were obtained from cotyledon with petiole. PCR and Southern blot assay confirmed the integration of SCK gene into the Chinese cabbage genome.

Bhattacharya *et al.* (2002) transferred a synthetic *cryIA(b)* of *B. thuringiensis* (Bt) to cabbage cultivar 'Golden Acre' via *Agrobacterium tumefaciens* using hypocotyl explants. Transformed plants resistant to kanamycin were regenerated. Hybridization experiments demonstrated gene integration and mRNA expression. Immunoblot analysis revealed high level expression of Bt toxin protein in the transgenic plants.

Wang *et al.* (2002) developed a reliable method for introduction of *Bt* gene into Chinese cabbage cv. Huayang 3 as an alternative method for controlling insect pests, as well

as the effectiveness of the Bt-transgenic plants on controlling beet armyworm (*Spodoptera exigua*).

Sretenovic *et al.* (2002) conducted *in vitro* culture technique for the micropropagation and genetic improvement of cabbage. *Agrobacterium tumefaciens*-mediated transformation of cabbage *in vitro* effectively introduced a *bar* gene for resistance to Basta, marker gene for kanamycin resistance and a *gus* reporter gene. *In vitro* selection resulted in cabbage local genotypes resistant to paraquat at low concentrations.

Kim *et al.* (2003) developed an effective plant regeneration procedure and gene transfer system via *Agrobacterium tumefaciens* strain LBA 4404 harbouring a binary vector pMBP-1 containing a synthetic *Ricinus communis* protein disulfide isomerase (PDI) gene with full codon modification from cotyledonary explants of 5 days old seedlings with two days preculturing. Polymerase chain reaction in southern blotting analysis were used to identify and characterize the transgenic plants with the integrated *pdi* gene.

Cho *et al.* (2003a) co-cultivated explants of Chinese cabbage (*Brassica campestris* L. subsp. *pekinensis*) inbred lines CC42, CC51 and CC52 with *Agrobacterium tumefaciens* strain LBA 4404 harbouring a binary vector pCAMBIA containing *hpt* gene for resistance to hygromycin and *GLOase* gene related to L-ascorbic acid biosynthesis from rat. A number of hygromycin resistant plantlets were regenerated from cotyledonary petioles. Polymerase chain reaction, Southern and Northern blotting analysis were used to identify and characterize the transgenic plants with the integrated *GLOase* gene.

Cho *et al.* (2003b) developed an effective plant regeneration procedure and a gene transfer system via *Agrobacterium tumefaciens* in the commercially important vegetable Chinese cabbage (*Brassica campestris* L. subsp. *perkinensis*). Hypocotyl explants from 5 days old seedlings with 2 days pre-culture were infected with *A. tumefaciens* strain LBA 4404 harbouring a binary vector pCAMBIA containing the MADS domain-containing transcription factor flowering locus C (FLC) gene with full codon modification. A number of hygromycin resistant plants were regenerated. Polymerase chain reaction and southern and northern blotting analysis were used to identify and characterize the transgenic plants.

Yang *et al.* (2003) verified that *gna* (Snowdrop lectin) gene integrated into Chinese cabbage genome by *Agrobacterium tumefaciens* through molecular tests and insect bioassay

with aphid (*Myzus persicae*) larvae. The results of the inheritance analysis showed that foreign gene in two transgenic Chinese cabbage plants, 282-3 and 282-9, was inherited in simple Mendelian fashion in their progenies.

Lee *et al.* (2003) isolated anther (tapetum) – specific gene BcA9 from Chinese cabbage [*Brassica campestris* L. subsp. *pekinensis* (*B. pekinensis*)] cv. Jangwar, using the *Arabidopsis* tapetum specific A9 gene as a probe. The DNA and amino acid sequences of the coding region of the BcA9 gene showed high homology with A9 genes from *Arabidopsis* and *B. napus*, a plant expression vector, pGR011, was constructed by fusing the BcA9 promoter and the cytotoxic diphtheria toxin-A-chain (DTX-A) gene. Several transgenic plants from cabbage, *B. oleracea* var *capitata*, were obtained by way of *Agrobacterium*–mediated transformation. Southern blot analysis indicated that the tapetum-specific BcA9 promoter and DTX-A gene were successfully integrated into the genome of the transgenic cabbage. Under the control of the BcA9 promoter, expression of the cytotoxic DTX-A gene in the tapetal cells of the transgenic plants resulted in male sterile cabbage.

Wang *et al.* (2003) studied the expression of a GUS reporter gene and *cryIac* gene under the control of the proprietary *tCUP* gene expression system in vegetable crucifers. The optimization and concomitant development of the effective transformation protocols, allowed a *cryIac* insect resistant gene to be introduced into an applied an inbred line of cabbage and a hybrid line of cauliflower. Also described the regeneration optimization approach and extend the observations by evaluating the expression of a GUS reporter gene and *cryIac* gene in different parts of the transgenic cabbage and cauliflower. A high level of expression was observed in all parts, especially in leaf tissue of cabbage and cauliflower and this high level of expression was consistent throughout plant development to senescence. The results, using ELISA showed that the *cryIac* gene, directed by the *tCUP* gene expression system, produced a high level of CRY protein. Insect bioassay showed no significant leaf damage after exposure to the larvae and 100 % mortality of three targets insect's larvae (Imported cabbage worm, Diamondback moth and Cabbage looper).

Bhattacharya *et al.* (2004) transferred bacterial bet A gene for biosynthesis of glycine to cabbage (*Brassica oleracea* L. var. *capitata*) cultivar 'Golden Acre' through *Agrobacterium*–mediated transformation of hypocotyl explants. Transgenic status was established through southern hybridization and mRNA expression in the shoots. The

transformants exhibited higher tolerance to NaCl stress compared to untransformed parent plants.

Yu and Cao (2004) isolated male sterility related antisense gene – CYP86MF and introduced to pBI121 generating pBI35S-AMF which contained CaMv35S promoter, and then pBI35S-AMF was transferred into *Agrobacterium tumefaciens* strain LBA 4404 and EHA 105 through the assistant plasmid pRK 2013/HB 101 by triparental cross. Results and PCR chain reaction amplification and restriction digestion showed that the fragment of antisense gene CYP86MF was introduced into pBI121, plasmid, and the expression vector pBI 35S-AMF was transferred into *A. tumefaciens* successfully. The antisense gene fragment was transferred into Chinese cabbage [*Brassica campestris* L. subsp. *chinensis* (*B. chinenensis*) cv. Shanghaiqing] and more than 130 kanamycin resistant plants were obtained.

Resistance to herbicide Basta [phosphinothricin] was introduced into pure inbred lines of Savoy cabbage (*Brassica oleracea* L. var. *sabauda*) by co-cultivation of cotyledon and hypocotyl explants with *Agrobacterium tumefaciens* strain AG11/pDM805 and LBA4404/pGKB5 (LB5-1) (Sretenovic *et al.*, 2004). Putative transformants that survived in the selective medium supplemented with 10mg/l PPT were confirmed by GUS assay and PCR analysis. The transformation rate was 58 % with AG11/pDM805 and 25 % with 2B5-1.

Zhang *et al.* (2004) carried out *in vitro* transformation of cotyledons with petiole of Chinese cabbage (*Brassica campestris* L. subsp. *Pekinensis* cv. Beijing 80) using *Agrobacterium tumefaciens* C58 carrying plasmid pBB Basta *pin II-bar*. Transgenic plants were confirmed by PCR, PCR-Southern and genomic southern blotting which showed that the *bar* and *pin-II* gene were co-integrated into the plant genome. Bioassay with *Pieris rapae* L. by feeding leaf tissue had higher mortality and retarded developments compared with those larvae fed with non-transgenic control plants.

Lee *et al.* (2004) transformed cabbage plants with the potato proteinase inhibitor II (*pin II*) gene, *bar* gene and *hpt* gene using *Agrobacterium*. The expression of the *pin II* gene was driven by its own promoter which was wound inducible. Ten transgenic plants were obtained from the medium containing hygromycin as a selection antibiotic. The integration and expression of *pin II* and *bar* genes were confirmed by Southern and Northern hybridization. Growth and development of diamondback moth (*Plutella xylostella*) and tobacco cutworm (*Spodoptera litura*) larvae were examined on T₁ plants.

A synthetic fusion gene of *Bacillus thuringiensis* encoding a translational product of *cryIB* and *cryIAb* β -endotoxins was transferred to a torpical cabbage breeding line by *Agrobacterium*-mediated transformation (Paul *et al.*, 2005). Polymerase chain reaction (PCR) analysis revealed that twelve of the putative transformants contained the transgene and Southern hybridization confirmed the gene integration in the DBM (diamondback moth) resistant plants. Double Antibody Sandwich Enzyme – linked Immunosorbent Assay (ELISA) analysis revealed the accumulation of fusion protein upto 0.16 % of total soluble protein in the leaves of transgenic plants. Insect bioassays carried out with the leaves of PCR-positive plants and neonate larvae of DBM showed that one of the transgenic plants was completely resistant to repeated infestation of larvae.

Li *et al.* (2005) used *Agrobacterium tumefaciens* to deliver the *vhb* gene into cabbage (*Brassica oleracea* L. var. *capitata*) cv. Xianguang's parent line 103, using hypocotyls and cotyledon petioles as explants for infection, and they obtained a transformation efficiency of 3–5 %. Molecular analysis indicated that the *vhb* gene was stably integrated into the cabbage genome and that the *vhb* gene was expressed at the RNA level. Characterization of the *vhb* over-expressing transgenic plants revealed that transgenic seeds germinated faster than the wild type controls. The transgenic plants showed tolerance to a prolonged submergence treatment, suggesting that the *vhb* gene may provide an excellent tool for creation of submergence/flooding – tolerant cultivars of agriculturally important crops.

Gribova *et al.* (2005) developed the selection scheme for obtaining significantly more efficient hybrid of cabbage. (*Brassica oleraceae* L. var. *capitata*) Transgenic fertile analogues of sterile parental types of 4 lines (Ges 2, Meg 2, Drv 2 and Zmu 7) of cabbage were obtained by *Agrobacterium*-mediated transformation, which were *bar* gene hemizygotic.

Lingling *et al.* (2005) transferred cowpea trypsin inhibitor (*CpTI*) gene into cauliflower by *Agrobacterium*-mediated transformation method, and 14 transgenic cauliflower plants were obtained. Cotyledons and hypocotyls were used as explants. The putative transformants were assayed by PCR and Southern blotting analysis. The results indicated that *CpTI* gene was transferred into cauliflower successfully. The result of preliminary insect-resistant assay showed that the transgenic plants were more resistant to *Pieris rapae* than non-transgenic plants.

The hypocotyl explants of cabbage were transformed with *Agrobacterium* strain EHA015 containing a plasmid vector with the gene IGF-I (insulin-like growth factor) flanked by matrix attachment regions isolated from genome of tobacco (Zhang and Wang, 2005). 25 resistant shoots were obtained from 10 transformation experiments with a total of 15200 explants. Some of the resistant regenerated plants were tested by PCR analysis and Southern blot, and results showed that the gene was integrated into the cabbage genome.

Lei *et al.* (2006) studied the transformation of cysteine proteinase inhibitor gene into cabbage (*Brassica oleracea* L. var. *capitata*). *Agrobacterium* strain LBA4404 containing the expression vector pBI121-OCI was constructed with *OC-I* gene under the control of CaMV35S promoter and Nos terminator is used for genetic transformation of cabbage. The putative transformants were assayed by PCR and Southern blot analysis. The result showed that *OC-I* gene was transferred into cabbage successfully. The resistance of transgenic plants to insects was stronger compared to the control in the field.

Zhao *et al.* (2006b) developed a transformation system for Chinese cabbage protoplasts using *Agrobacterium tumefaciens* strain LBA4404 (harbouring the plasmid pBin SCK and the plasmid pMOG 411 respectively). The plasmid pBin SCK contains a 415 bp insert derived from the Cowpea trypsin proteinase inhibitor gene and the plasmid pMOG 411 contains a 870 bp fragment which codes an anti-bacterial peptide gene. Freshly isolated protoplasts of Chinese cabbage (*Brassica campestris* L.subsp.*pekinensis*) lines were pre-treated at 4°C for 1 hour, then incubated at 25°C for 2–3 days in the dark. 3 drops of *A. tumefaciens* solution in log-phase were added to 10ml protoplasts and cocultivated for 48 hours at 25°C. Some kanamycin-resistant plants and a number of kanamycin-resistant calli were obtained. Southern blot hybridization analysis demonstrated the presence of the *CpTI* gene and the anti-bacterial peptide gene in the Chinese cabbage genome. Northern blot analysis of the kanamycin-resistant plantlets and calli confirmed the accumulation of the *CpTI* and the anti-bacterial peptide mRNAs.

Venkatesh, (2006) carried out genetic transformation studies to standardize a protocol for insect resistance gene (*cryIAb*) transfer in cabbage using *Agrobacterium*-mediated gene transfer technique. *Agrobacterium tumefaciens* strain containing *npt-II* and *cry IAb* genes in binary vector pBin Bt1 was used for genetic transformation studies. They studied effect of preincubation and cocultivation times on the transformation frequency in cotyledon and

hypocotyl explants of cabbage. Preincubation of 72 hours and cocultivation of 48 hours was found optimum as it gave maximum transgenic shoot regeneration on selective medium. 12 putative *Bt* transformants were randomly selected and subjected for molecular analysis. PCR with *cryIAb* gene specific primers amplified 1.0 kb fragments from genomic DNA of kanamycin resistant shoots, thereby indicating the presence of transgenes in the regenerated plantlets. Out of 12 putative transgenic plantlets, only 4 plantlets DNA (*cryIAb* gene) got amplified showing the integration of the gene in the genome of transgenic plantlets.

Min *et al.* (2007) adapted a mannose selection system for use in the *Agrobacterium*-mediated transformation of Chinese cabbage. Hypocotyl explant from 4-5 days old seedlings of Chinese cabbage inbred lines were pre-cultured for 2-3 days and then infected with *Agrobacterium*. Two genes (L-gulonolactone oxidase, GLOase and jasmonic methyl transferase, JMT) were transformed into Chinese cabbage. They found that supplementing the media with 7g/l mannose and 2 % sucrose provide the necessary conditions for the selection of transformed plants from non-transformed plants.

Lei *et al.* (2009) obtained transgenic cabbage lines with insect-resistance, *cryIa8* gene was transferred to cabbage (*Brassica oleracea* L. var. *capitata*) with the transformation of hypocotyl segments and cotyledon with petiole via *Agrobacterium tumefaciens*. Thirty-eight transformants with kanamycin-resistance were obtained. PCR and Southern blot analysis showed that *cryIa8* gene was integrated into the genome of cabbage. Moreover, RT-PCR and Western blot detection demonstrated that *cryIa8* gene was expressed in RNA and protein levels. The results of bioassay with the susceptible and resistant diamondback moth (DBM, *Plutella xylostella*) displayed that most of the transgenic plants were resistant to the larvae of susceptible DBM and also to the insect strain which is resistant to *cryIaC* toxin.

Genetic transformation for cabbage was studied using hypocotyls of 'Xianguang' variety and *Agrobacterium tumefaciens* GV3101 (with activation tagging plasmid pSKI015) by Hua *et al.* (2009). The transformation factors of cabbage, including pre-culture time, invading time and co-culture time were optimized. The results showed that pre-culture for two days, invading for six minutes and co-culture for one day was the best treatment for cabbage transformation. Two plantlets were obtained invading hypocotyls of cabbage with GV3101 and screening on medium added with 1.5mg/L glufosinate. Of those two plantlets, one with yellow-edge and green central leaves, the other with roll leaves. Results of PCR

amplification and southern blot showed that enhancer of pSKIOIS had been inserted into genome of these two plantlets and thus they were activation tagging mutants of cabbage.

Vanjildorj *et al.* (2009) produced transgenic Chinese cabbage (*Brassica campestris* L. subsp. *pekinensis*) with high tolerance to soft rot disease. Tolerance was conferred by expression of *N-acyl-homoserine lactonase* (AHL- lactonase) in Chinese cabbage through an efficient *Agrobacterium*-mediated transformation method. To synthesize and express the AHL- lactonase in Chinese cabbage, the plant was transformed with *aii* gene fused to Pin II signal peptide (protease inhibitor II from potato). Transgenic lines were selected by the growth on hygromycin- containing medium. Southern blot analysis showed that the transgene was stably integrated into the genome.

Rafat *et al.* (2010) evaluated a number of parameters to influence genetic transformation via *Agrobacterium*-mediated transformation method to increase the frequency of transformation of cabbage (*Brassica oleracea* L. subsp. *capitata*) cv. KY cross with *AtHSP101* gene. The binary vector pCAMHSP was designed and mobilised into two *Agrobacterium tumefaciens* strains C58 and GV2260. The study was carried out on hypocotyl and shoot tips explants of cabbage cv. KY cross. Transformation parameters optimized were pre-culture medium, acetosyringone application, bacterial density and inoculation time. The PCR assay and production of mRNA of *AtHSP101* gene were confirmed by RT-PCR. The expression of *LacZ* gene in the transgenic plants also showed that it could be applied as a plant transformation reporter gene in genetic transformation studies. Multiple shoot regeneration of hypocotyl and shoot tip explants of cabbage after co-cultivation with *Agrobacterium* was optimized and medium containing 2mg/l BAP was observed to be best for shoot regeneration after co-cultivation. In these studies, 45 % and 32.5 % transformation efficiencies were achieved for hypocotyl and shoot tip explants, respectively using the optimized procedure.

Deng-Xia *et al.* (2011) obtained transgenic cabbage line with broad insect resistance, a new synthetic *Bacillus thuringiensis cryIBa3* gene was introduced into white cabbage via *Agrobacterium tumefaciens*-mediated transformation and 37 transformants were obtained. Polymerase chain reaction (PCR) and Southern blot analyses confirmed that *cryIBa3* was successfully inserted into the genome of cabbage. Reverse transcription-polymerase chain reaction (RT-PCR) demonstrated that *cryIBa3* was expressed. Western blot results confirmed the production of insecticidal protein encoded by *cryIBa3*. Insect bioassays showed that

transgenic cabbages effectively controlled both susceptible and *cryIAC*-resistant diamondback moth (DBM) larvae.

Awasthi and Srivastava (2013) conducted an investigation to standardize the protocol for insect resistance gene (*cryIAb*) transfer in cauliflower (*Brassica oleracea* L. var. *botrytis* cv. Pusa Snowball K-I) using an *Agrobacterium*-mediated gene transfer technique. An *Agrobacterium tumefaciens* strain containing the insect resistance (*crIAb*) and marker (*npt-II*) gene in the binary vector pBinBt 1 was used for genetic transformation. They studied effect of preincubation and co-cultivation time of 72 hours of found optimal as it gave maximum transgenic shoot regeneration on the selective medium from the hypocotyl explants. However, from cotyledon explants, only a single shoot was obtained. Fifteen putative Bt-transformants were isolated and were subjected to molecular analysis. PCR with *cry IAb* gene specific primers amplified 1.0Kb fragments from genomic DNA in only 4 kanamycin resistant shoots, thereby indicating that the regenerated plantlets were transgenic. A bioassay was carried out by feeding insect larvae on the leaves of transgenic plantlets. They mentioned that studies are in progress to pyramid two Bt-gene (*cryIAb* and *cryIAa*) in cauliflower (*Brassica oleracea* L. var. *botrytis* cv. Pusa Snowball K-I).

2.2.4 Direct gene transfer technique-mediated desirable genes transfer in cabbage and related *Brassica* species

Direct gene transfer studies in *Brassica* species was initiated by Mukhopadhyay *et al.* (1991). There are only few reports on direct gene transfer technique-mediated desirable genes in *Brassica* species which are discussed below.

Mukhopadhyay *et al.* (1991) reported efficient regeneration (80 %) of *Brassica oleracea* L. and high genetic transformation frequency of 10–33 % through PEG-mediated gene transfer using isolated protoplasts from 6 days old seedlings. Three different plasmid vectors carrying marker genes for resistance to methoxyfraxate, hygromycin and phosphinothricin were constructed and used for genetic transformation. A high %age of 70 and 93 of normal, fertile transformants were obtained carrying *hpt* or *bar* genes, respectively.

Boyer *et al.* (1993) TMV RNA was inoculated into Chinese cabbage protoplast via electroporation and sensitive detection of viral RNA products were analysed by Northern blot analysis. It was observed that electroporation of TMV RNA was more efficient in *Brassica campestris* L. than in Chinese cabbage protoplasts.

Resistance to black rot disease (*Xanthomonas campestris* pv. *campestris*) was introduced into cabbage (*Brassica oleracea* var. *capitata*) cv. KY cross by transferring total genomic DNA from black rot resistant pI281552 into KY cross, via pollen tube gene transfer (Liao *et al.*, 1998).

Radchuk *et al.* (2002). Mesophyll protoplasts of *Brassica oleracea* L. var. *botrytis* were successfully transformed using polyethylene glycol (PEG). The success of plant transformation depended on both gene transfer and plant regeneration. Parameters, such as PEG and vector concentrations and heat shock conditions were tested in experiments on transient expression of the β -glucuronidase gene and the most suitable conditions for DNA uptake were determined. Two antibiotic resistance marker genes for neomycin phosphotransferase and hygromycin phosphotransferase, and three vector plasmids with different lengths were used to obtain stable transformants.

Yu and Cao (2004) introduced a plant expression vector pBIA9-AMF containing an antisense fragment of the CYP86MF gene and the tapetum - specific A9 promoter by floral - dipping and pollen tube transformation methods to Chinese cabbage and flowering Chinese cabbage. Kanamycin resistant seedlings would be obtained by the pollen-tube method through germination tests of T₁ progeny seeds, but not the floral dip method. One of the two kanamycin resistant seedlings proved that the antisense fragment of the CYP86MF gene was integrated into the plant genome and was confirmed by PCR amplification and Southern blotting.

Xu *et al.* (2004) Chinese cabbage (*Brassica campestris* L. subsp. *pekinensis*) carrying the *pin II* and *bar* gene generated by vacuum infiltration transformation and strict self crossing. PCR assay, Basta resistance and insect resistance analysis of the T₂ generation lines proved the stable inheritance and expression of the inserted genes.

Liu *et al.* (2008) used chloroplast genetic engineering approach for genetic transformation in cabbage. This was an environment friendly approach, where the foreign integrated gene was often expressed at a higher level than nuclear transformation. The *cryIAb* gene was successfully transferred into cabbage chloroplast genome. The *aadA* and *cryIAb* genes were inserted into the pASCC 201 vector and driven by *prn* promoter. The cabbage-specific plasmid vectors were transferred into chloroplast of cabbage via particle gun

mediated transformation. Regenerated plantlets were selected by their resistance to spectinomycin and streptomycin. The results of PCR, Southern, Northern hybridization and western analysis indicated that the *aadA* and *cryIAb* genes were not only successfully integrated into the chloroplast genome, but functionally expressed at the mRNA and protein level.

2.3 Factor effecting transformation frequency in different plant species

The frequency of *Agrobacterium*-mediated transformation is very low. For optimization of the transformation protocol, interaction between *Agrobacterium tumefaciens* and plant is probably the most important aspect to be considered. Several factors affect efficiency of transformation. For effective transformation various factors have to be optimized prior to transformation such as preculture of the explants, colonization period, wounding methods, lethal dose for each agent (antibiotics), quantity of bacteria during co-cultivation, long enough co-cultivation, use of *vir* gene inducer like acetosyringone and stringent selection pressure from the onset are important to obtain stable transformants (Sriskandarajah *et al.* 2004).

Mathias and Mukasa (1987) reported that the cephalosporin antibiotic cefotaxime increases growth, regeneration and embryogenesis in wheat calli. They investigated the effect of cefotaxime on callus initiated from immature embryos of four barley (*Hordeum vulgare* L.) varieties. In calli cultured in the presence of antibiotic callus growth was up to 45 % greater than in controls and the frequency of regenerating calli was increased by up to 80 %. There was an apparent interaction of the antibiotic with genotype and the 2,4-D in the medium.

Mathews and Litz (1990) used various concentration of kanamycin in the culture medium to check various developmental stages of Mango (*Mangifera indica* L.) somatic embryos. The level of kanamycin necessary for growth inhibition was dependent on the size and stage of the somatic embryos at the time of treatment and the kind of exposure. Growth of proembryos in liquid suspension was arrested at 0.125mg/ml, while the maturation of later stages of somatic embryos on solid medium was inhibited at 0.200mg/ml.

Yepes and Aldwinckle (1994) tested the effect of two antibiotics cefotaxime and carbenicillin which commonly used during transformation studies to eliminate *Agrobacterium tumefaciens* in apple leaf explants on morphogenesis. Cefotaxime at a dose of

250mg/l enhanced regeneration and shoot development, whereas carbenicillin at a dose of 500mg/l induced abundant callus formation and inhibited regeneration. Kanamycin, a widely used selection agent for plant transformation, strongly inhibited regeneration even at very low doses.

Rao *et al.* (1995) reported by the use of cefotaxime (50 and 100mg/l), a cephalosporin antibiotic and the amino acids asparagine and proline (200mg/l) enhanced the production of embryogenic callus, increased the frequency of plant regeneration, and delayed the loss of regeneration potential in immature embryo- derived callus cultures of *Sorghum bicolor* (L.) Moench. Although these compounds did not promote callus induction or growth of callus, they influenced plant regeneration considerably in 10 low responding genotypes of grain and high anthocyanin containing sweet sorghums.

Orlikowska *et al.* (1995) studied the effects of co-cultivation conditions on transformation efficiency and direct shoot regeneration from seedling explants of safflower cv. 'Centennial'. *Agrobacterium tumefaciens* strain EHA105/p35SGUSInt was more infective than LBA4404/pBI121 as determined by numbers of sectors expressing β -glucuronidase activity. Compared to nontransformed controls, efficiency of direct shoot regeneration was markedly decreased by co-cultivation with EHA105 and the decrease exacerbated by addition of acetosyringone, indicating that a hypersensitive response to bacterial infection may reduce organogenetic potential. Likewise exposure of co-cultivated explants to kanamycin or geneticin in selective media reduced regeneration efficiency. Addition of 500mg/l carbenicillin slightly increased numbers of regenerating shoots. Transformed shoots were obtained only when kanamycin selection was initiated 1 or 2 days after co-cultivation. Presence of transgenes in geneticin-resistant shoots was confirmed using polymerase chain reaction and Southern hybridization assays.

Bondt *et al.* (1996) previously developed a protocol for efficient gene transfer and regeneration of transgenic calli following co-cultivation of apple (cv. Jonagold) explants with *Agrobacterium tumefaciens*. Factors which were found to be essential for efficient shoot regeneration were the use of gelrite as a gelling agent and the use of the cytokinin-mimicking thidiazuron in the selective postcultivation medium. Improved transformation efficiencies were obtained by combining the hormones thidiazuron and zeatin and by using leaf explants from *in vitro* grown shoots not older than 4 weeks after multiplication. Attempts to use phosphinothricin acetyl transferase as a selectable marker were not successful. Using

selection on kanamycin under optimal postcultivation conditions, about 2 % of the leaf explants developed transgenic shoots or shoot clusters. The presence and expression of the transferred genes was verified by β -glucuronidase assays and Southern analysis. The transformation procedure was successfully applied to several other apple cultivars.

Hammerschlag *et al.* (1997) evaluated a range of antibiotics and short-term exposure to an acidified (pH 3.0) medium for their effects on eliminating *Agrobacterium tumefaciens*, supervirulent strain EIIA101 from leaf explants of 'Royal Gala' apple (*Malus X domestica* Borkh.) and on shoot regeneration. Exposure of leaf explants to regeneration and elongation media containing 100 μ g/ml concentrations of the antibiotics carbenicillin, cefotaxime and ceftioxin singly or in combinations for 52 days did not eliminate *A. tumefaciens* from the explants. The %age of regeneration on carbenicillin, cefotaxime and ceftioxin was 97 %, 11 %, and 50 %, respectively, compared to 67 % for the control. Short-term (1 to 19 hours) vacuum infiltration with 500 μ g/ml, of any of the above antibiotics did not inhibit regeneration and failed to eliminate *A. tumefaciens* from leaf explants. Cefotaxime (2000 μ g/ml) did not inhibit the %age of regeneration and was more effective than carbenicillin or ceftioxin in preventing growth of *A. tumefaciens* when vacuum infiltrated into apple leaf explants for 30 minutes.

Nauerby *et al.* (1997) investigated the influence of 150mg/l timentin on the regeneration potential of *Nicotiana tabacum* Petit Havana SRI leaf discs and cotyledon explants and compared with the effects of 500mg/l cefotaxime or 1000mg/l carbenicillin. The concentrations of the antibiotics tested were all efficient for the elimination of agrobacterial cells. They found a positive influence of timentin on shoot regeneration from leaf discs (127 % after 1 month of culture). There was no influence on shoot production from cotyledons after 1 month and generally no influence on the rooting ability. Cefotaxime did not affect shoot production from leaf discs, but had an inhibitory effect on cotyledon explants regeneration (81 % after 2 months of culture). Rooting of shoots from leaf discs was inhibited by adding cefotaxime to the regeneration medium (68 % after 2 months), whereas rooting of shoots from cotyledons was not affected. Carbenicillin influenced shoot formation negatively as the shooting frequency for leaf disc explants was reduced to 56 % and for cotyledons to 51 % after 2 months of culture. In general, it was demonstrated that shoots arising from cotyledon explants rooted more efficiently than shoots arising from leaf disc explants. It

could be concluded that the influence of timentin on tissue culture was negligible or positive as compared to those of cefotaxime or carbenicillin.

Pena *et al.* (1997) previously developed procedures for the efficient production of sweet orange (*Citrus sinensis* L. Osbeck) and Carrizo citrange (*C. sinensis* L. Osbeck×*Poncirus trifoliata* L. Raf.) transgenic plants using an *Agrobacterium tumefaciens*-mediated transformation and shoot tip grafting *in vitro* regeneration system. Now they reported on the optimization of the co-cultivation, regeneration and selection conditions for efficient and reliable production of transgenic lime (*C. aurantifolia* Swing.) plants. Improved transformation frequencies were obtained by cocultivating the explants with *Agrobacterium* on feeder plates. Optimum regeneration of transgenic shoots was obtained by exposing the explants to darkness for 2 weeks and by using kanamycin at 100mg/l as selective agent. Attempts to use geneticin as selection antibiotic were not successful. Shoot tip grafting of regenerated shoots on Troyer citrange seedlings resulted in 100 % successful production of transgenic plants. The presence and expression of the transferred genes in the regenerated plants was verified by β -glucuronidase histochemical and fluorimetric assays, neomycin phosphotransferase ELISA assays, PCR and Southern analyses.

Cervera *et al.* (1998) had investigated the co-cultivation with *Agrobacterium tumefaciens*, regeneration and selection conditions on the transformation efficiency of citrange (*Citrus sinensis* L. Osbeck×*Poncirus trifoliata* L. Raf.). Factors such as co-cultivation period, preculture of explants, use of acetosyringone or feeder plates during co-cultivation, co-cultivation on a medium rich in auxins, postcultivation in darkness, and different kanamycin concentrations for selection were assessed. A 3-days co-cultivation on a medium rich in auxins improved transformation frequencies, since it increased the number of dividing cells competent for transformation, at the cut ends of the explants. Exposure of explants to darkness for 4 weeks on selection medium resulted in further callus development and increased the regeneration frequency of transgenic shoots. Furthermore, this treatment drastically reduced the number of regenerated escape shoots. A transformation efficiency of 41.3 % was achieved using the optimized transformation procedure.

Cheng *et al.* (1998b) determined the effects of timentin on shoot regeneration of tobacco (*Nicotiana tabaccum*) and Siberian elm (*Ulmus pumila* L.) and its use for the suppression of *Agrobacterium tumefaciens* in *Agrobacterium*-mediated genetic transformation. Timentin was a mixture of ticarcillin and clavulanic acid, and at

concentrations of 200–500mg/l with ratios of ticarcillin: clavulanic acid of 50:1 and 100:1, it had little effect on shoot regeneration of tobacco or Siberian elm. Timentin was as effective in suppressing *A. tumefaciens* as carbenicillin and cefatoxime at concentrations commonly used in transformation. The disarmed *A. tumefaciens* strain LBA4404 in infected tobacco leaf tissues was visually undetectable after three subcultures on medium with 500mg/l of timentin and 250mg/l carbenicillin. Timentin was stable in solid agar medium and remained effective for at least 70 days, but was unstable when stored as a mixed stock solution or as separate ticarcillin and clavulanic acid stock solutions at –20°C or –80°C freezer for 4 weeks. Timentin may be an alternative antibiotic for the effective suppression of *A. tumefaciens* in genetic transformation.

Ling *et al.* (1998) studied the effect of antibiotics to eliminate growth of *Agrobacterium tumefaciens*. They found ticarcillin/potassium clavulanate was a very effective combination of antibiotics to eliminate *Agrobacterium tumefaciens* during tomato transformation. It showed no toxicity to tomato tissues at a concentration of 150mg/l and significantly promotes callus formation and shoots regeneration. The transformation frequency was raised more than 40 % in comparison to cefotaxime. Cefotaxime itself did not inhibit callus growth in culture medium, but it clearly decreased shoot differentiation. Together with kanamycin, cefotaxime showed a strong negative effect on callus growth, shoot regeneration and transformation efficiency. Unlike the widely used carbenicillin and cefotaxime, ticarcillin/potassium clavulanate was light stable and resistant to inactivation by β -lactamase. Furthermore, ticarcillin/potassium clavulanate was more economical than carbenicillin and cefotaxime. In conclusion, ticarcillin/potassium clavulanate was a very good alternative to eliminate *Agrobacterium tumefaciens* in plant transformation and has the potential to be widely used for plants which were sensitive to carbenicillin and cefotaxime.

Pierre Péros *et al.* (1998) initiated *in vitro* culture of 32 *Vitis vinifera* cultivars and intraspecific hybrids from axillary buds. The development of roots and shoots was followed during 14 subcultures on two hormone-free micropropagation media. One medium (M64) was used until the 8th sub-culture, after which it was replaced by G90 medium which was found more suitable for plantlet growth and permitted an increase in the time between subcultures. Large differences in plantlet growth between cultivars were demonstrated on both media. The number of roots had greatest variability between cultivars (CV=39 %) as compared with stem length (CV=21–22 %) and number of nodes (CV=12–14 %). The

number of nodes was positively correlated with shoot length whereas root number appeared to be poorly positively correlated with shoot development. Adventitious bud regeneration from leaves was studied for 20 cultivars and averaged 36.7 % of regenerative explants with large differences between cultivars (CV=47 %). However, organogenic competence was not correlated with micropropagation ability. High sensitivity of *Vitis vinifera* plantlets to kanamycin and hygromycin was demonstrated with a strong interaction between cultivar and antibiotic. At 0.8mg/l, hygromycin was lethal to plantlets. This effect was only observed at 4mg/l for kanamycin, whereas 1mg/l stimulated the development of plantlets.

Dixit and Srivastava (1999) studied resistance level of kanamycin in cauliflower (*Brassica oleracea* L. var. *botrytis* cv. Pusa Snow Ball). They had given increase dose of kanamycin (10, 20, 30, 40, 50mg/l) to hypocotyl and cotyledon explants to see minimum concentration of kanamycin required for selection of putative transformed cells during transformation. Decrease in fresh weight in both hypocotyl and cotyledon tissues were observed with increase in kanamycin concentrations. Even 50mg/l kanamycin did not completely inhibit the growth but callus formation and regeneration was affected.

Humara *et al.* (1999) reported a protocol for transfer and expression of foreign chimeric genes into cotyledons excised from *Pinus pinea* L. embryos. *Agrobacterium tumefaciens* EHA105 harbouring the plasmid p35SGUSint was more infective than LBA4404 or C58 GV3850, as determined by the %age of cotyledons showing *uidA* expression. Factors which significantly affected the T-DNA transfer included: (1) preinduction and concentration of bacteria, (2) days of coculture and (3) the wounding procedure applied. More efficient transfer of the *uidA* gene was achieved growing the bacteria in YEP medium at pH7, infecting the cotyledons according to the sonication-assisted *Agrobacterium*-mediated transformation procedure with a bacterial density of 1 (OD_{600nm}) for 5minutes, and coculture for 72hours. Using this protocol, 49.7 % of the cotyledons showed a diffuse blue staining 7 days after infection. However, all were necrotic 30 days after inoculation. Since a decrease in bacterial density to 0.01 allowed the recovery of about 4 % of cotyledons forming buds 1 month after inoculation, they concluded that the high mortality associated with the infection may be related to the hypersensitive response of the plant to bacterial infection.

Tang *et al.* (2000) evaluated four antibiotics for their effects on eliminating the hypervirulent *Agrobacterium tumefaciens* strain C58C1 ATHV Rif^R (pEHA101)/p35-*gus*-intron from walnut somatic embryos and on the production of secondary somatic embryos

and the transformed somatic embryos. Exposure to 100–1000mg/l of ampicillin, carbenicillin or cefotaxime respectively for up to 60 days did not eliminate the *A. tumefaciens* while timentin at 500–1000mg/l eradicated it from somatic embryos. One-hour acidified medium treatments and the addition of 100mg/l kanamycin to 500mg/l ampicillin, carbenicillin, cefotaxime or timentin were of little help in eliminating the *Agrobacterium*. All four antibiotics reduced somatic embryo production, carbenicillin minimally and cefotaxime maximally, especially at higher concentrations, in comparison with antibiotic-free medium. Putative transformed embryos were selected for continued proliferation on a 100mg/l kanamycin-containing medium. Histochemical assessments indicated that more *gus*-positive somatic embryos, particularly fully *gus*-positive embryos, regenerated from timentin-containing medium than from other antibiotic-containing media under equivalent conditions. Transformed embryos have been grown and converted into plants and *gus* activity was observed in whole plants.

In the study, Park *et al.* (2000) reported a simple and efficient system to regenerate Chinese cabbage plants and study of the effects of plant growth regulators, AgNO₃, initial dark treatment, various antibiotics, and herbicide on shoot induction from hypocotyl or cotyledon of Chinese cabbage. Shoots were induced at various combinations of naphthalene acetic acid (NAA) and benzylAdenine (BA) levels. The best combination of plant growth regulators was 2.0mg/l NAA and 1.0mg/l BA for cotyledon, and 1.0mg/l NAA and 5.0mg/l BA for hypocotyl. The experiment investigating the effect of AgNO₃ demonstrated that 16.7mg/l AgNO₃ was effective for inducing shoot regeneration from both of explants. Three to five days of initial dark treatments had significant effects for increasing the number of regenerated shoots; however, different growth regulator combinations showed various responses to duration of dark treatments. The effects of kanamycin, hygromycin, cefatoxime, carbenicillin and phosphinothricin (PPT) on shoot induction from cotyledon and hypocotyl were tested. Shoot induction was completely inhibited by kanamycin at 10mg/l, hygromycin at 5.0mg/l, PPT at 5.0mg/l or higher, but not by carbenicillin and cefatoxime.

Song *et al.* (2000) studied effects of kanamycin, cefotaxime, carbenicillin and ampicillin on morphogenesis of Chinese cabbage in tissue culture. Results showed that the 4 antibiotics had little effect on callus induction, but they had a great influence on shoot and root differentiation. Even at a very low concentration, kanamycin inhibited the differentiation of roots and shoots. Cefotaxime inhibited redifferentiation at lower concentrations and

postponed morphogenesis of root and shoot. Carbenicillin had no obvious effect on shoot and root differentiation with a frequency over 70 %. Shoot differentiation increased with higher concentrations of ampicillin. When the concentration reached 1000mg/litre, shoot differentiation frequency was as high as 96.67 %. Thus, carbenicillin and ampicillin could be used in tissue culture and genetic transformation of Chinese cabbage.

Estopà *et al.* (2001) determined the effect of several β -lactam antibiotics on shoot regeneration, growth and rooting of carnation (*Dianthus caryophyllus* L.), and their use in combination with kanamycin in *Agrobacterium*-mediated genetic transformation. Carbenicillin, cefotaxime and ticarcillin increased the regeneration rate when added alone in non-inoculated explants; whereas, with inoculated explants, this effect was only observed in ticarcillin-containing medium. Cefotaxime inhibited root growth in both transgenic and non-transgenic shoots. Rooting of non-transgenic shoots was completely inhibited in all culture media containing kanamycin. The different antibiotics used, alone or in combination, did not prevent the occurrence of false positive shoots, but it was possible to select transgenic shoots when rooting was induced in a kanamycin and ticarcillin-containing medium. Regenerated transformed shoots were free of *Agrobacterium* after culturing in rooting medium, as was proven by the PCR analysis for the *nptII* gene, the antibiogram and the culture of tissue pieces of transgenic shoots on LB broth. The use of kanamycin and timentin with or without carbenicillin, was very useful in the transformation procedure, for the elimination of *Agrobacterium* in regenerated shoots before their transfer to greenhouse conditions and also in the selection of transgenic versus false-positive shoots.

Hu and Phillips (2001) improved the transformation efficiency of cultivated tomato (*Lycopersicon esculentum* cv. UC82) using *Agrobacterium tumefaciens* from 14 % in a previous report to 25 % in the present study. Several variables potentially involved in the improvement of transformation efficiency were evaluated, including enhancements in the regeneration system, antibiotics used for *Agrobacterium*-overgrowth control, and method of applying kanamycin for selection. The most important variable identified was the influence of overgrowth-control antibiotics on both the regeneration response and transformation efficiency. The best transformant recovery and *Agrobacterium*-overgrowth control was obtained using 250mg/l claforan and 250mg/l ticarcillin as the overgrowth-control antibiotics in the media. Selfed T1 progeny plants showed Mendelian inheritance ratios in 77 % of the

independently transformed lines according to phenotype expression [β -glucuronidase (*gus*) assay results], and confirmed by polymerase chain reaction amplification of the transgene in progeny.

Agrobacterium-mediated plant genetic transformation required a two-step process for its success: selection and regeneration of transformed tissues, and the elimination of the transformation vector, *Agrobacterium*. Teixeira da Silva and Fukai (2001) used carbenicillin (CA), cefotaxime (CF) and vancomycin (VA) singly, or in combination, to eliminate *Agrobacterium tumefaciens* LBA4404 at transgenic plant selection levels (10 or 25 μ g/ml kanamycin for chrysanthemum and tobacco, respectively). The three antibiotics differed in their capacities to eliminate *Agrobacterium i.e.*, bacterial threshold survival levels (TSLs), depending on the strain, medium and light conditions. Plant TSLs differed from those for *Agrobacterium*, and were cultivar, species and light dependent, with CA>VA>CF in terms of phytotoxicity. Since over 90 % of plant transformation experiments used *Agrobacterium* as the transformation vector, with most of these containing an aminoglycoside antibiotic degrading gene, such as *nptII* or *hptII*, the morphogenic reaction of these two economically important plant species to these antibiotics had relevance to the various sectors involved with genetic transformation.

Carbenicillin and cefotaxime, two antibiotics commonly used for excluding *Agrobacterium tumefaciens* during plant transformation. Yu *et al.* (2001) were tested these antibiotics for their bacteriostatic effects as well as for their effects on plant regeneration in adventitious root explants of papaya following co-culture with *Agrobacterium*. A washing step with sterilized distilled water two days after co-culture enhanced the bacteria-suppressing effects of antibiotics. Proliferation of *Agrobacterium* was completely suppressed in the medium containing 125mg/l carbenicillin or cefotaxime. Callus fresh weight increase was apparently enhanced in the media with higher concentrations of carbenicillin (250-500mg/l), but was extremely inhibited in media with the same concentrations of cefotaxime. Higher %ages of somatic embryos were found in the medium with 125mg/l carbenicillin or 250mg/l cefotaxime; however larger numbers of somatic embryos from the individual callus were obtained in the medium with 125mg/l carbenicillin than in the medium with 250mg/l cefotaxime. %ages of abnormal somatic embryos were lower in the medium with lower concentrations of carbenicillin (125-250mg/l).

Alsheikh *et al.* (2002) studied the phytotoxic effect of different antibiotics that eliminated *Agrobacterium tumefaciens* with the least phytotoxic effects. An efficient and reproducible method of shoot regeneration from leaf discs was developed with optimal shoot regeneration obtained on medium supplemented with 0.25mg/l indole-3-butyric acid and 3mg/l benzylAdenine for both *F. vesca* forms. Petiole sections were also effective for *F. v. semperflorens* regeneration, and culture vessel capacity was found to influence shooting from both explant types and *F. vesca* forms. Of a range of antibiotics commonly used for *Agrobacterium* elimination, carbenicillin had the least inhibitory effect on shoot regeneration and was found to effectively control strain LBA4404. Both *F. vesca* forms showed a high sensitivity to kanamycin and, therefore, a selection regime ramped from 10mg/l to 25mg/l kanamycin over a period of 8 weeks was developed. Optimal transformation efficiency (15 %) was achieved by the appropriate use of explants type and age, leaf-disc orientation, *Agrobacterium* density and inoculation time and phenolic compounds for bacterial virulence induction. Southern analysis confirmed the integration of the β -glucuronidase reporter gene into the genomic DNA of both *F. vesca* L. forms.

Jayashree *et al.* (2003) reported *Agrobacterium tumefaciens*-mediated genetic transformation and the regeneration of transgenic plants in *Hevea brasiliensis*. Immature anther-derived calli were used to develop transgenic plants. These calli were co-cultured with *A. tumefaciens* harboring a plasmid vector containing the *H. brasiliensis* superoxide dismutase gene (*HbSOD*) under the control of the CaMV 35S promoter. The β -glucuronidase gene (*uidA*) was used for screening and the neomycin phosphotransferase gene (*nptII*) was used for selection of the transformed calli. Factors such as co-cultivation time, co-cultivation media and kanamycin concentration were assessed to establish optimal conditions for the selection of transformed callus lines. Transformed calli surviving on medium containing 300mg/l kanamycin showed a strong GUS-positive reaction. Somatic embryos were then regenerated from these transgenic calli on MS2 medium containing 2.0mg/l spermine and 0.1mg/l abscisic acid. Mature embryos were germinated and developed into plantlets on MS4 medium supplemented with 0.2mg/l gibberellic acid, 0.2mg/l kinetin (KIN) and 0.1mg/l indole-3-acetic acid. A transformation frequency of 4 % was achieved. The morphology of the transgenic plants was similar to that of untransformed plants. Histochemical GUS assay revealed the expression of the *uidA* gene in embryos as well as leaves of transgenic plants.

The presence of the *uidA*, *nptII* and *HbSOD* genes in the *Hevea* genome was confirmed by polymerase chain reaction amplification and genomic Southern blot hybridization analyses.

Li *et al.* (2003) developed a new transformation system for citrus mediated by *Agrobacterium tumefaciens* by using callus as an explants of Valencia sweet orange (*Citrus sinensis* (L.) Osbeck). Factors affecting *Agrobacterium*-mediated transformation efficiency included mode of pre-cultivation, temperature of co-cultivation and presence of acetosyringone (AS). The highest transformation efficiency was obtained with a 4 days pre-cultivation period in liquid medium. Transformation efficiency was higher when co-cultivation was performed for 3 days at 19°C than at 23 or 28°C. Almost no resistant callus was obtained if the co-cultivation medium lacked AS. The transformation procedure yielded transgenic Valencia plants containing the *pTA29-barnase* gene, as verified by PCR amplification and confirmed by Southern blotting.

Padilla *et al.* (2003) described an improved system for routinely developing transgenic plum plants (*Prunus domestica* L.) through the use of *Agrobacterium tumefaciens*. The production of non-transformed "escapes" has been virtually eliminated, and rates of plant establishment in the greenhouse have been dramatically improved. The system was based on the regeneration of shoots from hypocotyls extracted from mature seed. The shoot regeneration medium was Murashige and Skoog (MS) salts and vitamins supplemented with 7.5µM thidiazuron and 0.25µM indole-butyric acid. Transferring the explants after co-cultivation to shoot regeneration medium containing 80mg/l of kanamycin and 300mg/l of Timentin reduced the total number of regenerated shoots without affecting the transformation rate. Transformation rates using the described system averaged 1.2 % of the hypocotyl slices producing transgenic plants, with a range of 0–4.2 %. The transgenic shoots rooted at a rate of 90% on half-strength MS salts and vitamins supplemented with 5.0µM α-naphthaleneacetic acid and 0.01µM kinetin. Plantlets were transferred to a greenhouse directly from culture tubes with a 90 % average survival.

Wei *et al.* (2003) evaluated three antibiotics ampicillin, carbenicillin, and cefotaxime for their effects on induction, growth, and differentiation of organogenic calli, as well as rooting of regenerated shoots of three loblolly pine (*Pinus taeda* L.) genotypes. Of the antibiotics administered, cefotaxime maximally increased the frequency of callus formation and growth rate of organogenic calli, carbenicillin maximally increased the frequency of shoot regeneration and the average number of adventitious shoots per piece of organogenic

callus, ampicillin maximally decreased the rooting frequency of regenerated shoots and mean number of roots per regenerated shoot, in comparison with antibiotic-free media. Compared with the control, ampicillin minimally increased the frequency of callus formation, cefotaxime minimally increased the frequency of shoot regeneration, and carbenicillin minimally decreased the rooting frequency of regenerated shoots in three loblolly pine genotypes tested. All three antibiotics increased the frequencies of callus formation and shoot regeneration, and reduced the rooting frequency of regenerated shoots suggested that the establishment of an efficient *Agrobacterium tumefaciens*-mediated transformation protocol for stable integration of foreign genes into loblolly pine need to select a suitable antibiotic. This investigation could be useful for optimizing genetic transformation of conifers.

Petri *et al.* (2005) tested the effect of different concentrations of the aminoglycoside antibiotics, geneticin, paromomycin and streptomycin on adventitious regeneration from leaf explants of apricot to design an alternative procedure for selecting transgenic shoots. Streptomycin and paromomycin reduced shoot regeneration %age with increasing concentration of antibiotics. Almost a complete inhibition of regeneration was reached when 20 μ M paromomycin was used, although up to 40 μ M streptomycin was necessary to completely inhibit regeneration. Geneticin had a very toxic effect on apricot leaves and regeneration was inhibited at almost all concentrations tested. Addition of kanamycin hastened the development of adventitious buds although silver thiosulfate and not kanamycin was responsible for the observed increase in the consistency of the results from independent experiments. Kanamycin and paromomycin at the concentrations tested improved selection of transformed cells and resulted in a larger number of *gfp*-expressing regions. Paromomycin at 40 and 25.7 μ M kanamycin improved proliferation of transformed tissues as compared with the other antibiotics used and non-selected controls.

Costa *et al.* (2006) developed an improved almond transformation system with a 100 \times increase in efficiency (12.3 %) as compared to the existing transformation method (0.1 %) in spite of the lower regeneration ability of the explants used. Leaf transformation was performed with *Agrobacterium* EHA105/p35 SGUSINT. Main modifications introduced in the transformation protocol were the use of 150 μ M acetosyringone (AS) during the 21-days induction period, and a different selection strategy. Transformed shoots were assayed using PCR, GUS analyses and Southern blotting. The improved methodology was being applied for

transformation of *in vitro* propagated cultivars and opens the possibility of using almond as model for functional studies in *Prunoideae*.

Ding *et al.* (2006) evaluated four antibiotics, kanamycin, geneticin, carbenicillin and cefotaxime, for their effect on the regeneration of adventitious buds, shoot differentiation, rooting from regenerated shoots of *Populus euphratica* as well as on their control of *Agrobacterium*-mediated transformations. Results showed that the optimum concentration ranges of kanamycin and geneticin were 15–20 and 10–15mg/l at the stage of transgenic plantlet selection. The inhibitory effects of cefotaxime and carbenicillin varied among different genotypes of *Agrobacterium*. The inhibition of cefotaxime on *Agrobacterium* C58 was stronger than that of carbenicillin. The optimum concentration of cefotaxime was 100mg/l. Cefotaxime and carbenicillin had similar effects on *Agrobacterium* LBA4404 and their optimum concentrations were both 150mg/l.

Kumlehn *et al.* (2006) developed a novel genetic transformation method for barley (*Hordeum vulgare* L.), based on infection of androgenetic pollen cultures with *Agrobacterium tumefaciens*. Winter-type barley cv. 'Igri' was amenable to stable integration of transgenes mediated by *A. tumefaciens* strain LBA4404 harbouring a vector system that confers hypervirulence, or by the non-hypervirulent strain GV3101 with a standard binary vector. The efficacy of gene transfer was substantially influenced by pollen pre-culture time, choice of *Agrobacterium* strain and vector system, *Agrobacterium* population density, medium pH and the concentrations of acetosyringone, CaCl₂ and glutamine. After co-culture, rapid removal of viable agrobacteria was crucial for subsequent development of the pollen culture. The growth of agrobacteria was suppressed by the concerted effects of appropriate antibiotics, low pH, reduced level of glutamine and high concentrations of CaCl₂ and acetosyringone. Following infection with LBA4404 and GV3101, about 31 % and 69 %, respectively, of the primary transgenic (T₀) plants carried a single copy of the sequence integrated.

Tereso *et al.* (2006) studied the effects of antibiotics commonly used in *Agrobacterium*-mediated transformation on *Pinus pinaster* tissues. Embryogenic tissue growth from three embryogenic lines and adventitious bud induction from cotyledons from three open-pollinated seed families were analysed. Cefotaxime, carbenicillin and timentin commonly used for *Agrobacterium* elimination, at concentrations of 200–400mg/l did not inhibit the embryogenic tissue growth on filter paper nor as clumps. Adventitious bud

induction and bud number were significantly reduced for one of the tested families when using 400mg/l cefotaxime or timentin. The selection agent kanamycin significantly inhibited growth of embryogenic tissue on filter paper in all the embryogenic lines and concentrations tested (20–50mg/l). Kanamycin also inhibited growth of embryogenic clumps after two subcultures at 5–50mg/l. In cotyledons, kanamycin inhibited adventitious bud formation in the three seed families used, regardless of the concentrations tested (5–25mg/l). There was a significant effect of the seed family on the bud induction and the number of adventitious buds produced. From the results obtained, they proposed the use of timentin to eliminate *Agrobacterium* in transformation experiments, at concentrations of 400mg/l for embryogenic tissues and of 300mg/l for cotyledons. For selection of transformed tissues carrying the kanamycin resistance gene, kanamycin should be used at 20mg/l for embryogenic tissues on filter paper, at 5.0mg/l when clumps were in direct contact with the selection medium, and below 5.0mg/l for adventitious bud induction.

An efficient transformation system for Chinese cabbage cotyledon explants was developed using *Agrobacterium tumefaciens* strains LBA4404 harbouring the plasmid pMOG 411 and the plasmid pBinSCK respectively by Zhao *et al.* (2006a). Various factors affecting the transformation efficiency and subsequent regeneration were identified. The age of seedlings, growth conditions and status of *Agrobacterium* suspension, preculture of explants, co-cultivation time, ratio between *Agrobacterium* and explants, acetosyringone and concentration of kanamycin had a significant influence on transformation frequency and plant regeneration. The presences of the antibacterial peptide gene and the cowpea trypsin inhibitor gene in those selected shoots in kanamycin medium were confirmed by PCR, Southern blotting and Northern blotting.

Saini and Jaiwal (2007) transformed *Vigna mungo* cotyledonary node explants with *Agrobacterium tumefaciens* strain EHA105 carrying a binary vector pCAMBIA2301, which contains a neomycin phosphotransferase gene (*npt-II*) and a β -glucuronidase (*gus*) gene (*uidA*) interrupted with an intron. Various factors such as preculture and wounding of explants, manipulations in inoculation and co-cultivation conditions were found to play a significant role in influencing tissue competence, *Agrobacterium* virulence and compatibility of both, for achieving the maximum transformation frequencies. The stable transformation with 4.31 % efficiency was achieved using the optimized conditions. The transformed green shoots that were selected and rooted on medium containing kanamycin and tested positive

for *nptII* gene by polymerase chain reaction were established in soil to collect seeds. GUS activity was detected in leaves, roots, pollen grains and T₁ seedlings. Southern analysis of T₀ plants showed the integration of *npt-II* into the plant genome.

Agrobacterium tumefaciens strain EHA105/p35SGUSInt, effective in expressing β -glucuronidase activity, was used to standardize the pre-culture of explants, bacterial dilution, and co-cultivation period, besides evaluating the effect of acetosyringone and post cultivation in darkness, and to assess the optimum concentration of kanamycin as selection agent for transformation of *Zingiber officinale* Rosc. By Suma *et al.* (2008). Transformants were recovered on selection media containing 100mg/l kanamycin and a combination of 2,4-D 1.0mg/l and BA 0.5mg/l and regenerated in half strength MS media of BA 3.0mg/l and 2,4-D 0.5mg/l. Successful transformation was confirmed by histochemical GUS assay and polymerase chain reaction analysis.

Öz *et al.* (2009) investigated an efficient selection system and *Agrobacterium* mediated transformation for chickpea (*Cicerarietinum* L.) cotyledonary nodes. Effect of selective agents and antibiotics on multiple shoot and root induction of cotyledonary nodes and effects of mechanical injury and vacuum infiltration on transformation efficiency were evaluated. Selective agents and antibiotics were applied to explants at different concentrations for one month and numbers of regenerated shoots and roots were recorded. Kanamycin at 100mg/l, hygromycin at 20mg/l, phosphinotricin at 3.0mg/l and glyphosate at 5.0mg/l were found to be appropriate to select chickpea transformants. Lowest concentrations of all selective agents (50mg/l kanamycin, 10mg/l hygromycin, 3.0mg/l phosphinotricin, 1.0mg/l glyphosate) totally inhibited rooting of the regenerated shoots. Among the *Agrobacterium*-eliminating-antibiotics, timentin significantly increased and carbenicillin significantly decreased shoot induction after 4 weeks of culture. On the other hand, cefotaxime at all concentrations significantly decreased root induction. Sulbactam, a β -lactamase inhibitor, in combination with carbenicillin and cefotaxime displayed effective inhibition of *Agrobacterium tumefaciens* growth. Histochemical GUS staining was performed 4 and 16 days after transformation to analyze putative transgenics. Mechanical injury prior to transformation and vacuum infiltration at 200mm Hg for 40 minutes, during bacterial inoculation might be employed to increase the efficiency of chickpea transformation.

Kavitha *et al.* (2010) used *Agrobacterium tumefaciens* strains LBA 4404 harbouring a binary plasmid pCambar (containing Basta resistance (*bar*) gene, neomycin

phosphotransferase (*npt II*) gene and β -glucuronidase (*gus*) gene) to optimize the transformation efficiency in *Citrullus lanatus* cv. Round Dragon. In this study, the ability of *Agrobacterium tumefaciens* to mediate a gene transfer in *Citrullus lanatus* was highly dependent on various transformation factors. In current investigation, they have established factors influencing gene expression including explants age of cotyledon (5 days old cotyledon), preculturing conditions (2 days), wounding technique (multi wired with massive wounding technique), *Agrobacterium* concentration (A_{600} 0.8), co-incubation period of explants with *Agrobacterium* (30 minutes) and acetosyringone concentration (200 μ M) in co-cultivation medium. Confirmation was done by histochemical GUS assay activity after 3 days of co-cultivation period. By combining the best condition from each evaluated factors, we successfully established an efficient and reproducible *Agrobacterium*-mediated transformation protocol for *Citrullus lanatus* cv. Round Dragon which yield 100 % of transgene expression with 181.18 ± 0.57 blue spots per responding explants.

Husaini (2010) studied the importance of some parameters affecting transformation, Following previously described *Agrobacterium tumefaciens*-mediated transformation procedures for *Fragaria* \times *ananassa* Duch. 'Chandler'. The most important factor that increased the % recovery of transformants was the introduction of a pre-selection phase, in-between co-cultivation and selection, in which leaf disks were cultured on pre-selection regeneration medium containing validamycin A, timentin, and cefotaxime. The average %age of leaf disks forming shoots on selection medium containing cefotaxime (250mg/l) + timentin (250mg/l) was 5.4 % and about three shoots per regenerating leaf disk. Maximum transformation % age, based on polymerase chain reaction, was 31.25 %. Transgene integration and copy number were assessed by Southern hybridization confirming single copy as well as multiple copies of transgene integration in shoots as well as roots separately. This confirmed the non-chimeric nature of these transgenic plants. The system was very promising for the regeneration of genetically transformed cells and obtaining transgenic strawberry plants at high efficiency.

Rashid *et al.* (2010) developed high efficiency *Agrobacterium tumefaciens*-mediated transformation system of wheat (*Triticum aestivum* L.) cv. Inqilab-91 by exploiting bacterial culture density and acetosyringone concentration. *Agrobacterium* strain EHA101 harboring binary vector pIG121Hm, containing gene for GUS activity and hygromycin resistance was used in transformation experiments. Different optical densities (0, 0.25, 0.5, 0.75 and 1.0) of

bacterial suspension and different concentrations (0, 50, 100 μ M) of acetosyringone were evaluated in transformation studies. Maximum transformation efficiency (4.16 %) was obtained with bacterial suspension of O.D._{600nm} = 0.5. Contamination could not be controlled when O.D._{600nm} = 0.75 and 1 was used. In case of concentration of acetosyringone, 100 μ M was found to be better for transformation where transformation efficiency was 15.62 %. Transformation results were confirmed with GUS analysis.

Yang *et al.* (2010b) developed a protocol for *Agrobacterium*-mediated transformation for embryogenic callus of an excellent climber species, *Parthenocissus tricuspidata*. *A. tumefaciens* strain EHA105 or C58 harboring the pCAMBIA2301 binary vector with the neomycin phosphotransferase (*nptII*) and β -glucuronidase (*uidA*) gene was used. Factors affecting the transformation efficiency, including the *Agrobacterium* strains, co-cultivation time, *Agrobacterium* concentration, and infection time, were evaluated. Strain EHA105 proved to be significantly better than C58, and 4 days of co-culture was critical for transformation. An *Agrobacterium* suspension at a concentration of 0.5–0.7 $\times 10^8$ cells ml⁻¹ (OD₆₀₀ = 0.5–0.7) and an infection time of 40 minutes was optimal for transformation. By applying these optimized parameters, we recovered six independent transformed shoots that were kanamycin resistant and contained the *npt-II* gene, as verified by polymerase chain reaction (PCR) analysis. Southern blot analysis confirmed that T-DNA was stably integrated into the genome of three out of six PCR-positive lines. Furthermore, histochemical GUS assay revealed the expression of the *uidA* gene in kanamycin-resistant calli, somatic embryos, and leaves of transgenic plants.

Sujana and Naidu (2011) examined the effect of different concentrations of bavistin, cefotaxime and kanamycin along with the ethylene inhibitor STS for *in vitro* plant regeneration using axillary bud explants of *Mentha piperita*. Filter sterilized bavistin, cefotaxime and kanamycin were added separately in the range of 0–300mg (bavistin); 10, 20, 30, 40, 50, 100, or 120 μ M (cefotaxime or kanamycin) to growth regulator free MS medium containing 3 % sucrose. In a separate experiment bavistin (100mg/l) along with growth regulators such as BAP and TDZ were added to MS medium containing 3 % sucrose. Similarly the regeneration medium was supplemented with 10, 20, 30, 40, 50, 80, 100 or 120 μ M of STS. Maximum number of shoots 6.85 ± 0.18 was seen with 150mg/l bavistin with 100 % regeneration. These plantlets were further maintained for root emergence on a rooting medium supplemented with growth regulators such as IAA, IBA and NAA. The rooted plants were acclimatized and transferred to field plots with 95 % of plants surviving in the field.

Rashid *et al.* (2011) exploited explant source and acetosyringone concentration for the efficient development of *Agrobacterium*-mediated gene delivery system in wheat cultivars Inqilab 91 and Chakwal 97. Seedlings, mature embryos and calli used as explants sources for transformation in both the cultivars showed positive response. It was further observed that mature embryos produced maximum transformation efficiencies of 40.0 and 36.25 % with 37.5 and 31.03 % of regeneration frequencies of transgenic plants for Chakwal 97 and Inqilab 91, respectively. Seedlings produced 33.75 and 27.5 % while calli produced 26.25 and 22.5 % transformation efficiencies for both the cultivars. Acetosyringone concentration was also a limiting factor in transformation experiments especially in the case of cereals crop. Different concentrations of acetosyringone were used at the time of co-cultivation for optimization of the transformation protocol and maximum transformation efficiencies of 52.44 and 47.56 % were obtained with 50µM of acetosyringone from the cultivars Chakwal 97 and Inqilab 91, respectively.

Sharma *et al.* (2011) used cefotaxime with combination of kanamycin to eliminate *Agrobacterium* LBA 4404 and selection of transgenic plant. They used 100-500mg/l cefotaxime with same concentration (20mg/l) of kanamycin to select transformed cells. They concluded that 500mg/l cefotaxime and 20mg/l kanamycin showed maximum shoot regeneration (20.00 %) and complete inhibition of *Agrobacterium* growth.

Ahmad *et al.* (2012) did optimization of different factors affecting *Agrobacterium*-mediated transformation of *chitinase* gene. Nodes were used as explants of potato cultivars Desiree and Sh-5. *Agrobacterium tumefaciens* strain LBA4404 harboring a pB1333-EN4-RCG3 plasmid having *chitinase* gene and selectable marker hygromycin gene under the control of the CaMV 35S promoter was used. The parameters optimized for the potato transformation includes co-cultivation time, cefotaxime concentration, and days to pre-selection. The results showed that the infection time (2 minutes) gave best mean value of transformation efficiency that was, 2.9 and 2.1 in Desiree and Sh-5, respectively. The over growth of *Agrobacterium* were controlled with a concentration of 200mg/l of cefotaxime and obtained maximum mean value of transformation efficiency of 3.38 and 3.10 in Desiree and Sh-5, respectively. The pre-selection period seven days prior to selection were considered effective for regeneration of explants and high transformation efficiency. The high mean value of regeneration (3.08 and 2.82) and transformation efficiency (3.00 and 2.60) was observed in Desiree and Sh-5, respectively. The putative transgenic plants were analyzed through PCR by using RCG-3 specific primers.

Table 1: Plant regeneration studies in cabbage (*Brassica oleracea* L. var. *capitata*) and related *Brassica* species through direct organogenesis

S. No.	Plant species/cultivar	Explants used	Reference
1	<i>B. oleracea</i> L. var. <i>capitata</i>	Roots, stem, cotyledon and leaves	Bajaj and Nietsh (1975)
2	<i>B. oleracea</i> L. var. <i>capitata</i>	Leaf explants	Primo-Millo and Harada (1975)
3	<i>B. oleracea</i> L. var. <i>capitata</i>	Hypocotyl and cotyledon	Dai <i>et al.</i> (1994)
4	<i>B. oleracea</i> L. var. <i>capitata</i>	Cotyledon and hypocotyl	Petrova and Antonova (1996)
5	<i>B. campestris</i> L. subsp. <i>pekinensis</i>	Cotyledon	Zhang <i>et al.</i> (1997)
6	<i>B. oleracea</i> L. var. <i>capitata</i>	Nodal segment	Souza <i>et al.</i> (1998)
7	<i>B. campestris</i> L. subsp. <i>pekinensis</i>	Cotyledonary	Zhang <i>et al.</i> (1998)
8	<i>B. oleracea</i> L. var. <i>capitata</i> <i>B. oleracea</i> L. var. <i>botrytis</i> <i>B. oleracea</i> L. var. <i>italica</i>	Hypocotyl	Pua <i>et al.</i> (1999)
9	<i>B. oleracea</i> L. var. <i>capitata</i>	Shoot cutting	Souza <i>et al.</i> (1999)
10	<i>B. campestris</i> L.	Petiole with cotyledon	Cao <i>et al.</i> (2000)
11	<i>B. campestris</i> L. subsp. <i>chinensis</i>	Cotyledon	Xie <i>et al.</i> (2000)
12	<i>B. oleracea</i> L. var. <i>acephala</i> (common cabbage)	Young leaves	Donato <i>et al.</i> (2001)
13	<i>B. campestris</i> L. subsp. <i>pekinensis</i>	Cotyledon and hypocotyl	Kang <i>et al.</i> (2001)
14	<i>B. oleracea</i> L. var. <i>capitata</i>	Protoplasts from fully expanded leaves	Kondratenko (2001)
15	<i>B. oleracea</i> L. var. <i>capitata</i>	Various tissues	Cheng <i>et al.</i> (2001a)
16	<i>B. campestris</i> L.	Cotyledon and hypocotyl	Kang <i>et al.</i> (2002)
17	<i>B. campestris</i> L. subsp. <i>chinensis</i>	Cotyledon	Zhang <i>et al.</i> (2002)
18	<i>B. campestris</i> L. subsp. <i>chinensis</i>	Cotyledon	Zhang <i>et al.</i> (2002)
19	<i>B. campestris</i> L.	Cotyledon	Lu <i>et al.</i> (2003)
20	<i>B. campestris</i> L.	Cotyledon	Zhao <i>et al.</i> (2004)
21	<i>B. campestris</i> L. subsp. <i>pekinensis</i>	-	Wang <i>et al.</i> (2005)
22	<i>B. campestris</i> L.	Cotyledon	Fan <i>et al.</i> (2005)
23	<i>B. campestris</i> L.	Cotyledon with petiole	Yu <i>et al.</i> (2005b)
24	<i>B. campestris</i> L.	Hypocotyl	Zhu <i>et al.</i> (2006)
25	<i>B. campestris</i> L.	Cotyledon with petiole	Chen and Hou (2008)

26	<i>B. oleracea</i> L. var. <i>capitata</i> <i>B. oleracea</i> L. var. <i>botrytis</i> <i>B. oleracea</i> L. var. <i>italica</i>	Cotyledon, hypocotyl and root	Pavlovic <i>et al.</i> (2010)
27	<i>B. campestris</i> L.	Leaf	Liu <i>et al.</i> (2011b)
28	<i>B. oleracea</i> L. var. <i>capitata</i>	Anther	Cristea <i>et al.</i> (2012)
29	<i>B. oleracea</i> L. var. <i>capitata</i>	Lateral bud	Pavlovic <i>et al.</i> (2012)

Table 2: Plant regeneration studies in cabbage (*Brassica oleracea* L. var. *capitata*) and related *Brassica* species through indirect organogenesis

S. No.	Plant species/cultivar	Explants used	Reference
1	<i>B. oleracea</i> L. var. <i>capitata</i>	Different explants of seedling	Mascarenhans <i>et al.</i> (1978)
2	<i>B. oleracea</i> L. var. <i>capitata</i>	Protoplast and root segment	Lillo and Shahin (1986)
3	<i>B. oleraceae</i> L. var. <i>capitata</i>	Protoplast	Koda <i>et al.</i> (1988)
4	<i>B. oleracea</i> L. var. <i>capitata</i>	Cotyledon/Hypocotyl/Leaf/Petiole	Bel'-Skaya <i>et al.</i> (1990)
5	<i>B. oleracea</i> L. var. <i>capitata</i> <i>B. campestris</i>	Hypocotyl	Hagazi and Matsubara (1991)
6	<i>B. oleracea</i> L. var. <i>capitata</i>	Protoplast	Wang <i>et al.</i> (1991)
7	<i>B. oleracea</i> L. var. <i>capitata</i> <i>B. oleracea</i> L. var. <i>italica</i>	Hypocotyl	Eisner <i>et al.</i> (1992)
8	<i>B. oleracea</i> L. var. <i>capitata</i>	Hypocotyl	Havel <i>et al.</i> 1994
9	<i>B. campestris</i> L. subsp. <i>chinensis</i>	Hypocotyl protoplast	Hou <i>et al.</i> (2000a)
10	<i>B. campestris</i> L. subsp. <i>chinensis</i>	Cotyledonary protoplast	Hou <i>et al.</i> (2000b)
11	<i>B. oleracea</i> L. var. <i>capitata</i> <i>B. oleracea</i> L. var. <i>botrytis</i> <i>B. oleracea</i> L. var. <i>italica</i>	Mesophyll protoplast	Kirti <i>et al.</i> (2001)
12	<i>B. oleracea</i> L. <i>capitata</i>	Protoplast	Chen <i>et al.</i> (2004)
13	<i>B. oleracea</i> L. var. <i>capitata</i>	Hypocotyl	Wang and Wang (2007)
14	<i>B. oleracea</i> L. var. <i>italica</i>	Leaf and shoot tip	Farzinebrahimi <i>et al.</i> (2012)
15	<i>B. oleracea</i> L. var. <i>capitata</i>	Cotyledon and hypocotyl	Sharma <i>et al.</i> (2013)

Table 3: Plant regeneration studies in cabbage (*Brassica oleracea* L. var. *capitata*) and related *Brassica* species through somatic embryogenesis

S. No.	Plant species/cultivar	Explants used	Reference
1	<i>B. oleracea</i> X <i>B. campestris</i>	Hypocotyl protoplast	Terada <i>et al.</i> (1987)
2	<i>B. oleracea</i> L. var. <i>capitata</i>	Microspores	Cao <i>et al.</i> (1990)
3	<i>B. campestris</i> L. subsp. <i>pekinensis</i>	Cotyledon	Choi <i>et al.</i> (1996)
4	<i>B. oleracea</i> L. var. <i>capitata</i>	Leaf	Donato <i>et al.</i> (2000)
5	<i>B. oleracea</i> L. var. <i>capitata</i>	Microspores	Jiang <i>et al.</i> (2005)
6	<i>B. oleracea</i> L. var. <i>capitata</i>	Anther	Gorecka and Krzyzanowska (2007)
7	<i>B. campestris</i> L.	Microspores	Gao <i>et al.</i> (2009)
8	<i>B. oleracea</i> L. var. <i>italica</i>	Cotyledon, hypocotyl and root	Yang <i>et al.</i> (2010a)
9	<i>B. oleracea</i> L. var. <i>capitata</i>	Microspores	Yuan <i>et al.</i> (2012)
10	<i>B. campestris</i> L.	Microspores	Wang <i>et al.</i> (2012)

Table 4: *Agrobacterium*-mediated reporter/marker gene transfer in cabbage (*Brassica oleracea* L. var. *capitata*) and related *Brassica* species

S. No.	Plant species/cultivar	Technique of gene transfer	Gene transferred	Explants used	Reference
1	<i>B. oleracea</i> L. var. <i>capitata</i>	<i>Agrobacterium tumefaciens</i> -mediated gene transfer	<i>npt-II</i>	Cotyledon	Shahin and Yashar (1986)
2	<i>B. oleracea</i> L. and <i>B. campestris</i>	<i>Agrobacterium tumefaciens</i> -mediated gene transfer	<i>npt-II</i> and <i>gus</i>	Peduncle	Christey and Earle (1988)
3	<i>B. oleracea</i> L. var. <i>capitata</i>	<i>Agrobacterium tumefaciens</i> -mediated gene transfer	<i>nos</i> and <i>npt-II</i>	Hypocotyl	Yu and Shao (1988)
4	<i>B. oleracea</i> L. var. <i>botrytis</i>	<i>Agrobacterium rhizogenes</i> -mediated gene transfer	T-DNA	Hypocotyl	David and Tempe (1988)
5	<i>B. oleracea</i> L. var. <i>acephala</i>	<i>Agrobacterium rhizogenes</i> -mediated gene transfer	T-DNA	Leaf tissue	Hosoki <i>et al.</i> (1989)
6	<i>B. oleracea</i> L. var. <i>capitata</i>	<i>Agrobacterium rhizogenes</i> -mediated gene transfer	T-DNA segment (<i>npt-II</i>)	Hypocotyl	He (1990)

7	<i>B. oleracea</i> L. var. <i>capitata</i>	<i>Agrobacterium rhizogenes</i> -mediated gene transfer	<i>npt-II</i>	Induction of root on hypocotyl	He <i>et al.</i> (1991)
8	<i>B. oleracea</i> L. var. <i>capitata</i>	<i>Agrobacterium rhizogenes</i> -mediated gene transfer	<i>npt-II</i> and <i>hpt</i>	Axenic root	Berthomieu and Jouanin (1992)
9	<i>B. oleracea</i> L. var. <i>capitata</i>	<i>A. rhizogenes</i> -mediated gene transfer	<i>aux-2</i>	Hypocotyl	Beclin <i>et al.</i> (1993)
10	<i>B. oleracea</i> L. var. <i>capitata</i>	<i>Agrobacterium tumefaciens</i> -mediated gene transfer	<i>gus</i>	Cotyledon, hypocotyl and petiole	Berthomieu <i>et al.</i> (1994)
11	<i>B. oleracea</i> and <i>B. campestris</i>	<i>Agrobacterium rhizogenes</i> -mediated gene transfer	<i>npt-II</i>	Leaf and cotyledon with petiole	Christey <i>et al.</i> (1997)
12	<i>B. oleracea</i> L. var. <i>botrytis</i>	<i>Agrobacterium tumefaciens</i> -mediated gene transfer	<i>npt-II</i>	Cotyledon and hypocotyl	Bhalla and Smith (1998b)
13	<i>B. campestris</i> L. subsp. <i>pekinensis</i>	<i>Agrobacterium tumefaciens</i> -mediated gene transfer	<i>npt-II</i>	Cotyledon	Cai <i>et al.</i> (1999)
14	<i>B. oleracea</i> L. var. <i>capitata</i>	<i>Agrobacterium tumefaciens</i> -mediated gene transfer	<i>gus</i> and <i>npt</i>	Cotyledon	Pius and Achar (2000)
15	<i>B. campestris</i> L.	<i>Agrobacterium tumefaciens</i> and <i>Agrobacterium rhizogenes</i> -mediated gene transfer	<i>npt-II, hpt</i> and <i>gus</i>	Cotyledon	Zhang <i>et al.</i> (2000)
16	<i>B. oleracea</i> L. (Broccoli, cabbage, cauliflower and Kale)	<i>Agrobacterium rhizogenes</i> -mediated gene transfer	<i>gfp</i> gene	Transgenic root	Cogan <i>et al.</i> (2001)
17	<i>B. oleracea</i> L. var. <i>botrytis</i>	<i>Agrobacterium</i> -mediated gene transfer	<i>gus</i>	Hypocotyl	Chakarbarty <i>et al.</i> (2002)
18	<i>B. oleracea</i> L. var. <i>capitata</i>	<i>Agrobacterium tumefaciens</i> -mediated gene transfer	<i>npt-II</i>	Shoot induction from hypocotyl	Tsukazaki <i>et al.</i> (2002)
19	<i>B. oleracea</i> L. var. <i>capitata</i>	<i>Agrobacterium tumefaciens</i> -mediated gene transfer	<i>npt-II</i> and <i>gus</i>	Hypocotyl	Sharma and Srivastava (2003)

20	<i>B. oleracea</i> L. var. <i>capitata</i>	<i>Agrobacterium tumefaciens</i> -mediated gene transfer	<i>npt-II</i> and <i>gus</i>	Hypocotyl	Singh and Srivastava (2003)
21	<i>B. oleracea</i> L. var. <i>capitata</i>	<i>Agrobacterium</i> -mediated gene transfer	<i>bar</i>		Gribova <i>et al.</i> (2005)
22	<i>B. campestris</i> L. var. <i>chinensis</i>	<i>Agrobacterium tumefaciens</i> -mediated gene transfer	<i>npt-II</i>	Seedling	Yu <i>et al.</i> (2005a)
23	<i>B. oleracea</i> L. var. <i>botrytis</i>	<i>Agrobacterium tumefaciens</i> -mediated gene transfer	<i>npt-II</i> and <i>gus</i>	Cotyledon and hypocotyl	Sharma <i>et al.</i> (2012b)

Table 5: Direct gene transfer technique-mediated reporter/marker gene transfer in cabbage (*Brassica oleracea* L. var. *capitata*) and related *Brassica* species

S. No.	Plant species/cultivar	Technique of gene transfer	Gene transferred	Explants used	Reference
1	<i>B. oleracea</i> L. var. <i>botrytis</i>	Leaf disc transformation, PEG-mediated gene transfer and Electroporation	<i>npt-II</i>	Protoplast	Eimert and Siegemund (1992)
2	<i>B. oleracea</i> L. var. <i>botrytis</i>	Microinjection and <i>Agrobacterium tumefaciens</i>	<i>npt-II</i>	Seedling	Eimert <i>et al.</i> (1992)
3	<i>B.campestris</i> L. subsp. <i>pekinensis</i>	Particle bombardment	<i>gus</i>	Leaf disc	Cho <i>et al.</i> (1994)
4	<i>Brassica</i> species	Particle Bombardment	<i>gus</i>	Cotyledon and hypocotyl	Tuan and Garg (2001)
5	<i>B. oleracea</i> L. var. <i>botrytis</i>	PEG- mediated gene transfer	<i>npt-II, hpt and gus</i>	Mesophyll protoplast	Radchuk <i>et al.</i> (2002)
6	<i>B. campestris</i> L.	Microinjection	T-DNA	Flower bud	Yan <i>et al.</i> (2004)

Table 6: *Agrobacterium*-mediated desirable gene transfer in cabbage (*Brassica oleracea* L. var. *capitata*) and related *Brassica* species

S. No.	Plant species/cultivar	Technique of gene transfer	Gene transferred	Explants used	Reference
1	<i>B. oleracea</i> L. var. <i>capitata</i>	<i>Agrobacterium rhizogenes</i> -mediated gene transfer	<i>npt-II</i> and <i>hph</i> gene	Leaf petiole	Berthomieu and Jouanin (1992)
2	<i>B. oleracea</i> L. var. <i>capitata</i>	<i>Agrobacterium tumefaciens</i> -mediated gene transfer	<i>cryIA(c)</i>	Cotyledon	Bai <i>et al.</i> (1992)
3	<i>B. oleracea</i> L. var. <i>capitata</i>	<i>Agrobacterium tumefaciens</i> -mediated gene transfer	<i>cryIA(c)</i>	Cabbage seedling	Metz <i>et al.</i> (1995)
4	<i>B. campestris</i> L. subsp. <i>pekinensis</i>	<i>Agrobacterium tumefaciens</i> -mediated gene transfer	<i>npt-II</i> and TMV coat protein gene	Cotyledonary petiole	Jun <i>et al.</i> (1995)
5	<i>B. campestris</i> L. subsp. <i>chinensis</i>	<i>Agrobacterium tumefaciens</i> -mediated gene transfer	<i>CpTI</i> & <i>npt-II</i>	Cotyledon	Cai <i>et al.</i> (1997)
6	<i>B. campestris</i> L. subsp. <i>chinensis</i>	<i>Agrobacterium tumefaciens</i> -mediated gene transfer	<i>bar</i>	Microspore derived embryos	Liu <i>et al.</i> (1998)
7	<i>B. campestris</i> L. subsp. <i>pekinensis</i>	<i>Agrobacterium tumefaciens</i> -mediated gene transfer	<i>bar</i>	Hypocotyl	Lim <i>et al.</i> (1998)
8	<i>B. oleracea</i> L.	<i>Agrobacterium tumefaciens</i> -mediated gene transfer	<i>cryIB</i> & <i>cryIAb</i>	Hypocotyl	Gonzalez <i>et al.</i> (1998)
9	<i>B. oleracea</i> L. var. <i>capitata</i>	<i>Agrobacterium tumefaciens</i> -mediated gene transfer	<i>Bt</i> -gene	Cotyledon	Cai <i>et al.</i> (1999a)
10	<i>B. oleracea</i> L. var. <i>italica</i>	<i>Agrobacterium tumefaciens</i> -mediated gene transfer	<i>cryIC</i>	Hypocotyl and petiole	Cao <i>et al.</i> (1999b)
11	<i>B. oleracea</i> L. var. <i>italica</i>	<i>Agrobacterium rhizogenes</i> -mediated gene transfer	<i>Antisense ACC oxidase</i>	Leaf and cotyledon	Henzi <i>et al.</i> (1999)
12	<i>B. campestris</i> L. subsp. <i>chinensis</i>	<i>Agrobacterium tumefaciens</i> -mediated gene transfer	<i>npt-II</i> & <i>api</i>	Cotyledon with petiole	Zhang <i>et al.</i> (1999)
13	<i>B. campestris</i> L. subsp. <i>chinensis</i>	<i>Agrobacterium tumefaciens</i> -mediated gene transfer & <i>A. rhizogenes</i>	<i>npt-II</i> , <i>hpt</i> and <i>gus</i>	Cotyledon	Zhang <i>et al.</i> (2000)

14	<i>B. campestris</i> L. subsp. <i>chinensis</i>	<i>Agrobacterium tumefaciens</i> -mediated gene transfer	Barnase and <i>npt-II</i>	Stem tip callus of young seedlings	Yu <i>et al.</i> (2000)
15	<i>B. oleracea</i> L. var. <i>capitata</i>	<i>Agrobacterium tumefaciens</i> -mediated gene transfer	<i>cryIAb3/cryIIa3</i>	Hypocotyl	Jin <i>et al.</i> (2000)
16	<i>B. campestris</i> L.	<i>Agrobacterium tumefaciens</i> -mediated gene transfer	<i>hpt</i> and <i>cryIC</i>	Hypocotyl	Cho <i>et al.</i> (2001)
17	<i>B. campestris</i> L.	<i>Agrobacterium tumefaciens</i> -mediated gene transfer	TuMV-CP gene	Cotyledonary petiole	Zhu <i>et al.</i> (2001)
18	<i>B. campestris</i> L. subsp. <i>pekinensis</i>	<i>Agrobacterium tumefaciens</i> -mediated gene transfer	<i>LMV-CP</i> gene		Cheng <i>et al.</i> (2001b)
19	<i>B. oleracea</i> L. var. <i>botrytis</i>	<i>Agrobacterium</i> -mediated gene transfer	<i>CryIA(b)</i>	Hypocotyl	Chakrabarty <i>et al.</i> (2002)
20	<i>B. campestris</i> L. subsp. <i>pekinensis</i>	<i>Agrobacterium tumefaciens</i> -mediated gene transfer	Modified CpTI (SCK) gene	Cotyledon with petiole	Yang <i>et al.</i> (2002)
21	<i>B. oleracea</i> L. var. <i>capitata</i>	<i>Agrobacterium tumefaciens</i> -mediated gene transfer	<i>cryIA(b)</i>	Hypocotyl	Bhattacharya <i>et al.</i> (2002)
22	<i>B. campestris</i> L. subsp. <i>pekinensis</i>	<i>Agrobacterium tumefaciens</i> -mediated gene transfer	<i>Bt</i> -gene	-	Wang <i>et al.</i> (2002)
23	<i>B. oleracea</i> L. var. <i>capitata</i>	<i>Agrobacterium tumefaciens</i> -mediated gene transfer	<i>npt-II, gus & bar</i>	-	Sretenovic <i>et al.</i> (2002)
24	<i>B. campestris</i> L. subsp. <i>pekinensis</i>	<i>Agrobacterium tumefaciens</i> -mediated gene transfer	<i>pdi</i>	Cotyledon	Kim <i>et al.</i> (2003)
25	<i>B. campestris</i> L. subsp. <i>pekinensis</i>	<i>Agrobacterium tumefaciens</i> -mediated gene transfer	<i>npt-II</i> and <i>GLOase</i>	Cotyledonary petiole	Cho <i>et al.</i> (2003)
26	<i>B. campestris</i> L. subsp. <i>pekinensis</i>	<i>Agrobacterium tumefaciens</i> -mediated gene transfer	<i>npt-II & flowering locus C (FLC)</i>	Hypocotyl	Cho <i>et al.</i> (2003)
27	<i>B. campestris</i> L.	<i>Agrobacterium tumefaciens</i> -mediated gene transfer	<i>gna</i>	-	Yang <i>et al.</i> (2003)
28	<i>B. oleracea</i> L. subsp. <i>capitata</i>	<i>Agrobacterium tumefaciens</i> -mediated gene transfer	<i>DTX-A</i>	Hypocotyl	Lee <i>et al.</i> (2003)

29	<i>B. oleracea</i> L. var. <i>capitata</i> <i>B. oleracea</i> L. var. <i>botrytis</i>	<i>Agrobacterium tumefaciens</i> -mediated gene transfer	<i>gus</i> and <i>cryIAc</i>	Different organs	Wang <i>et al.</i> (2003)
30	<i>B. oleracea</i> L. var. <i>botrytis</i>	<i>Agrobacterium tumefaciens</i> -mediated gene transfer	<i>cryIAb</i>	Cotyledon and hypocotyl	Awasthi (2003)
31	<i>B. oleracea</i> L. var. <i>capitata</i>	<i>Agrobacterium tumefaciens</i> -mediated gene transfer	<i>bet A</i>	Hypocotyl	Bhattacharya <i>et al.</i> (2004)
32	<i>B. campestris</i> L. subsp. <i>chinensis</i>	<i>Agrobacterium tumefaciens</i> -mediated gene transfer	<i>CYP86MF</i>	-	Yu and Cao (2004)
33	<i>B. oleracea</i> L. var. <i>sabauda</i>	<i>Agrobacterium tumefaciens</i> -mediated gene transfer	<i>bar</i>	Cotyledon and hypocotyl	Sretenovic <i>et al.</i> (2004)
34	<i>B. campestris</i> L. subsp. . <i>pekinensis</i>	<i>Agrobacterium tumefaciens</i> -mediated gene transfer	<i>bar</i> & <i>pin-II</i>	Cotyledonary petiole	Zhang <i>et al.</i> (2004)
35	<i>B. oleracea</i> L. var. <i>capitata</i>	<i>Agrobacterium tumefaciens</i> -mediated gene transfer	<i>pin-II</i> , <i>bar</i> and <i>npt</i>	Microspores	Lee <i>et al.</i> (2004)
36	<i>B. oleracea</i> L. var. <i>capitata</i>	<i>Agrobacterium tumefaciens</i> -mediated gene transfer	<i>cryIB</i> and <i>cryIAb</i>	Hypocotyl	Paul <i>et al.</i> (2005)
37	<i>B. oleracea</i> L. var. <i>capitata</i>	<i>Agrobacterium tumefaciens</i> -mediated gene transfer	<i>vhb</i>	Hypocotyl and cotyledon	Li <i>et al.</i> (2005)
38	<i>B. oleracea</i> L. var. <i>capitata</i>	<i>Agrobacterium tumefaciens</i> -mediated gene transfer	<i>bar</i>	-	Gribova <i>et al.</i> (2005)
39	<i>B. oleracea</i> L. var. <i>botrytis</i>	<i>Agrobacterium tumefaciens</i> -mediated gene transfer	<i>CpTI</i>	Cotyledon and hypocotyl	Lingling <i>et al.</i> (2005)
40	<i>B. oleracea</i> L. var. <i>capitata</i>	<i>Agrobacterium tumefaciens</i> -mediated gene transfer	<i>IGF-I</i>	Hypocotyl	Zhang and Wang (2005)
41	<i>B. oleracea</i> L. var. <i>capitata</i>	<i>Agrobacterium tumefaciens</i> -mediated gene transfer	<i>OC-1</i>	Hypocotyl	Lei <i>et al.</i> (2006)
42	<i>B. campestris</i> L.	<i>Agrobacterium tumefaciens</i> -mediated gene transfer	<i>CpTI</i>	Protoplast	Zhao <i>et al.</i> (2006b)
43	<i>B. oleracea</i> L. var. <i>capitata</i>	<i>Agrobacterium tumefaciens</i> -mediated gene transfer	<i>cryIAb</i>	Cotyledon and hypocotyl	Venkatesh (2006)

44	<i>B. campestris</i> L.	<i>Agrobacterium tumefaciens</i> -mediated gene transfer	<i>GLOase and JMT</i>	Hypocotyl	Min <i>et al.</i> (2007)
45	<i>B. oleracea</i> L. var. <i>capitata</i>	<i>Agrobacterium tumefaciens</i> -mediated gene transfer	<i>cryIa8</i>	Cotyledon and hypocotyl	Lei <i>et al.</i> (2009)
46	<i>B. oleracea</i> L. var. <i>capitata</i>	<i>Agrobacterium tumefaciens</i> -mediated gene transfer	<i>pSKIOIS</i>	Hypocotyl	Hua <i>et al.</i> (2009)
47	<i>B. campestris</i> L. subsp. <i>pekinensis</i>	<i>Agrobacterium</i> -mediated gene transfer	<i>Aii</i>	-	Vanjildorj <i>et al.</i> (2009)
48	<i>B. oleracea</i> L. var. <i>capitata</i>	<i>Agrobacterium</i> -mediated gene transfer	<i>AtHSP 101</i>	Hypocotyl and shoot tip	Rafat <i>et al.</i> (2010)
49	<i>B. oleracea</i> L. var. <i>capitata</i>	<i>Agrobacterium tumefaciens</i> -mediated gene transfer	<i>cryIBa3</i>	-	Deng-Xia <i>et al.</i> (2011)
50	<i>B. oleracea</i> L. var. <i>botrytis</i>	<i>Agrobacterium tumefaciens</i> -mediated gene transfer	<i>cryIAb</i>	Cotyledon and hypocotyl	Awasthi and Srivastava (2013)

Table 7: Direct gene transfer technique-mediated desirable gene transfer in cabbage (*Brassica oleracea* L. var. *capitata*) and related *Brassica* species

S. No.	Plant species/cultivar	Technique of gene transfer	Gene transferred	Explants used	Reference
1	<i>B. oleracea</i> L. var. <i>kunwari</i>	PEG mediated gene transfer	<i>dhfr</i> , <i>npt</i> and <i>bar</i>	Hypocotyl	Mukhopadhyay <i>et al.</i> (1991)
2	<i>B. campestris</i> L. subsp. <i>chinensis</i>	Electroporation	TMV RNA	Protoplast	Boyer <i>et al.</i> (1993)
3	<i>B. oleracea</i> L. var. <i>capitata</i>	Pollen tube gene transfer	Black rot resistance gene	-	Liao <i>et al.</i> (1998)
4	<i>B. oleracea</i> L. var. <i>botrytis</i>	PEG mediated gene transfer	<i>gus</i> , <i>npt</i> , <i>hpt</i>	Mesophyll protoplast	Radchuk <i>et al.</i> (2002)
5	<i>B. campestris</i> L. subsp. <i>pekinensis</i>	Microinjection	Anti-sense <i>BcpIH</i> and <i>npt-II</i>	Flower bud	Yan and Cao (2003)
6	<i>B. campestris</i> L. subsp. <i>chinensis</i>	Pollen tube transformation	Anti-sense fragment of <i>CYP86MF</i>	Cotyledon and hypocotyl	Yu <i>et al.</i> (2004)
7	<i>B. campestris</i> L. subsp. <i>pekinensis</i>	Vacuum infiltration transformation	<i>pin-II</i> and <i>bar</i>		Xu <i>et al.</i> (2004)
8	<i>B. oleracea</i> L. var. <i>capitata</i>	Particle gun method	<i>aadA</i> and <i>cryIAb</i>	Chloroplast	Liu <i>et al.</i> (2008)

Table 8: Factors effecting transformation frequency in different plant species

Sr. No.	Plant	Technique	Factors studied	Reference
1	<i>Hordeum vulgare</i> L	Plant regeneration	Effect of antibiotic cephalosporin and cefotaxime	Mathias and Mukasa (1987)
2	<i>Mangifera indica</i> L	<i>Agrobacterium</i> -mediated gene transfer	Effect of kanamycin	Mathews and Litz (1990)
3	<i>MalusX domestica</i>	<i>Agrobacterium</i> -mediated gene transfer	Effect of antibiotics cephalosporin, cefotaxime and kanamycin	Yepes and Aldwinckle (1994)
4	<i>Sorghum bicolor</i> L. Moench	Plant regeneration	Effect of antibiotics (cephalosporin and cefotaxime) and amino acids (asparagine and proline)	Rao <i>et al.</i> (1995)
5	<i>Carthamus tinctorius</i> cv. Centennial	<i>Agrobacterium</i> -mediated gene transfer	Effect of co-cultivation conditions, acetosyringone and antibiotics	Orlikowska <i>et al.</i> , (1995)
6	<i>MalusX domestica</i>	<i>Agrobacterium</i> -mediated gene transfer	Postcultivation conditions	Bondt <i>et al.</i> , (1996)
7	<i>MalusX domestica</i>	<i>Agrobacterium</i> -mediated gene transfer	Effect of antibiotics and acidified medium	Hammerschlag <i>et al.</i> , (1997)
8	<i>Nicotiana tabacum</i>	<i>Agrobacterium</i> -mediated gene transfer	Effect of antibiotics	Nauerby <i>et al.</i> , (1997)
9	<i>Citrus sinensis</i> L. cv. Osbeck	<i>Agrobacterium</i> -mediated gene transfer	Optimization of cocultivation, regeneration and selection conditions	Pena <i>et al.</i> , (1997)
10	<i>Citrus sinensis</i> L. Osbeck X <i>Poncirus trifoliata</i> L. Raf.	<i>Agrobacterium</i> -mediated gene transfer	Effect of co-cultivation conditions, acetosyringone and antibiotics	Cervera <i>et al.</i> , (1998)
11	<i>Nicotiana tabacum</i> and <i>Ulmus pumila</i> L.	<i>Agrobacterium</i> -mediated gene transfer	Effect of antibiotics	Cheng <i>et al.</i> , (1998b)
12	<i>Solanum lycopersicum</i>	<i>Agrobacterium</i> -mediated gene transfer	Effect of antibiotics	Ling <i>et al.</i> , (1998)

13	<i>Vitis vinifera</i>	<i>Agrobacterium</i> -mediated gene transfer	Effect of antibiotics	Pierre peros <i>et al.</i> , (1998)
14	<i>Brassica oleracea</i> L. var. <i>botrytis</i>	<i>Agrobacterium</i> -mediated gene transfer	Effect of kanamycin	Dixit and Srivastava (1999)
15	<i>Pinus pinea</i> L.	<i>Agrobacterium</i> -mediated gene transfer	Preinduction and concentration of bacteria, days of coculture and wounding procedure	Humara <i>et al.</i> , (1999)
16	<i>Juglans regia</i>	<i>Agrobacterium</i> -mediated gene transfer	Effect of antibiotics	Tang <i>et al.</i> , (2000)
17	<i>Brassica campestris</i> L.	-	Effects of plant growth regulators, AgNO ₃ , initial dark treatment, various antibiotics, and herbicide	Park <i>et al.</i> (2000)
18	<i>Brassica campestris</i> L.	Tissue culture and genetic transformation	Effects of kanamycin, cefotaxime, carbenicillin and ampicillin	Song <i>et al.</i> (2000)
19	<i>Dianthus caryophyllus</i> L.	<i>Agrobacterium</i> -mediated gene transfer	Effect of antibiotics	Estopa <i>et al.</i> , (2001)
20	<i>Solanum lycopersicum</i> cv. UC82	<i>Agrobacterium</i> -mediated gene transfer	Effect of antibiotics	Hu and Phillips (2001)
21	<i>Chrysanthemum indicum</i> and <i>Nicotiana tabaccum</i>	<i>Agrobacterium</i> -mediated gene transfer	Effect of antibiotics	Teixeira da Silva and Fukaiuses (2001)
22	<i>Carica papaya</i>	<i>Agrobacterium</i> -mediated gene transfer	Effect of antibiotics	Yu <i>et al.</i> , (2001)
23	<i>F. vesca</i>	<i>Agrobacterium</i> -mediated gene transfer	Phytotoxic effect of antibiotics	Alsheikh <i>et al.</i> , (2002)
24	<i>Hevea brasiliensis</i>	<i>Agrobacterium</i> -mediated gene transfer	Cocultivation conditions and effect of antibiotics	Jayashree <i>et al.</i> , (2003)
25	<i>Citrus sinensis</i> (L.) Osbeck	<i>Agrobacterium</i> -mediated gene transfer	Mode of preculturing, temperature of cocultivation and presence of acetosyringone	Li <i>et al.</i> (2003)

26	<i>Prunus domestica</i> L.	<i>Agrobacterium</i> -mediated gene transfer	Effect of antibiotics	Padilla <i>et al.</i> , (2003)
27	<i>Pinus taeda</i> L.	<i>Agrobacterium</i> -mediated gene transfer	Effect of antibiotics	Wei <i>et al.</i> , (2003)
28	<i>Prunus armeniaca</i>	-----	Effect of antibiotics	Petri <i>et al.</i> , (2005)
29	<i>Prunus amygdalus</i>	<i>Agrobacterium</i> -mediated gene transfer	Effect of acetosyringone	Costa <i>et al.</i> , (2006)
30	<i>Populus euphratica</i>	-----	Effect of antibiotics	Ding <i>et al.</i> , (2006)
31	<i>Hordeum vulgare</i> L.	<i>Agrobacterium</i> -mediated gene transfer	Preinduction and concentration of bacteria, days of coculture and acetosyringone	Kumlehn <i>et al.</i> , (2006)
32	<i>Pinus pinaster</i>	<i>Agrobacterium</i> -mediated gene transfer	Effect of antibiotics	Tereso <i>et al.</i> , (2006)
33	<i>Brassica campestris</i> L.	<i>Agrobacterium</i> -mediated gene transfer	Age of seedlings, growth conditions and status of <i>Agrobacterium</i> suspension, preculture of explants, cocultivation time, acetosyringone and concentration of kanamycin	Zhao <i>et al.</i> (2006a).
34	<i>Vigna mungo</i>	<i>Agrobacterium</i> -mediated gene transfer	Preinduction and concentration of bacteria, days of coculture and wounding procedure	Saini and Jaiwal (2007)
35	<i>Zingiber officinale</i> Rosc.	<i>Agrobacterium</i> -mediated gene transfer	Preculturing time, bacterial dilution, cocultivation period, the effect of acetosyringone and kanamycin concentration	Suma <i>et al.</i> (2008)
36	<i>Cicer arietinum</i> L.	<i>Agrobacterium</i> -mediated gene transfer	Effect of selective agent and antibiotics	Öz <i>et al.</i> (2009)

37	<i>Citrullus lanatus</i> cv. Round Dragon	<i>Agrobacterium</i> -mediated gene transfer	explants age, preculturing conditions, wounding technique, <i>Agrobacterium</i> concentration, co-incubation period and acetosyringone concentration	Kavitah <i>et al.</i> (2010)
38	<i>Fragaria X ananassa</i> Duch. Chandler	<i>Agrobacterium</i> -mediated gene transfer	Effect of co-cultivation conditions, acetosyringone and antibiotics	Husaini (2010)
39	<i>Triticum aestivum</i> L.)	<i>Agrobacterium</i> -mediated gene transfer	Different optical densities of bacterial suspension and different concentrations of acetosyringone	Rashid <i>et al.</i> (2010)
40	<i>Parthenocissus tricuspidata</i>	<i>Agrobacterium</i> -mediated gene transfer	<i>Agrobacterium</i> strains, co-cultivation time, <i>Agrobacterium</i> concentration, and infection time	Yang <i>et al.</i> (2010b)
41	<i>Mentha piperita</i>	-	Effect of bavistin, cefotaxime, kanamycin and ethylene inhibitor STS	Sujana and Naidu (2011)
42	<i>Triticum aestivum</i>	<i>Agrobacterium</i> -mediated gene transfer	Explant source and acetosyringone concentration	Rashid <i>et al.</i> (2011)
43	<i>Solanum lycopersicum</i>	<i>Agrobacterium</i> -mediated gene transfer	Petiole	Sharma <i>et al.</i> (2011)
44	<i>Solanum tuberosum</i> L.	<i>Agrobacterium</i> -mediated gene transfer	Co-cultivation time, cefotaxime concentration, and days to pre-selection	Ahmad <i>et al.</i> (2012)

Chapter-3

MATERIAL AND METHODS

The present investigation on “*Agrobacterium*-mediated insect resistance gene transfer in cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India) and molecular analysis of regenerated plantlets” was carried out in the Department of Biotechnology of Dr Y. S. Parmar University of Horticulture and Forestry, Nauni, Solan. The details of material and methods used have been described under the following headings

3.1 Plant material

3.2 Source of explants

3.3 Nutrient medium

3.3.1 Murashige and Skoog’s medium

3.3.2 YMB medium

3.4 Sterilization of glasswares and instruments

3.5 Culture conditions

3.6 Plant regeneration in cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India)

3.6.1 Shoot regeneration from cotyledon explants

3.6.2 Shoot regeneration from hypocotyl explants

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3.7.1 Preparation of normal shoot regeneration medium and selective shoot regeneration media for leaf and petiole explants

3.7.2 Inoculation of leaf and petiole explants on the normal and the selective media

3.7.3 Morphological observations and measurement of relative growth of cultured tissues of leaf and petiole explants

3.8 Effect of cefotaxime on the regeneration potential of cotyledon and hypocotyl tissues of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India) (without co-cultivation)

3.8.1 Preparation of selective shoot regeneration media for cotyledon and hypocotyl explants

3.8.2 Inoculation of cotyledon and hypocotyl explants on the selective shoot regeneration media

3.8.3 Morphological observations and adventitious shoot regeneration

3.9 Effect of cefotaxime and kanamycin on the growth of agrobacterial cells and regeneration potential of cotyledon and hypocotyl tissues of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India) (after co-cultivation)

3.9.1 Preparation of selective shoot regeneration media for cotyledon and hypocotyl explants

3.9.2 Inoculation of cotyledon and hypocotyl explants on the selective shoot regeneration media

3.9.3 Morphological observations and adventitious shoot regeneration

3.10 Genetic transformation in cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India)

3.10.1 Permission from Institutional Biosafety Committee (IBSC) of our University to transfer insect resistance gene (*cryIAa*) into cabbage

3.10.2 Development of putative transgenic plantlets from cotyledon and hypocotyl explants

- 3.10.2.1 *Agrobacterium* strain and plasmid
- 3.10.2.2 Maintenance of *Agrobacterium* strain
- 3.10.2.3 Preparation of fresh cultures of *Agrobacterium* strain
- 3.10.2.4 Preculturing of cotyledon and hypocotyl explants
- 3.10.2.5 Co-cultivation of cotyledon and hypocotyl explants
- 3.10.2.6 Effect of acetosyringone on shoot regeneration and transformation frequency from cotyledon and hypocotyl explants in cabbage
 - a) Preparation of selective shoot regeneration medium supplemented with different concentrations of acetosyringone
 - b) Inoculation of cotyledon and hypocotyl explants
- 3.10.2.7 Selection of transformed cells on selective media
 - a) Preparation of selective shoot regeneration media
 - b) Transfer of cotyledon and hypocotyl explants on the selective shoot regeneration media after co-cultivation
 - c) Regeneration of putative transgenic shoots from cotyledon and hypocotyl explants
 - d) Root regeneration from *in vitro* developed putative transgenic shoots and development of complete plantlets
- 3.10.2.8 Hardening of *in vitro* developed putative transgenic plantlets

3.11 Molecular analyses of putative transgenic shoots / plantlets of cabbage (*Brassica oleracea* L. var. *capitata* cv. **Pride of India)**

3.11.1 Confirmation of presence/integration of the transgenes i.e. *cryIAa* (insect resistance gene) and *npt-II* (kanamycin resistance gene) into the genome of cabbage by PCR analysis using designed primers

- 3.11.1.1 Isolation of plasmid DNA
- 3.11.1.2 Isolation and purification of genomic DNA from the putative transgenic shoots/plantlets and control plantlets (non-transformed) of cabbage
- 3.11.1.3 Quantification of DNA
- 3.11.1.4 Amplification of *cryIAa* and *npt-II* genes by polymerase chain reaction using designed primers

3.11.1.5 Gel electrophoresis of amplified product

3.11.1.6 Viewing the gel and photography

3.11.2 Confirmation of integration of the transgene i.e. *cryIAa* (insect resistance gene) into the genome of cabbage by Southern hybridization

3.11.2.1 Isolation of plasmid DNA

3.11.2.2 Radiolabelling of DNA probe

3.11.2.3 Isolation and purification of genomic DNA from putative transgenic and control shoots/ plantlets of cabbage

3.11.2.4 Southern Blotting and Autoradiography

3.11.3 Confirmation of expression of the transgenes i.e. *cryIAa* (insect resistance gene) and *npt-II* (kanamycin resistance gene) in the transgenic cabbage by using reverse transcriptase-polymerase chain reaction (RT-PCR)

3.11.3.1 Isolation of total RNA from PCR positive transgenic shoots/ plantlets of cabbage

3.11.3.2 cDNA synthesis by RT-PCR

3.11.3.3 Amplification of cDNA by using specific designed primers

3.11.3.4 Gel electrophoresis of amplified cDNA i.e. *cryIAa* and *npt-II*

3.11.3.5 Viewing the gel and photography

3.11.4 Confirmation of expression of transgene i.e. *cryIAa* (insect resistance) in the transgenic cabbage by using Real Time polymerase chain reaction (RealTime-PCR) technique

3.11.4.1 Isolation of total RNA

3.11.4.2 cDNA synthesis by RealTime-PCR and amplification of cDNA by using SYBER green system

3.11.4.3 Real Time-PCR amplification

3.11.5 Bioassay of transgenic and control plantlets of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India)

3.12 Statistical analysis



Fig. 1a: Aseptically germinated 7-9 days old seedlings of cabbage (*Brassica oleracea* L. var. *capitata* cv. **Pride of India**).



Fig. 1b: *In vivo* grown 20-25 days old seedlings of cabbage (*Brassica oleracea* L. var. *capitata* cv. **Pride of India**).

3.1 Plant material

The certified seeds of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India) were procured from the Department of Vegetable Science, Dr. Y.S. Parmar University of Horticulture and Forestry, Nauni, Solan (H.P.). The seeds of cabbage were thoroughly washed under tap water and teepol and were soaked for an hour. After soaking, the seeds were surface sterilized with 0.1 % mercuric chloride (HgCl₂) for 1-2 minutes and then thoroughly washed (3-4 times) with sterilized distilled water under laminar air flow chamber. The seeds were then inoculated on half strength of MS basal medium containing 0.5 % sucrose. The pH of the medium was adjusted to 5.8. The culture tubes inoculated with seeds then covered with carbon paper in order to maintain the dark condition for seed germination and were kept in culture room at 26 ± 2°C temperature.

3.2 Source of explants

Seven to nine days old *in vitro* grown seedlings and 20-25 days old glass house grown seedlings were used as a source of explants i.e. cotyledon, hypocotyl, leaf and petiole, respectively, to carry out plant regeneration and genetic transformation studies in cabbage (Fig. 1a and 1b).

3.3 Nutrient medium

3.3.1 Murashige and Skoog's medium

Nutrient medium and concentration of plant growth regulators for the optimum growth and differentiation of cells and tissues *in vitro* may vary with species or cultivar. Even the tissues from different parts of the same plant may have different nutritional requirement and different concentration of plant growth regulators may be required by them for optimum plant growth and differentiation.

MS (Murashige and Skoog, 1962) medium supplemented with 100mg/l mesoinositol, 3 % sucrose and 0.8 % agar-agar was used as basal medium. Different concentrations and combinations of auxins and cytokinins (BAP+IAA, BAP+NAA, Kn+IAA, Kn+IAA, TDZ, TDZ+Adenine, TDZ+NAA and TDZ+IAA) were used in MS basal medium for plant regeneration experiment. The pH of the medium was adjusted to 5.8 with the help of 0.1N NaOH and 0.1N HCl before adding agar-agar. The medium was poured in culture vessels and sterilized by autoclaving at 15 pound per square inch pressure for 15-20 minutes.

3.3.2 YMB Medium

YMB (Yeast Mannitol Broth) medium was used for the maintenance and preparation of fresh cultures of genetically engineered *Agrobacterium tumefaciens* strain.

Composition of YMB medium

- i) 1 % mannitol
- ii) 0.04 % yeast extract
- iii) 0.01 % NaCl
- iv) 0.02 % MgSO₄.7H₂O
- v) 0.05 % K₂HPO₄
- vi) 1.5 % agar-agar
- vii) 50mg/l Kanamycin (by filter sterilization)

The pH of the medium was adjusted to 7.5 with 0.1N NaOH or 0.1N HCl before adding agar-agar into the medium. The medium was sterilized at 15 pound per square inch for 15-20 minutes in an autoclave. Kanamycin was added into sterilized molten or liquid YMB medium by filter sterilization using the Whatman[®] membrane filter of 0.22 μm pore size (Whatman limited, Maid Stone, England).

3.4 Sterilization of glasswares and instruments

All the glasswares were cleaned in a solution of 10 % teepol with test tube brush and rinsed in tap water. Finally they were rinsed in distilled water and dried in hot dry oven for 3 to 4 hours. Glasswares and instruments such as flasks, petri dishes, beakers, test tubes, scalpels, forceps, filter assembly etc. were wrapped with paper/plugged with cotton plug and sterilized at 15 lbs/inch² for 15-20 minutes.

3.5 Culture conditions

All the aseptic manipulations were carried out under vertical laminar air flow chamber. Laminar flow was pre-sterilized with ultraviolet light for 15 minutes and sterilized instruments were used for inoculation. Defined precautions were taken to maintain aseptic conditions inside the cabinet. After inoculation (of explants), all the culture vessels were kept in culture room at 26±2°C under 16 hours of photoperiod.

3.6 Plant regeneration in cabbage (*Brassica oleracea* L. var. *capitata* cv. *Pride of India*)

3.6.1 Shoot regeneration from cotyledon explants

For obtaining high efficiency shoot regeneration, the cotyledons were excised from seven to nine days old aseptically grown seedlings and cut into 0.5-1.0cm pieces with the help of sterilized scalpel blade. These explants were cultured in flasks containing full strength MS basal medium supplemented with various combinations and concentrations of plant growth regulators such as BAP+IAA (mg/l), BAP+NAA (mg/l), Kn+IAA (mg/l), Kn+NAA (mg/l), TDZ (mg/l), TDZ+Adenine (mg/l), TDZ+NAA (mg/l) and TDZ+IAA (mg/l) (Table 9, 11, 12, 13 and 14). For every combination, 5 flasks with 5 explants each were inoculated and each experiment was repeated thrice. Observations were taken at the interval of 7 days till shoot regeneration. Explants were evaluated for average number of shoots per explants and percentage shoot regeneration.

3.6.2 Shoot regeneration from hypocotyl explants

To obtain high frequency shoot regeneration, the hypocotyls were excised from seven to nine days old aseptically grown seedlings and cut into 0.5-1.0cm pieces and inoculated on full strength MS medium supplemented with varying concentrations and combinations of plant growth regulators such as BAP+IAA (mg/l), BAP+NAA (mg/l), Kn+IAA (mg/l), Kn+NAA (mg/l), TDZ (mg/l), TDZ+Adenine (mg/l), TDZ+NAA (mg/l) and TDZ+IAA (mg/l) (Table 10, 11, 12, 13 and 14). For every combination 5 flasks with 6 explants were inoculated and each experiment was repeated thrice. Observations were taken at the interval of 7 days till shoot regeneration. Explants were evaluated for average number of shoots per explants and percentage shoot regeneration.

3.6.3 Shoot regeneration from leaf explants

For obtaining high efficiency shoot regeneration, leaf explants were excised from glass house grown 20-25 days old seedlings and surface sterilized. The explants were cut into small pieces and their surface was gently tapped with the scalpel blade to injure them. These explants were cultured in flasks containing full strength MS basal medium supplemented with various combinations and concentrations of plant growth regulators such as BAP+IAA

(mg/l), BAP+NAA (mg/l), Kn+IAA (mg/l), Kn+NAA (mg/l), TDZ (mg/l), TDZ+Adenine (mg/l), TDZ+NAA (mg/l) and TDZ+IAA (mg/l) (Table 9, 11,12, 13 and 14). For every combination 5 flasks with 5 explants were inoculated and each experiment was repeated thrice. Observations were taken at the interval of 7 days till shoot regeneration. Explants were evaluated for average number of shoots per explants and percentage shoot regeneration.

3.6.4 Shoot regeneration from petiole explants

To obtain high frequency shoot regeneration, petiole explants were excised from glass house grown 20-25 days old seedlings and surface sterilized. The explants were cut into small pieces and their surface was gently tapped with the scalpel blade to injure them. These explants were cultured in flasks containing full strength MS basal medium supplemented with various combinations and concentrations of plant growth regulators such as BAP+IAA (mg/l), BAP+NAA (mg/l), Kn+IAA (mg/l), Kn+NAA (mg/l), TDZ (mg/l), TDZ+Adenine (mg/l), TDZ+NAA (mg/l) and TDZ+IAA (mg/l) (Table 10, 11, 12, 13 and 14). For every combination 5 flasks with 6 explants were inoculated and each experiment was repeated thrice. Observations were taken at the interval of 7 days till shoot regeneration. Explants were evaluated for average number of shoots per explants and percentage shoot regeneration.

Table 9: Different combinations and concentrations of BAP+IAA, BAP+NAA, Kn+IAA and Kn+NAA used in MS medium for shoot regeneration from cotyledon and leaf explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. *Pride of India*)

Sr. No.	Medium code	Medium composition
1	A1	MS basal + 1.0mg/l Kn + 0.25mg/l IAA
2	A2	MS basal + 1.0mg/l Kn + 0.50mg/l IAA
3	B7	MS basal + 2.0mg/l Kn + 0.25mg/l NAA
4	B8	MS basal + 2.0mg/l Kn + 0.5mg/l NAA
5	C7	MS basal + 2.0mg/l BAP+ 0.25mg/l IAA
6	C8	MS basal + 2.0mg/l BAP+ 0.50mg/l IAA
7	D5	MS basal + 1.5mg/l BAP+ 0.50mg/l NAA
8	D7	MS basal + 2.0mg/l BAP+ 0.25mg/l NAA
9	C9	MS basal + 2.0mg/l BAP+ 0.75mg/l IAA
10	D2	MS basal + 1.0mg/l BAP+ 0.50mg/l NAA

Table 10: Different combinations and concentrations of BAP+IAA, BAP+NAA, Kn+IAA and Kn+NAA used in MS medium for shoot regeneration from hypocotyl and petiole explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. *Pride of India*)

Sr. No.	Medium code	Medium composition
1	A6	MS basal + 1.5mg/l Kn + 0.75mg/l IAA
2	A4	MS basal + 1.5mg/l Kn + 0.25mg/l IAA
3	B7	MS basal + 2.0mg/l Kn + 0.25mg/l NAA
4	B9	MS basal + 2.0mg/l Kn + 0.75mg/l NAA
5	C7	MS basal + 2.0mg/l BAP + 0.25mg/l IAA
6	C9	MS basal + 2.0mg/l BAP + 0.75mg/l IAA
7	D2	MS basal + 2.0mg/l BAP + 0.50mg/l NAA
8	D7	MS basal + 2.0mg/l BAP + 0.25mg/l NAA
9	B4	MS basal + 1.5mg/l Kn + 0.25mg/l NAA
10	D7	MS basal + 2.0mg/l BAP + 0.25mg/l NAA

Table 11: Different concentrations of TDZ (Thidiazuron) used in MS medium for shoot regeneration from cotyledon, hypocotyl, leaf and petiole explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. *Pride of India*)

Sr. No.	Medium code	Medium composition
1	E1	MS basal + 0.110mg/l TDZ
2	E2	MS basal + 0.220mg/l TDZ
3	E3	MS basal + 0.330mg/l TDZ
4	E4	MS basal + 0.440mg/l TDZ
5	E5	MS basal + 0.550mg/l TDZ
6	E6	MS basal + 0.660mg/l TDZ
7	E7	MS basal + 0.770mg/l TDZ
8	E8	MS basal + 0.880mg/l TDZ
9	E9	MS basal + 0.990mg/l TDZ
10	E10	MS basal + 1.100mg/l TDZ

Table 12: Different concentrations and combinations of TDZ (Thidiazuron) and Adenine used in MS medium for shoot regeneration from cotyledon, hypocotyl, leaf and petiole explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. *Pride of India*)

Sr. No.	Medium code	Medium composition
1	F1	MS basal + 0.110mg/l TDZ + 79.7 mg/l Adenine
2	F2	MS basal + 0.220mg/l TDZ + 79.7 mg/l Adenine
3	F3	MS basal + 0.330 mg/l TDZ + 79.7mg/l Adenine
4	F4	MS basal + 0.440 mg/l TDZ + 79.7mg/l Adenine
5	F5	MS basal + 0.550 mg/l TDZ + 79.7mg/l Adenine
6	F6	MS basal + 0.660 mg/l TDZ + 79.7mg/l Adenine
7	F7	MS basal + 0.770 mg/l TDZ + 79.7mg/l Adenine
8	F8	MS basal + 0.880 mg/l TDZ + 79.7mg/l Adenine
9	F9	MS basal + 0.990 mg/l TDZ + 79.7mg/l Adenine
10	F10	MS basal + 1.10 mg/l TDZ + 79.7mg/l Adenine

Table 13: Different concentrations and combinations of TDZ (Thidiazuron) and NAA used in MS medium for shoot regeneration from cotyledon, hypocotyl, leaf and petiole explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. *Pride of India*)

Sr. No.	Medium code	Medium composition
1	G1	MS basal + 0.110 mg/l TDZ + 0.02 mg/l NAA
2	G2	MS basal + 0.220 mg/l TDZ + 0.02 mg/l NAA
3	G3	MS basal + 0.330mg/l TDZ + 0.02 mg/l NAA
4	G4	MS basal + 0.440mg/l TDZ + 0.02 mg/l NAA
5	G5	MS basal + 0.550mg/l TDZ + 0.02 mg/l NAA
6	G6	MS basal + 0.660mg/l TDZ + 0.02 mg/l NAA
7	G7	MS basal + 0.770mg/l TDZ + 0.02 mg/l NAA
8	G8	MS basal + 0.880mg/l TDZ + 0.02 mg/l NAA
9	G9	MS basal + 0.990mg/l TDZ + 0.02 mg/l NAA
10	G10	MS basal + 1.10 mg/l TDZ + 0.02 mg/l NAA

Table 14: Different concentrations and combinations of TDZ (Thidiazuron) and IAA used in MS medium for shoot regeneration from cotyledon, hypocotyl, leaf and petiole explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India)

Sr. No.	Medium code	Medium composition
1	H1	MS basal + 0.110 mg/l TDZ + 0.088 mg/l IAA
2	H2	MS basal + 0.220 mg/l TDZ + 0.088 mg/l IAA
3	H3	MS basal + 0.330mg/l TDZ + 0.088 mg/l IAA
4	H4	MS basal + 0.440mg/l TDZ + 0.088 mg/l IAA
5	H5	MS basal + 0.550mg/l TDZ + 0.088 mg/l IAA
6	H6	MS basal + 0.660mg/l TDZ + 0.088 mg/l IAA
7	H7	MS basal + 0.770mg/l TDZ + 0.088 mg/l IAA
8	H8	MS basal + 0.880mg/l TDZ + 0.088 mg/l IAA
9	H9	MS basal + 0.990mg/l TDZ + 0.088 mg/l IAA
10	H10	MS basal + 1.10 mg/l TDZ + 0.088 mg/l IAA

3.6.5. Root regeneration from *in vitro* developed shoots and development of complete plantlets

The regenerated shoots which were obtained from cotyledon, hypocotyl, leaf and petiole explants were transferred to root regeneration media containing MS basal medium with various concentrations of different auxins (IAA, NAA and IBA) (Table 15, 16 and 17) for root induction to get complete plantlets.

3.6.6 Hardening of *in vitro* regenerated plantlets

i) Washing of roots

After proper *in vitro* development, the plantlets were taken out of the tubes in such a way that no damage was caused to their root system. The roots were washed gently under running tap water to remove adhering medium. After removal of the medium, plantlets were kept in running tap water for a few minutes so that they do not wilt after transfer to soil. The plantlets were treated with 0.5 % bavistin for 2-3 minutes.

ii) Planting in cocopeat mixture

The *in vitro* regenerated plantlets after washing were transferred to the cocopeat mixture which was sterilized by autoclaving at 15lbs/inch² for 30 minutes at 121°C (after

sterilization of the cocopeat mixture, the pots were filled with this mixture). After transfer of the plantlets to cocopeat mixture they were watered with 0.5 % bavistin solution and covered with polythene bags to maintain relative humidity.

iii) Planting in potting mixture

The plantlets from cocopeat were transferred to pots containing presterilized potting mixture (consisting of sand + soil + FYM). The potted plantlets were kept under varying conditions of humidity and light intensity and observed for growth/survival.

Table 15: Composition of root regeneration medium having various concentrations of IAA

S. No.	Medium code	Medium composition
1.	I-RR ₁	MS basal + 0.05mg/l IAA
2.	I-RR ₂	MS basal + 0.10mg/l IAA
3.	I-RR ₃	MS basal + 0.20mg/l IAA

Table 16: Composition of root regeneration medium having different concentrations of NAA

S. No.	Medium code	Medium composition
1.	II-RR ₁	MS basal + 0.05mg/l NAA
2.	II-RR ₂	MS basal + 0.10mg/l NAA
3.	II-RR ₃	MS basal + 0.20mg/l NAA

Table 17: Composition of root regeneration medium having different concentrations of IBA

S. No.	Medium code	Medium composition
1.	III-RR ₁	MS basal + 0.05mg/l IBA
2.	III-RR ₂	MS basal + 0.10mg/l IBA
3.	III-RR ₃	MS basal + 0.20mg/l IBA

3.7 Kanamycin sensitivity in cultured tissues (leaf and petiole explants) of cabbage (*Brassica oleracea* L. var. *capitata* cv. *Pride of India*)

3.7.1 Preparation of normal shoot regeneration medium and selective shoot regeneration media for leaf and petiole explants

MS salts (macro and micro salts) and vitamins supplemented with 100mg/l meso-inositol, 3.0 % sucrose and 0.8 % agar-agar for solidification of medium were used as basal medium. The selective media for kanamycin sensitivity experiment was prepared by adding kanamycin (kanamac, Macleods Laboratory Pvt. Ltd., Mumbai, India) into each pre-sterilized

molten MS regeneration medium of cabbage cv. Pride of India, under aseptic conditions by filter sterilization through 0.22µm pore size, Whatman® membrane filter. Different concentrations (0, 10, 20, 30, 40, 50 and 60mg/l) of kanamycin was added into the medium to study the effect of antibiotic on the relative growth of cultured explants/tissues (leaf and petiole) of cabbage.

3.7.2 Inoculation of leaf and petiole explants on the normal and the selective media

The leaf and petiole explants were excised from glasshouse grown seedlings of cabbage, surface sterilized and cut into small pieces and weighed on an electronic balance under aseptic conditions in the laminar flow cabinet. The initial fresh weight of the explants was recorded. The explants were cultured on the normal regeneration media as control and on the selective regeneration media containing different concentrations of kanamycin (Table 18 and 19).

3.7.3 Morphological observations and measurement of relative growth of cultured tissues of leaf and petiole explants

Morphological changes were observed in leaf and petiole explants from 0 to 35 days in culture. Fresh weight of both the explants (leaf and petiole) was measured at the interval of 7 days, from 0 day to 35 days in culture. Relative growth at 7 days interval was calculated on the normal as well as on the selective media.

Table 18: Selective shoot regeneration media used for kanamycin sensitivity in leaf explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. *Pride of India*)

S.No.	Medium Code	Medium composition
1	DLK1	MS basal + 1.5mg/l BAP + 0.50mg/l NAA – without Kanamycin (control)
2	DLK2	MS basal + 1.5mg/l BAP + 0.50mg/l NAA + 10mg/l Kanamycin
3	DLK3	MS basal + 1.5mg/l BAP + 0.50mg/l NAA + 20mg/l Kanamycin
4	DLK4	MS basal + 1.5mg/l BAP + 0.50mg/l NAA + 30mg/l Kanamycin
5	DLK5	MS basal + 1.5mg/l BAP + 0.50mg/l NAA + 40mg/l Kanamycin
6	DLK6	MS basal + 1.5mg/l BAP + 0.50mg/l NAA + 50mg/l Kanamycin
7	DLK7	MS basal + 1.5mg/l BAP + 0.50mg/l NAA + 60mg/l Kanamycin

Table 19: Selective shoot regeneration media used for kanamycin sensitivity in petiole explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. *Pride of India*)

S.No.	Medium Code	Medium composition
1	BPK1	MS basal + 2.0mg/l Kn + 0.25mg/l NAA – without Kanamycin (control)
2	BPK2	MS basal + 2.0mg/l Kn + 0.25mg/l NAA + 10mg/l Kanamycin
3	BPK3	MS basal + 2.0mg/l Kn + 0.25mg/l NAA + 20mg/l Kanamycin
4	BPK4	MS basal + 2.0mg/l Kn + 0.25mg/l NAA + 30mg/l Kanamycin
5	BPK5	MS basal + 2.0mg/l Kn + 0.25mg/l NAA + 40mg/l Kanamycin
6	BPK6	MS basal + 2.0mg/l kn + 0.25mg/l NAA + 50mg/l Kanamycin
7	BPK7	MS basal + 2.0mg/l kn + 0.25mg/l NAA + 60mg/l Kanamycin

3.8 Effect of cefotaxime on the regeneration potential of cotyledon and hypocotyl tissues of cabbage (*Brassica oleracea* L. var. *capitata* cv. *Pride of India*) (without co-cultivation)

3.8.1 Preparation of selective shoot regeneration media for cotyledon and hypocotyl explants

The best shoot regeneration medium was selected from the plant regeneration studies in cabbage with respect to cotyledon and hypocotyl explants, respectively in which high frequency shoot regeneration was obtained. The selective shoot regeneration media were prepared by adding different concentrations of cefotaxime into pre-sterilized molten shoot regeneration media for cotyledon and hypocotyl explants under aseptic condition by filter sterilization. Different concentrations (100, 200, 300, 400 and 500mg/l) of cefotaxime were added into the media to study the effect of antibiotic on the regeneration potential of cotyledon and hypocotyl explants (Tables 20 and 21, respectively).

3.8.2 Inoculation of cotyledon and hypocotyl explants on the selective shoot regeneration media

The cotyledon and hypocotyl explants were excised from aseptically grown seedlings (seven to nine days old), cut into small pieces and cultured on selective shoot regeneration medium (MS medium + 2.0mg/l Kn + 0.50mg/l NAA) for cotyledon explants and (MS medium + 1.5mg/l Kn + 0.25mg/l IAA) for hypocotyl explants containing different concentrations of cefotaxime. The growth and differentiation of the explants were recorded.

3.8.3 Morphological observations and adventitious shoot regeneration

Morphological changes were observed in these tissues/explants at interval of seven days till shoot regeneration in culture. The effect of cefotaxime on growth and regeneration potential of cotyledon and hypocotyl tissue was recorded after one month.

Table 20: Selective shoot regeneration medium with different concentrations of cefotaxime used to study its effect on the regeneration potential of cotyledon tissues of cabbage (*Brassica oleracea* L. var. *capitata* cv. *Pride of India*)

S.No.	Medium Code	Medium composition
1	BCC1	MS basal + 2.0mg/l Kn + 0.50mg/l NAA – without Cefotaxime(control)
2	BCC2	MS basal + 2.0mg/l Kn + 0.50mg/l NAA + 100mg/l Cefotaxime
3	BCC3	MS basal + 2.0mg/l Kn + 0.50mg/l NAA + 200mg/l Cefotaxime
4	BCC4	MS basal + 2.0mg/l Kn + 0.50mg/l NAA + 300mg/l Cefotaxime
5	BCC5	MS basal + 2.0mg/l Kn + 0.50mg/l NAA + 400mg/l Cefotaxime
6	BCC6	MS basal + 2.0mg/l Kn + 0.50mg/l NAA + 500mg/l Cefotaxime

Table 21: Selective shoot regeneration medium with different concentrations of cefotaxime used to study its effect on the regeneration potential of hypocotyl tissues of cabbage (*Brassica oleracea* L. var. *capitata* cv. *Pride of India*)

S. No.	Medium Code	Medium composition
1	AHC1	MS basal + 1.5mg/l Kn + 0.25mg/l IAA – without Cefotaxime (control)
2	AHC2	MS basal + 1.5mg/l Kn + 0.25mg/l IAA + 100mg/l Cefotaxime
3	AHC3	MS basal + 1.5mg/l Kn + 0.25mg/l IAA + 200mg/l Cefotaxime
4	AHC4	MS basal + 1.5mg/l Kn + 0.25mg/l IAA + 300mg/l Cefotaxime
5	AHC5	MS basal + 1.5mg/l Kn + 0.25mg/l IAA + 400mg/l Cefotaxime
6	AHC6	MS basal + 1.5mg/l kn + 0.25mg/l IAA + 500mg/l Cefotaxime

3.9 Effect of cefotaxime and kanamycin on the growth of abgrobacterial cells and regeneration potential in cotyledon and hypocotyl tissues of cabbage (*Brassica oleracea* L. var. *capitata* cv. *Pride of India*)(after co-cultivation)

3.9.1 Preparation of selective shoot regeneration media for cotyledon and hypocotyl explants

The best shoot regeneration medium was selected from the plant regeneration studies in cabbage for cotyledon and hypocotyl explants, in which high frequency shoot regeneration was obtained. The selective media were prepared by adding cefotaxime and kanamycin into presterilized molten shoot regeneration medium under aseptic condition by filter sterilization through 0.22µm pore size Whatman® membrane filter. Different concentrations (0, 100, 200, 300, 400, 500mg/l) of cefotaxime with 50mg/l kanamycin were added into the medium to

study the effect of antibiotics on the growth of agrobacterial cells and regeneration potential of cotyledon and hypocotyl explants (Table 22 and 23, respectively).

3.9.2 Inoculation of cotyledon and hypocotyl explants on the selective shoot regeneration media

The cotyledon and hypocotyl explants were excised from aseptically grown seedlings (seven to nine days old) cut into small pieces and cultured on selective shoot regeneration medium (MS medium + 2.0mg/l Kn + 0.50mg/l NAA) for cotyledon explants and (MS medium + 1.5mg/l Kn + 0.25mg/l IAA) for hypocotyl explants containing different concentrations of cefotaxime with 50mg/l kanamycin.

3.9.3 Morphological observations and adventitious shoot regeneration

Morphological changes were observed in cotyledon and hypocotyl explants after 30 days in culture. The effect of cefotaxime and kanamycin on the growth of agrobacterial cells and plant cells were recorded till shoot regeneration.

3.10 Genetic transformation in cabbage (*Brassica oleracea* L. var. *capitata* cv. **Pride of India**)

Genetic transformation studies in cabbage were carried out by using *Agrobacterium*-mediated gene transfer technique.

Table 22: Selective shoot regeneration medium containing different concentrations of cefotaxime and kanamycin (50mg/l) used to study its effect on the regeneration potential of cotyledon tissues in cabbage (*Brassica oleracea* L. var. *capitata* cv. **Pride of India) after co-cultivation**

S.No.	Medium composition
1	MS basal + 2.0mg/l Kn + 0.50mg/l NAA + 0mg/l cefotaxime(control) + 50mg/l kanamycin
2	MS basal + 2.0mg/l Kn + 0.50mg/l NAA + 50mg/l Kanamycin + 100mg/l Cefotaxime
3	MS basal + 2.0mg/l Kn + 0.50mg/l NAA + 50mg/l Kanamycin + 200mg/l Cefotaxime
4	MS basal + 2.0mg/l Kn + 0.50mg/l NAA + 50mg/l Kanamycin + 300mg/l Cefotaxime
5	MS basal + 2.0mg/l Kn + 0.50mg/l NAA + 50mg/l Kanamycin + 400mg/l Cefotaxime
6	MS basal + 2.0mg/l Kn + 0.50mg/l NAA + 50mg/l Kanamycin + 500mg/l Cefotaxime

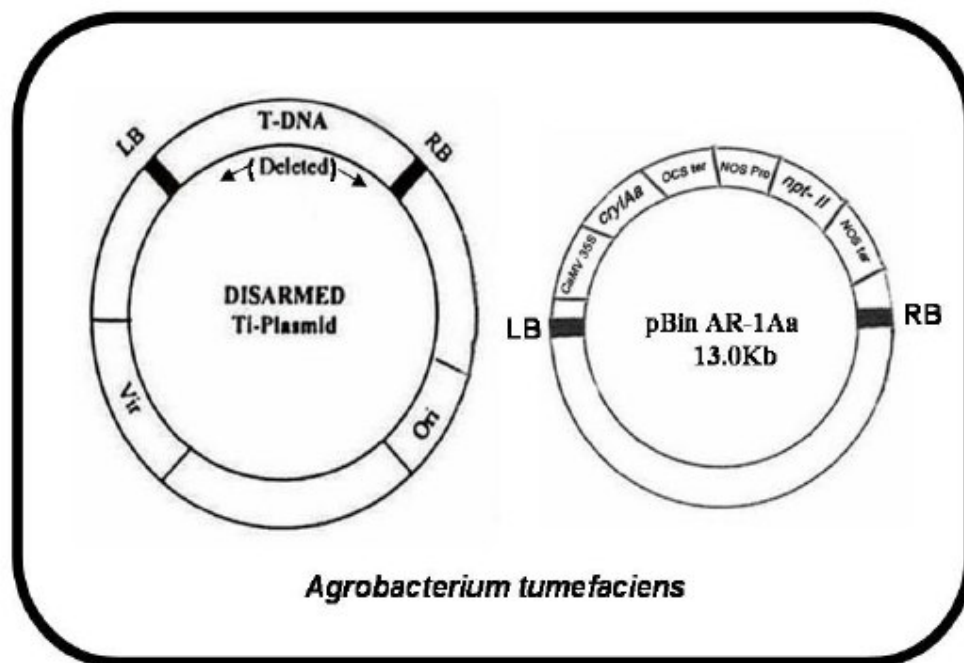


Fig. 2: The outline diagram of disarmed Ti-plasmid and binary vector pBin AR-1Aa containing *cryIAa* and *npt-II* genes in *Agrobacterium tumefaciens* strain. This strain was used for genetic transformation of cabbage (*Brassica oleracea* L. var. *capitata* cv. *Pride of India*).

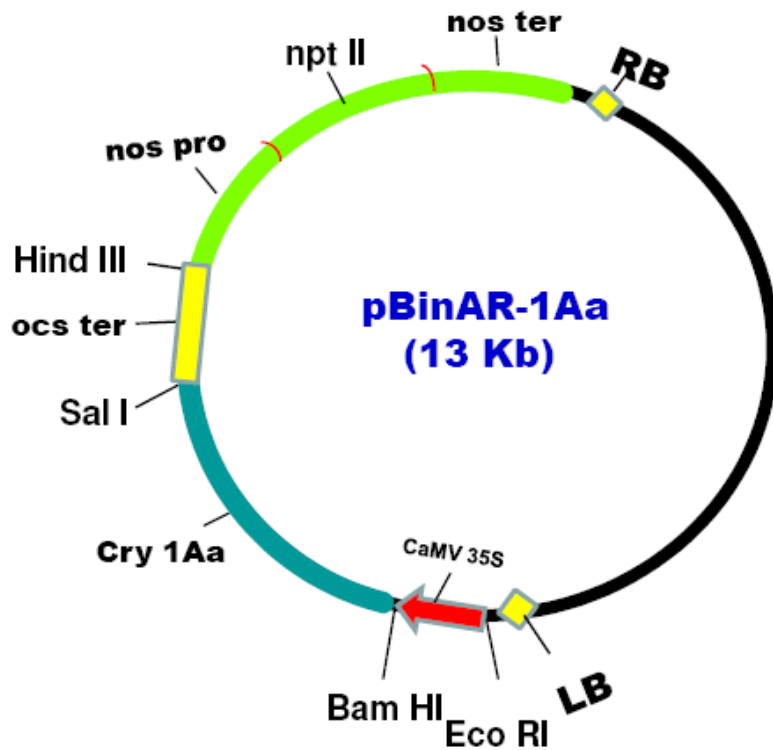


Fig. 3a: The detailed diagram of binary vector pBin AR-1Aa containing *cryIAa* (insect resistance) gene along with *npt-II* (kanamycin resistance) gene for selection in both bacteria and plant.



Fig. 3b: Schematic diagram of gene construct: T-DNA of pBinAR-1Aa containing transcriptional fusion of CaMV 35S promoter with the coding region of *cryIAa* gene and NOS promoter with the coding region of *npt-II* gene.

LB	-	Left border of T-DNA
RB	-	Right border of T-DNA
35S	-	CaMV 35S promoter
NOS ter	-	Nopaline synthase terminator
NOS pro	-	Nopaline synthase promoter
<i>npt-II</i>	-	Neomycin phosphotransferase-II
<i>cryIAa</i>	-	Insect resistance gene
OCS ter	-	Octopine synthase terminator

Table 23: Selective shoot regeneration medium containing different concentrations of cefotaxime and kanamycin (50mg/l) used to study its effect on the regeneration potential of hypocotyl tissues in cabbage (*Brassica oleracea* L. var. *capitata* cv. *Pride of India*) after co-cultivation

S.No.	Medium composition
1	MS basal + 1.5mg/l Kn + 0.25mg/l IAA + 50mg/l Kanamycin + 0mg/l Cefotaxime
2	MS basal + 1.5mg/l Kn + 0.25mg/l IAA + 50mg/l Kanamycin + 100mg/l Cefotaxime
3	MS basal + 1.5mg/l Kn + 0.25mg/l IAA + 50mg/l Kanamycin + 200mg/l Cefotaxime
4	MS basal + 1.5mg/l Kn + 0.25mg/l IAA + 50mg/l Kanamycin+ 300mg/l Cefotaxime
5	MS basal + 1.5mg/l Kn + 0.25mg/l IAA + 50mg/l Kanamycin + 400mg/l Cefotaxime
6	MS basal + 1.5mg/l kn + 0.25mg/l IAA + 50mg/l Kanamycin + 500mg/l Cefotaxime

3.10.1 Permission from Institutional Biosafety Committee (IBSC) of our University to transfer insect resistance gene (*cryIAa*) into cabbage

The permission to transfer insect resistance (*cryIAa*) gene into cabbage and their testing under laboratory and greenhouse conditions was obtained from Institutional Biosafety Committee (IBSC) of Dr Y.S. Parmar University of Horticulture and Forestry, Nauni-Solan. All the instructions of “Recombinant DNA Biosafety Guidelines” of the Department of Biotechnology, Govt. of India, New Delhi and Revised Guidelines for “Research in Transgenic Plants” were strictly followed.

3.10.2 Development of putative transgenic plantlets from cotyledon and hypocotyl explants

Development of transgenic plantlets was carried out as follows:

3.10.2.1 *Agrobacterium* strain and plasmid used

Genetically engineered disarmed *Agrobacterium tumefaciens* strain containing binary vector pBin-1Aa with *cryIAa* (insect resistance gene) and *npt-II* (kanamycin resistance gene) genes for selection in both bacteria and plant was used for co-cultivation experiment to transfer *cryIAa* and *npt-II* genes in cabbage. The *Agrobacterium tumefaciens* strain containing *cryIAa* and *npt-II* genes in binary vector was obtained from National Research Centre for Plant Biotechnology, IARI, PUSA, New Delhi (Fig. 2, 3a and 3b).

3.10.2.2 Maintenance of *Agrobacterium* strain

Agrobacterium strain was maintained by subculturing bacterial colonies on the selective YMB medium containing 50mg/l kanamycin. After proper growth of *Agrobacterium* the plates/slants were kept at low temperature (4⁰C) for storage.

3.10.2.3 Preparation of fresh cultures of *Agrobacterium* strain

Overnight fresh cultures of *Agrobacterium* strain were prepared by inoculating small colony into the 10 ml selective YMB broth. After inoculation the test tubes were kept at 28⁰C on orbital shaking incubator for proper growth. *Agrobacterium* suspension having 10⁸ cells /ml had an OD of 0.521 at 540 nm which was fixed and used for genetic transformation experiment.

3.10.2.4 Preculturing of cotyledon and hypocotyl explants

The best shoot regeneration medium was selected from the plant regeneration studies in cabbage with respect to the respective explants i.e. cotyledon and hypocotyl, in which highest frequency shoot regeneration was obtained. The explants (cotyledon and hypocotyl) were cut into small pieces of 0.5-1.0cm and inoculated on shoot regeneration media, respectively to study the effect of preincubation/preculturing on transformation frequency. The explants were pre-incubated for 24, 48 and 72 hours and then subsequently co-cultivated with fresh cultures of *agrobacterium* cells.

3.10.2.5 Co-cultivation of cotyledon and hypocotyl explants

Fresh cultures of the *Agrobacterium* strain were centrifuged for 10 minutes at 5000 rpm and supernatant was discarded. The pellet was resuspended in liquid MS basal medium to get the concentration of 10⁸cells/ml. The pre-incubated explants were immersed in bacterial suspension for 15-20 seconds and blotted on pre-sterilized filter paper and then inoculated on the respective shoot regeneration medium for co-cultivation. The pre-incubated explants were co-cultivated at different intervals of i.e. 48, 72 and 96 hours to study the effect of co-cultivation on transformation frequency.

3.10.2.6 Effect of acetosyringone on shoot regeneration and transformation frequency from cotyledon and hypocotyl explants in cabbage

a) Preparation of selective shoot regeneration medium supplemented with different concentrations of acetosyringone

To study effect of acetosyringone on per cent shoot regeneration of putative transformants, the best shoot regeneration medium for cotyledon and hypocotyl explants was

supplemented with different concentrations (50, 75, 100, 125 and 150 μ M) of acetosyringone. The acetosyringone was added by filter sterilization into the molten shoot regeneration media (Table 24, 25, 26 and 27).

b) Inoculation of cotyledon and hypocotyl explants

The cotyledon and hypocotyl explants were excised from aseptically grown seedlings (seven to nine days old) cut into small pieces and cultured on selective shoot regeneration medium for cotyledon and hypocotyl explants containing different concentrations of acetosyringone. The explants were pre-incubated and co-cultivated for standardized time interval to study effect of acetosyringone on transformation frequency.

Table 24: Effect of different concentrations of acetosyringone on shoot regeneration and transformation frequency from cotyledon explants in cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India)

S. No.	Medium Code	Medium composition
1	BCA1	MS basal + 2.0mg/l Kn + 0.50mg/l NAA + 50 μ M Acetosyringone
2	BCA2	MS basal + 2.0mg/l Kn + 0.50mg/l NAA + 75 μ M Acetosyringone
3	BCA3	MS basal + 2.0mg/l Kn + 0.50mg/l NAA + 100 μ M Acetosyringone
4	BCA4	MS basal + 2.0mg/l Kn + 0.50mg/l NAA + 125 μ M Acetosyringone
5	BCA5	MS basal + 2.0mg/l Kn + 0.50mg/l NAA + 150 μ M Acetosyringone

Table 25: Effect of different concentrations of acetosyringone on shoot regeneration and transformation frequency from cotyledon explants in cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India)

S. No.	Medium Code	Medium composition
1	GCA1	MS basal + 0.330mg/l TDZ and 79.7mg/l Adenine + 50 μ M Acetosyringone
2	GCA2	MS basal + 0.330mg/l TDZ and 79.7mg/l Adenine + 75 μ M Acetosyringone
3	GCA3	MS basal + 0.330mg/l TDZ and 79.7mg/l Adenine + 100 μ M Acetosyringone
4	GCA4	MS basal + 0.330mg/l TDZ and 79.7mg/l Adenine + 125 μ M Acetosyringone
5	GCA5	MS basal + 0.330mg/l TDZ and 79.7mg/l Adenine + 150 μ M Acetosyringone

Table 26: Effect of different concentrations of acetosyringone on shoot regeneration and transformation frequency from hypocotyl explants in cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India)

S.No.	Medium Code	Medium composition
1	AHA1	MS basal + 1.5mg/l Kn + 0.25mg/l IAA + 50µM Acetosyringone
2	AHA2	MS basal + 1.5mg/l Kn + 0.25mg/l IAA + 75µM Acetosyringone
3	AHA3	MS basal + 1.5mg/l Kn + 0.25mg/l IAA + 100µM Acetosyringone
4	AHA4	MS basal + 1.5mg/l Kn + 0.25mg/l IAA + 125µM Acetosyringone
5	AHA5	MS basal + 1.5mg/l Kn + 0.25mg/l IAA + 150µM Acetosyringone

Table 27: Effect of different concentrations of acetosyringone on shoot regeneration and transformation frequency from hypocotyl explants in cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India)

S.No.	Medium Code	Medium composition
1	HHA1	MS basal + 0.220mg/l TDZ and 0.088mg/l IAA + 50µM Acetosyringone
2	HHA2	MS basal + 0.220mg/l TDZ and 0.088mg/l IAA + 75µM Acetosyringone
3	HHA3	MS basal + 0.220mg/l TDZ and 0.088mg/l IAA + 100µM Acetosyringone
4	HHA4	MS basal + 0.220mg/l TDZ and 0.088mg/l IAA + 125µM Acetosyringone
5	HHA5	MS basal + 0.220mg/l TDZ and 0.088mg/l IAA + 150µM Acetosyringone

3.10.2.7 Selection of transformed cells on selective media

a) Preparation of selective shoot regeneration media

For the preparation of selective shoot regeneration media kanamycin (Kanamac, Macleods Laboratory Pvt. Ltd., Mumbai, India) and cefotaxime (Stancef, Ranbaxy Laboratory Ltd., India) were added at concentration of 50mg/l and 400mg/l, respectively, by filter sterilization into the molten shoot regeneration media (Table 28).

b) Transfer of cotyledon and hypocotyl explants on the selective shoot regeneration media after co-cultivation

After co-cultivation the cotyledon and hypocotyl explants were transferred to the fresh selective shoot regeneration media, respectively for selection of transformed cells and inhibition of further agrobacterial growth. The cultures were kept in a culture room at $26\pm 2^{\circ}\text{C}$ for further growth and differentiation. The explants were subsequently sub-cultured on the fresh selective shoot regeneration media in order to check excessive agrobacterial growth.

Table 28: Selective shoot regeneration media for cotyledon and hypocotyl explants used for genetic transformation studies

S. No.	Explants	Medium code	Medium composition
1.	Cotyledon	GTC1	MS basal + 2.0mg/l Kn + 0.50mg/l NAA+ 50mg/l Kanamycin + 400mg/l Cefotaxime
2.	Cotyledon	GTC2	MS basal + 0.330mg/l TDZ + 79.7mg/l Adenine + 50mg/l Kanamycin + 400mg/l Cefotaxime
3.	Hypocotyl	GTH1	MS basal + 1.5mg/l Kn + 0.25mg/l IAA + 50mg/l Kanamycin + 400mg/l Cefotaxime
4.	Hypocotyl	GTH2	MS basal + 0.220mg/l TDZ + 0.088mg/l IAA + 50mg/l Kanamycin + 400mg/l Cefotaxime

c) Regeneration of putative transgenic shoots from cotyledon and hypocotyl explants

The explants (cotyledon and hypocotyl) which were able to survive on the selective shoot regeneration media had undergone slight callusing and adventitious bud/shoot formation. Percentage shoot regeneration was recorded in all the experiments to find out the effect of pre-incubation, co-cultivation and acetosyringone on the transformation frequency.

d) Root regeneration from *in vitro* developed putative transgenic shoots and development of complete plantlets

Regenerated putative transgenic shoots which were obtained from both the explants were transferred to the selective root regeneration medium (MS basal + 0.10 mg/l IBA + 50mg/l kanamycin + 400mg/l cefotaxime) for root induction to get complete plantlets.

3.10.2.8 Hardening of *in vitro* developed putative transgenic plantlets

In vitro regenerated transgenic plantlets were transferred to sterilized cocopeat for hardening. The survival and establishment of the plantlets were studied after transplanting the plantlets in potting mixture.

3.11 Molecular analyses of putative transgenic shoots / plantlets of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India)

3.11.1 Confirmation of presence/integration of the transgenes i.e. *cryIAa* (insect resistance gene) and *npt-II* (kanamycin resistance gene) into the genome of cabbage by PCR analysis using designed primers

3.11.1.1 Isolation of plasmid DNA

Plasmid DNA was isolated by using Genei Ultrapure Plasmid Mini Purification Kit

Growth of bacterial cells

1. The bacterial cells were grown and harvested from 10ml of YMB culture medium by centrifugation at 10,000 rpm for 15 minutes at 4⁰C.

Cell lysis

2. Resuspended the bacterial pellet in 0.8ml of solution I with RNase A.
3. Added 0.8ml solution II to the suspension. Mixed gently by inverting the tube 6-8 times. Incubated the mixture at room temperature for 5 minutes.
4. Added pre- chilled 0.8ml solution III to the suspension. Immediately mixed the lysate gently by inverting the tubes 6-8 times until a homogenous suspension was formed. Incubated the suspension on ice for 5 minutes.

Equilibration of the column

5. Added 1ml of equilibration buffer to the column.

Clarification of the lysate

6. The bacterial lysate was clarified by centrifugation of the suspension at 12000 rpm for 20 minutes at 4⁰C.

Binding

7. Loaded the clarified lysate from step 6 onto the column. Allowed the column to empty by gravity flow.

Washing

8. Washed the column twice with 2ml wash buffer

Elution

9. Eluted the plasmid DNA with 1ml elution buffer.

Precipitation

10. Added 0.75ml of isopropanol to precipitate the eluted plasmid DNA. Mixed carefully and centrifuged at 12,000 rpm for 30 minutes at room temperature. Carefully discarded the supernatant.

Wash and dry DNA pellet

11. Added 1ml of 70 % ethanol to the pellet. Mixed and centrifuged at 12,000 rpm for 10 minutes at room temperature. Carefully discarded the supernatant.

12. Repeated step 11 to ensure complete removal of residual salt. After decanting air dried the pellet for 5-10 minutes.

Reconstitute DNA

13. Redissolved the DNA pellet in 50µl 1X TE buffer.

3.11.1.2 Isolation and purification of genomic DNA from putative transgenic shoots/ plantlets and control plantlets (non-transformed) of cabbage

The leaves of putative transgenic and non-transgenic (control) cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India) were taken for the isolation of genomic DNA by modified CTAB method (Doyle and Doyle, 1990).

i) Solutions used for DNA extraction

1. 10% (w/v) CTAB (N-cetyl N,N,N-trimethyl ammonium bromide)
2. 0.5 M EDTA (pH 8.0)
3. 5 M NaCl
4. 1 M Tris-HCl
5. Chloroform : isoamyl alcohol (24:1)
6. Isopropanol
7. 70% Ethanol

ii) DNA extraction buffer

The DNA extraction buffer contained 100mM Tris base (pH 8.0), 20mM EDTA (pH 8.0), 1.4M NaCl, 2.0 % (w/v) CTAB and 0.2 % (v/v) 2-mercaptoethanol.

Procedure

1. Approximately 1.0g of tissue was ground to a fine power in liquid nitrogen in a pre-chilled pestle and mortar.
2. The powdered tissue was transferred to centrifuge tubes containing 7.0ml of DNA extraction buffer (prewarmed at 65⁰C). This was mixed by gentle inversion and incubated at 65⁰C for 1 hour.
3. Added 7ml (7 volume) chloroform: isoamyl alcohol (24:1, v/v) and mixed by inversion to emulsify.
4. Spun at 10,000 rpm for 10 minutes at room temperature.

5. The aqueous phase was pipetted out gently without disturbing the interphase to another pre-sterilized eppendorf tube. Added 2/3rd volume of isopropanol and kept it overnight.
6. The precipitated DNA was spun down to form a pellet.
7. DNA pellet was washed with 70 % ethanol and spun at 10,000 rpm for 5-10 minutes.
8. DNA pellet was dried in vacuum dry oven at 37⁰C for 10-15 minutes.
9. DNA pellet was dissolved in Autoclaved Distilled Water

iii) Reagents of DNA purification

1. RNase
2. Phenol: chloroform: isoamyl alcohol (25:24:1,v/v/v)
3. Chloroform: isoamyl alcohol (24:1,v/v)
4. 3.0M sodium acetate (pH 5.2)
5. Absolute ethanol (95%)
6. 70 % ethanol

Procedure

1. 2µl of RNase (10mg/ml) was added, mixed gently and incubated for 1 hour in a heating water bath at 37⁰C
2. Added equal volume of phenol: chloroform: isoamyl alcohol (25:24:1,v/v/v) and mixed thoroughly to emulsify.
3. The resultant solution was spun at 10,000 rpm for 10 minutes and upper aqueous phase was taken out carefully.
4. Added 1/10th volume of 3M sodium acetate (pH 5.2) and mixed thoroughly. Now two volumes of absolute ethanol (95 %) were added, mixed by gentle inversion and kept in ice for 20 minutes.
5. Spun at 10,000 rpm for 10 minutes to pellet the DNA.
6. The DNA pellet was washed with 70 % ethanol.
7. DNA pellet was dried and dissolved in 50µl Autoclaved Distilled Water

iv) Examining the quality of DNA

The DNA was electrophoresed at 0.8 % agarose gel by employing 80 volts current to examine the quality of DNA. The approximate purity of double standard DNA was estimated by determination of the ratio of absorbance at 260 and 280 nm (A_{260}/A_{280}). This ratio is 1.8

for pure double standard DNA. A_{260}/A_{280} ratio greater than 1.8 suggests RNA contamination, whereas one less than 1.8 suggest protein in the sample. The sample deviating considerably from this value was subjected to another cycle of DNA purification.

3.11.1.3 Quantification of DNA

DNA in the sample was quantified by taking absorbance at 260 nm and then equating it with the thumb rule that if absorbance at 260 nm is one then concentration of DNA in the sample must be equal to 50µg/ml. After quantifying DNA each sample was diluted with Autoclaved Distilled Water to bring the concentration to 25µg/µl.

3.11.1.4 Amplification of *cryIAa* and *npt-II* genes by polymerase chain reaction using designed primers

DNA (the gene) amplification of the cabbage transformants for molecular analysis was conducted using two specific designed primers (one forward primer, one reverse primer) (Table 29) for *cryIAa* and *npt-II* gene. The polymerase chain reactions were performed in a Thermocycler (BioRad, USA).

Table 29: Specific designed primers used for the amplification of *cryIAa* and *npt-II* genes in the genome of transgenic cabbage plantlets

Sequence of designed primers (forward and reverse) for <i>cryIAa</i> gene		
1.	Forward primer	5'GGGATGGCTAACAACCCAAAC 3'
2.	Reverse primer	5'GGCAAACCTCTGGTCCAGAGAGAAACC 3'
Sequence of designed primers (forward and reverse) for <i>npt-II</i> gene		
1.	Forward primer	5'TTGAACAAGATGGATTGCAC3'
2.	Reverse primer	5'GATGACAGGAGATCCTGCCC3'

a) Master mix used for *cryIAa* and *npt-II* genes amplification:

1.	Autoclaved double distilled water	:	15.7µl
2.	PCR buffer – 10X	:	2.5µl
3.	dNTPs – 1.25 mM each	:	1.5µl
5.	Forward primer – 25 pico moles (Table 29)	:	0.5µl
6.	Reverse primer – 25 pico moles (Table 29)	:	0.5µl
7.	Taq DNA polymerase – 1 unit	:	0.3µl
8.	DNA sample – 50 µg	:	4.0µl

b) *In vitro* amplification of *cryIAa* and *npt-II* gene using PCR

The reaction mixture prepared was mixed gently and dispensed 21µl of each to autoclaved PCR tubes. 4µl DNA was added separately to each PCR tube to make a final reaction mixture of 25µl. The following thermocycler programme was used for the amplification of *cryIAa* and *npt-II* gene.

Stage	Temperature	Time	Cycle(s)
1. Initial denaturation	94°C	4 mins	1
2. a) Denaturation	94°C	1 min	35
b) Annealing	50°C	1 min 30 sec	
c) Extension	72°C	2 mins	
3. Final extension	72°C	4 mins	1

Temperature profile

PCR was carried out with a total of 35 cycles. Initial denaturation was carried out for 4 minutes at 94°C. Further each cycle consisted of denaturation of 1 minute at 94°C, annealing of 1 minute and 30 seconds at 50°C and extension of 2 minutes at 72°C and a final extension of 4 minutes at 72°C.

3.11.1.5 Gel electrophoresis of amplified product

(i) The solutions for agarose gel electrophoresis

1. Ethidium bromide
2. 6X loading dye
3. 50X Tris acetate EDTA Buffer
4. Agarose gel (1.2%)

ii) Running conditions

Amplified DNA was electrophoresed in 1.2 % agarose gel under submerged conditions. 1X TAE buffer was used as gel tray buffer. Before loading the samples in the wells, 2µl of loading dye was added to each sample. About 8-10µl of sample was loaded into each well and amplified DNA was fractioned at 80 volts constant current till tracking dye reaches the other end. High range DNA ladder (Genei, Bangalore) was loaded in the wells along with the negative control.

3.11.1.6 Viewing the gel and photography

The amplified DNA products in the gel were viewed under UV light with the help of transilluminator and the gel was photographed by Alpha Imager[®] EC gel documentation

system (U.S.A). The images were stored in the computer software (Alpha imager) for further studies.

The plant which showed amplified DNA in PCR were marked as positive transgenic plantlets, whereas, the samples not showing amplified DNA for a particular gene i.e. *cryIAa* and *npt-II* were marked as non-transformed plantlets.

Plasmid DNA containing *npt-II* and *cryIAa* genes was used as +ve control and genomic DNA of *in vitro* regenerated plantlets of cabbage used as -ve control were also amplified by polymerase chain reaction.

3.11.2 Confirmation of integration of transgene i.e. *cryIAa* (insect resistance gene) into the genome of cabbage using Southern hybridization

3.11.2.1 Isolation of plasmid DNA

Plasmid DNA was isolated from recombinant *Agrobacterium*-strain by using Genei Ultrapure Plasmid Mini Purification Kit.

3.11.2.2 Radio-labelling of DNA probe

1. Isolated plasmid DNA was used for the preparation of radio labeled DNA probe.
2. Plasmid DNA was restricted with restriction enzyme Bam HI and Sal I in heating water bath at 37⁰C for 1 hour.
3. After restriction digestion the plasmid DNA was electrophoresed on 0.8 % agarose gel and the specific fragment of *cryIAa* gene (1.8Kb) was eluted for labeling of DNA probe.
4. Added the following component into eppendorf tube:

DNA template (probe)	:	100 ng
Decanucleotide in 5X reaction buffer	:	10 µl
Water (nuclease free)	:	40 µl
5. Vortexed the tube and microcentrifuged for 3-5 seconds.
6. The tube was incubated in boiling water bath for 5-10 minutes and cooled on ice.
7. Added the following components into the same tube:

Mix C-CTP	:	3 µl
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α P ³² dCTP	:	6 μ l
Klenow fragment, exo (5U)	:	1 μ l

8. Vortexed the tube and microcentrifuged and tubes were incubated at 37⁰C for 5 minutes.
9. 4 μ l of dNTP mix was added and incubated for 5 minutes at 37⁰C.
10. The reaction was stopped by addition of 1 μ l of 0.5M EDTA (pH 8.0). After adding EDTA the reaction was kept in boiling water bath for 10 minutes and cooled on ice.
11. Now probe was radiolabelled and used for hybridization.

3.11.2.3 Isolation and purification of genomic DNA from putative transgenic and control shoots/plantlets of cabbage

The leaves of putative transgenic and non-transgenic (control) cabbage (*Brassica oleracea* L. var *capitata* cv. Pride of India) were taken for the isolation of genomic DNA by modified CTAB method (Doyle and Doyle, 1990).

3.11.2.4 Southern Blotting and Autoradiography

For Southern blotting isolated genomic DNA from PCR +ve transgenic plantlets of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India) were used.

i) Solutions Used

1. 0.25N HCl
2. 5M NaCl
3. 5M NaOH
4. Neutralization Solution
5. 20X SSC Buffer
6. Pre-Hybridization Buffer
7. Hybridization Buffer

ii) Procedure

1. High molecular weight genomic DNA of cabbage was cut into smaller fragments by Bam HI Restriction enzyme.
2. Then DNA fragments were electrophoresed on an agarose gel of 0.8 % to separate them according to size.

3. Prior to blotting, the gel was treated with 0.25N HCl for 15 to 20 minutes, which depurinates the DNA fragments, breaking the DNA into smaller pieces, thus allowing more efficient transfer from the gel to membrane.
4. After depurination, the gel was washed 2-3 times with Double Distilled Water. Then gel was placed into an alkaline solution (5M NaCl and 5M NaOH) to denature the double-stranded DNA for 1 hour.
5. After denaturation, the gel was placed in neutralization solution for 1 hour and gel was kept for blotting action.
6. For blotting the gel was kept in contact with 20X SSC buffer for 16–18 hours. A sheet of nylon membrane was placed on top of the gel. Pressure was applied evenly to the gel (either using suction, or by placing a stack of paper towels and a weight on top of the membrane and gel), to ensure good and even contact between gel and membrane. Buffer transfer by capillary action from a region of high water potential to a region of low water potential.
7. After blotting the nylon membrane was taken out and washed with 2X SSC buffer for 15-20 minutes and dried on whatmann filter paper.
8. After drying, membrane was exposed to ultraviolet radiation to permanently attach the transferred DNA to the membrane.
9. The membrane was then kept in hybridization bottle containing pre-hybridization buffer in hybridization oven at 65°C for 2 to 3 hours. Then pre-hybridization buffer was replaced by hybridization buffer containing radiolabelled DNA probe and incubate the membrane for 16-18 hours at 65°C.
10. After hybridization, excess probe was washed from the membrane and the pattern of hybridization was visualized on X-ray film by autoradiography.

The southern Hybridization experiment was carried out at NRCPB, IARI, New Delhi.

3.11.3 Confirmation of expression of the transgenes i.e. *cryIAa* (insect resistance gene) and *npt-II* (kanamycin resistance gene) in the transgenic cabbage by using reverse transcriptase-polymerase chain reaction (RT-PCR)

3.11.3.1 Isolation of total RNA from PCR positive transgenic shoots/plantlets of cabbage

Intact RNA was isolated from PCR positive transgenic shoots/plantlets of cabbage by using NucleoSpin Total RNA isolation kit.

a) Homegenization

1. 100mg (net weight) leaves were taken from the PCR positive plantlets
2. Placed the tissue in a pre-chilled autoclaved mortar and pestle. Added small volumes of liquid nitrogen and grinded the sample to a fine powder with intermittent mixing.

b) Cell lysis

3. Added 350 μ l lysis buffer RA1 and 3.5 μ l of β -mercaptoethanol to the ground tissue. With the help of pestle, mix the sample thoroughly to make a homogeneous lysate. Allowed the sample to thaw with intermittent grinding. Transferred the lysate in a fresh 1.5ml vial.

c) Filtration of the Cell Lysate

4. To reduce the viscosity of the lysate, passed it through the NucleoSpin Filter (Violet ring) placed in a new 2ml collection tube, and centrifuged for 1 minute at 10,000 rpm at room temperature.
5. Discarded the column and transferred the flow through to a new 1.5ml vial.

d) RNA Binding

6. Added 350 μ l of ethanol (70 %) to the flow through. Mixed immediately by repeated pipetting.
7. Transferred the lysate, including any precipitate that may have formed to the NucleoSpin RNA column (light blue ring) placed in a 2ml collection tube. Closed the lid gently and centrifuged for 30 seconds at 11,000 rpm at room temperature. Discarded the flow through.

e) Desalting Silica Membrane

8. Added 350 μ l of MBD (Membrane Desalting Buffer) to the NucleoSpin RNA column. Closed the lid gently and centrifuged the tube at 11,000 rpm for 1 minute to dry the column membrane. Discarded the flow through.

f) Digest DNA

9. Added 95 μ l of DNase reaction mixture directly onto the centre of the silica membrane of the RNA column. Incubated at room temperature for 15 minutes.

g) Wash and dry silica membrane

10. Added 200µl of Buffer RA2 to the NucleoSpin RNA plant column. Centrifuged for 30 seconds at 11,000 rpm at room temperature to clean the remaining salt bound to the silica column. Discarded the flow through.
11. After centrifugation, removed the column carefully from collection tube so that the column does not contact the flow through. Placed the column in a new 2 ml collection tube.
12. Added 600µl of buffer RA3 to the NucleoSpin RNA plant column. Centrifuged for 30 seconds at 11,000 rpm. Discarded the flow through and placed the column back into the collection tube.
13. Added 250µl of buffer RA3 to the NucleoSpin RNA plant column. Centrifuged for 2 minutes at 11,000 rpm to dry the membrane completely.

h) Elution of RNA

14. Placed the NucleoSpin RNA column into a nuclease free 1.5ml vial. To elute highly pure RNA, added 60µl of RNase-free water at the center of the membrane. Centrifuged the column at 11,000 rpm for 1 minute. Stored the eluted RNA at -70°C as small aliquots.

3.11.3.2 cDNA synthesis by RT-PCR

cDNA synthesis was carried out by using AuPreP™ Gold cDNA Synthesis Kit.

Procedure

1. Thaw each tube in the AuPreP™ Gold cDNA Synthesis Kit and placed on ice.
2. Briefly centrifuge all the reagents and returned them on ice
3. Mixed the following component in a nuclease-free microcentrifuge tube on ice.

Total RNA sample	7 µl
Oligo (dT) Primer (40 µM)	1 µl
DEPC Treated Water	2 µl

3. Mixed all the component gently and heated the mixture at 70°C for 5 minutes. Removed the tubes and placed them rapidly on ice.

4. Added the following components to the reaction tube:

Reaction Mix (2.5X)	8 μ l
DTT (100mM)	2 μ l
5. Now incubated the tubes in heating water bath at 42⁰C for 1 hour.
6. After 1 hour cDNA synthesized and the tubes were stored at -20⁰C.

3.11.3.3 Amplification of cDNA by using specific designed primers

DNA (the gene) amplification of the cabbage transformants for molecular analysis was conducted using two specific designed primers (one forward primer, one reverse primer) (Table 29) for *cryIAa* and *npt-II* genes. The polymerase chain reactions were performed in a Thermocycler (BioRad, USA).

a) Master mix used for *cryIAa* and *npt-II* genes amplification:

- | | | | |
|----|---|---|--------------|
| 1. | Autoclaved double distilled water | : | 15.7 μ l |
| 2. | PCR buffer – 10X | : | 2.5 μ l |
| 3. | dNTPs – 1.25 mM each | : | 1.5 μ l |
| 5. | Forward primer – 25 pico moles (Table 21) | : | 0.5 μ l |
| 6. | Reverse primer – 25 pico moles (Table 21) | : | 0.5 μ l |
| 7. | Taq DNA polymerase – 1 unit | : | 0.3 μ l |
| 9. | cDNA | : | 4.0 μ l |

b) *In vitro* amplification of *cryIAa* and *npt-II* gene using PCR

All the components mentioned above, were mixed gently and dispensed 21 μ l of reaction mixture to each autoclaved PCR tubes. 4 μ l cDNA was added separately to each PCR tube to make a final reaction mixture of 25 μ l. The following thermocycler programme was used for the amplification of *cryIAa* and *npt-II* genes.

Stage	Temperature	Time	Cycle(s)
1. Initial denaturation	94 ⁰ C	4 mins	1
2. a) Denaturation	94 ⁰ C	1 min	35
b) Annealing	50 ⁰ C	1 min 30 sec	
c) Extension	72 ⁰ C	2 mins	
3. Final extension	72 ⁰ C	4 mins	1

Temperature profile

PCR was carried out with a total of 35 cycles. Initial denaturation was carried out for 4 minutes at 94°C. Further each cycle consisted of denaturation of 1 minute at 94°C, annealing of 1 minute and 30 seconds at 50°C and extension of 2 minutes at 72°C and a final extension of 4 minutes at 72°C.

3.11.3.4 Gel electrophoresis of amplified cDNA product i.e. *cryIAa* and *npt-II*

Amplified DNA was electrophoresed in 1.2% agarose gel under submerged conditions. 1X TAE buffer was used as gel tray buffer. Before loading the samples on the well and amplified DNA was fractioned at 80 volts constant current till tracking dye reaches the other end. Step up 1Kb DNA ladder was loaded in the wells along with the negative control.

3.11.3.5 Viewing the gel and photography

The amplified DNA products (by RT-PCR) in the gel were viewed under UV with the help of transilluminator and the gel was photographed by Alpha Imager[®] EC gel documentation system (U.S.A).

The plantlets which showed amplified cDNA *cryIAa* (the insect resistance gene) and *npt-II* (kanamycin resistance gene) in RT-PCR were marked as positive transgenic plantlets expressing the genes, whereas the samples not showing amplified DNA for a particular *cryIAa* and *npt-II* genes were marked as transgenic plantlets not expressing the (integrated / presence of) genes at transcriptional level.

3.11.4 Confirmation of expression of transgene i.e. *cryIAa* (insect resistance gene) in the transgenic cabbage by using Real Time polymerase chain reaction (Real Time-PCR) technique

3.11.4.1 Isolation of total RNA

Intact RNA was isolated from PCR positive transgenic shoots/plantlets of cabbage by NucleoSpin Total RNA isolation kit.

3.11.4.2 cDNA synthesis by RealTime-PCR and amplification of cDNA by using SYBER green system

cDNA synthesis was carried out by AuPreP[™] Gold cDNA synthesis kit.

Procedure

1. Thaw each tube in the AuPreP™ Gold cDNA synthesis kit and placed on ice.
2. Briefly centrifuged all the reagents and returned on ice
3. Mixed the following components in a nuclease-free microcentrifuge tube on ice.

Total RNA sample	2.0 µg
Oligo (dT) Primer (40 µM)	1 µl
DEPC Treated Water	10 µl

4. Mixed the components gently and heated the mixture at 70⁰C for 5 minutes. Removed the tubes and placed them rapidly on ice.
5. Added the following components to the reaction tube:
Reaction Mix (2.5X) 8 µl
DTT (100mM) 2 µl
6. Now incubated the tubes in heating water bath at 42⁰C for 1 hour.
7. Now the tubes contain cDNA and stored the tubes at -20⁰C.

3.11.4.3 Real Time PCR amplification

The cDNA synthesized by AuPreP™ Gold cDNA synthesis kit was used for Real Time PCR amplification. For Real Time PCR RBC Real Sens™ Real Time PCR mastermix (SYBR Green System) kit was used. Final reaction volume of 25µl was used.

Procedure:

1. The following components were added to sterile PCR tube

Component	Volume per reaction
2X Real Time PCR mastermix	12.5 µl
Forward Primer (10 µM) (Table 21)	0.75 µl
Reverse Primer (10 µM) (Table 21)	0.75 µl
RNase Free Water	9 µl

2. Mixed gently the above components and dispensed 23µl of reaction mixture to autoclaved PCR tubes.
3. 2µl of cDNA was added separately to each PCR tube to make a final reaction mixture of 25µl.

4. The reaction parameters are as follows:

Stage	Temperature	Time	Cycle(s)
1. Initial denaturation	94°C	10 mins	1
2. a) Denaturation	94°C	45 Secs.	45
b) Annealing	50°C	1 min.	
c) Extension	72°C	45 Secs.	

5. PCR tubes were kept in Real Time PCR and started the cycling program.

6. Performed the melting curve analysis of PCR products.

The Real Time-PCR experiment was carried out at NRCPB, IARI, New Delhi.

3.11.5 Bioassay of transgenic and control plantlets of cabbage (*Brassica oleracea* L. var. *capitata* cv. **Pride of India)**

The young leaves of transformed and non-transformed cabbage were cut and placed in small petridishes containing moistened filter paper. On each leaf disc, five late second- instar (6-days-old) larvae of diamondback moth (*Plutella xylostella*) were released and reared at 26–28°C with 60 % relative humidity. Each assay consisted of three replicates using larvae originating from the same egg cluster. For each replicate of either transformed or control plants, the percentage of larval mortality was calculated after three and six days after the start of bioassay.

The bioassay experiment was conducted at Department of Entomology, Dr Y.S.Parmar University of Horticulture and Forestry.

3.12 Statistical analysis

The data recorded for the different parameters were subjected to Completely Randomized Design (Gomez and Gomez, 1984). The statistical analysis based on mean values per treatment was made using analysis of variance for completely randomized design (CRD).

Chapter-4

EXPERIMENTAL RESULTS

All the experiments were conducted in research laboratory of the Department of Biotechnology, Dr. Y. S. Parmar University of Horticulture and Forestry, Nauni, Solan. The results obtained during present investigation are presented under the following headings.

4.1 Plant regeneration in cabbage (*Brassica oleracea* L. var. *capitata* cv. **Pride of India)**

4.1.1 Source of explants

4.1.2 Plant regeneration studies in cotyledon explants

- I. Inoculation of cotyledon explants
- II. Effect of BAP+IAA, BAP+NAA, Kn+IAA and Kn+NAA (in MS medium) on shoot bud regeneration
- III. Effect of TDZ (in MS medium) on shoot regeneration
- IV. Effect of TDZ and Adenine (in MS medium) on adventitious shoot bud regeneration
- V. Effect of TDZ and NAA (in MS medium) on shoot bud regeneration
- VI. Effect of TDZ and IAA (in MS medium) on shoot regeneration

4.1.3 Plant regeneration studies in hypocotyl explants

- I. Inoculation of hypocotyl explants
- II. Effect of BAP+IAA, BAP+NAA, Kn+IAA and Kn+NAA (in MS medium) on adventitious shoot bud regeneration
- III. Effect of TDZ (in MS medium) on shoot regeneration
- IV. Effect of TDZ and Adenine (in MS medium) on adventitious shoot bud regeneration
- V. Effect of TDZ and NAA (in MS medium) on shoot bud regeneration
- VI. Effect of TDZ and IAA (in MS medium) on shoot regeneration

4.1.4 Plant regeneration studies in leaf explants

- I. Inoculation of leaf explants
- II. Effect of BAP+IAA, BAP+NAA, Kn+IAA and Kn+NAA (in MS medium) on adventitious shoot bud regeneration
- III. Effect of TDZ (in MS medium) on shoot regeneration
- IV. Effect of TDZ and Adenine (in MS medium) on adventitious shoot bud regeneration
- V. Effect of TDZ and NAA (in MS medium) on shoot bud regeneration
- VI. Effect of TDZ and IAA (in MS medium) on shoot regeneration

4.1.5 Plant regeneration studies in petiole explants

- I. Inoculation of petiole explants
- II. Effect of BAP+IAA, BAP+NAA, Kn+IAA and Kn+NAA (in MS medium) on adventitious shoot bud regeneration
- III. Effect of TDZ (in MS medium) on shoot regeneration
- IV. Effect of TDZ and Adenine (in MS medium) on adventitious shoot bud regeneration
- V. Effect of TDZ and NAA (in MS medium) on shoot bud regeneration
- VI. Effect of TDZ and IAA (in MS medium) on shoot regeneration

4.1.6 Root regeneration in *in vitro* developed shoots and development of complete plantlets

- I. Effect of different concentrations of IAA, NAA and IBA on root regeneration from *in vitro* developed shoots

4.1.7 Hardening of the regenerated plantlets of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India)

4.2 Kanamycin sensitivity in cultured tissues of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India)

- I. Kanamycin sensitivity in leaf tissues of cabbage
- II. Kanamycin sensitivity in petiole tissues of cabbage

- 4.3 Effect of antibiotics on the regeneration potential of explants in cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India)**
- 4.3.1 Effect of cefotaxime on the regeneration potential in cotyledon tissues of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India) (without co-cultivation)**
- I. Inoculation of cotyledon explants
 - II. Effect of different concentrations of cefotaxime on shoot regeneration
- 4.3.2 Effect of cefotaxime on the regeneration potential in hypocotyl tissues of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India) (without co-cultivation)**
- I. Inoculation of hypocotyl explants
 - II. Effect of different concentrations of cefotaxime on shoot regeneration
- 4.3.3 Effect of cefotaxime and kanamycin (50mg/l) on the regeneration potential in cotyledon tissues of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India) (after co-cultivation)**
- 4.3.4 Effect of cefotaxime and kanamycin (50mg/l) on the regeneration potential in hypocotyl tissues of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India) (after co-cultivation)**
- 4.4 Genetic transformation studies in cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India)**
- 4.4.1 Development of putative transgenic plantlets from cotyledon explants of cabbage**
- I. Preculturing of cotyledon explants
 - II. Co-cultivation of cotyledon explants with *Agrobacterium* strain
 - III. Selection of transformed cells on the selective medium and regeneration of putative transgenic shoots
- 4.4.2 Development of putative transgenic plantlets from hypocotyl explants of cabbage**
- I. Preculturing of hypocotyl explants
 - II. Co-cultivation of hypocotyl explants with *Agrobacterium* strain
 - III. Selection of transformed cells on the selective medium and regeneration of putative transgenic shoots

- 4.4.3 Effect of different concentrations of acetosyringone on shoot regeneration from cotyledon explants in cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India) (after co-cultivation)
- 4.4.4 Effect of different concentrations of acetosyringone on shoot regeneration from hypocotyl explants in cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India) (after co-cultivation)
- 4.4.5 Shoot multiplication, elongation and maintenance of *in vitro* developed putative transgenic shoots
- 4.4.6 *In vitro* root regeneration from putative transgenic shoots of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India) and development of complete plantlets
- 4.4.7 Hardening of *in vitro* developed putative transgenic plantlets of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India)
- 4.5 Molecular analyses of putative transgenic shoots/ plantlets of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India)
 - A) Presence/Integration of transgenes i.e. *cryIAa* and *npt-II*
 - 4.5.1 Confirmation of presence of transgenes i.e. *npt-II* (kanamycin resistance gene) and *cryIAa* (insect resistance gene) into the genome of cabbage using polymerase chain reaction using gene specific primers
 - 4.5.2 Confirmation of integration of transgene i.e. *cryIAa* (insect resistance gene) into the genome of cabbage using Southern hybridization
 - B) Expression of *cryIAa* and *npt-II* genes
 - 4.5.3 Confirmation of expression of the transgenes i.e. *npt-II* (kanamycin resistance gene) and *cryIAa* (insect resistance gene) by using reverse transcriptase polymerase chain reaction (RT-PCR) technique
 - 4.5.4 Confirmation of expression of the transgene i.e. *cryIAa* (insect resistance gene) by using Real Time polymerase chain reaction (Real Time-PCR) technique
 - 4.5.5 Bioassay of transgenic and control plantlets of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India)

4.1 Plant regeneration in cabbage (*Brassica oleracea* L. var. *capitata* cv. **Pride of India)**

4.1.1 Source of explants

Seven to nine days old aseptically grown seedlings were used for cotyledon and hypocotyl as a source of explants. Eighty per cent seed germination was observed, during aseptic seed germination. Young and tender petioles and leaves were excised from glass house grown plants, surface sterilized and used for plant regeneration studies (Fig. 1a and 1b).

4.1.2 Plant regeneration studies in cotyledon explants

I. Inoculation of cotyledon explants

Cotyledon explants were excised from aseptically grown seedlings and cut into small pieces of 0.5–1.0cm in size, which were cultured on MS medium supplemented with various combinations and concentrations of plant growth regulators such as BAP+IAA (mg/l), BAP+NAA (mg/l), Kn+IAA (mg/l), Kn+NAA (mg/l), TDZ (mg/l), TDZ+Adenine (mg/l), TDZ+NAA (mg/l) and TDZ+IAA (mg/l) .

II. Effect of BAP+IAA, BAP+NAA, Kn+IAA and Kn+NAA (in MS medium) on shoot bud regeneration

Ten different media (Table 9) were tried for shoot regeneration, which showed different percentage of shoot regeneration and average number of shoots per explants. During early days of cultures, the explants increased in size about two times. No change in the colour of explants and the media was observed. Slight callusing was observed around cut surfaces of explants after 12-15 days. Adventitious shoot bud formation/shoot regeneration from cotyledon explant started after 25-30 days of culturing. Maximum per cent shoot regeneration (84.44 %) and maximum number of shoots per explant (2.59) were obtained from the cotyledon explants on BC8 medium. Shoot multiplication was achieved on the same shoot regeneration medium. Shoot regeneration was not observed on some of the media (Table 30, Fig. 4).

Table 30: Effect of various concentrations and combinations of BAP+IAA, BAP+NAA, Kn+IAA and Kn+NAA (in MS medium) on shoot regeneration from cotyledon explants of cabbage (*Brassica oleracea* L.var. *capitata* cv. *Pride of India*)

S. No	Medium Code	Callus formation	Shoot regeneration	Average number of shoots regenerated per explants	Percent shoot regeneration
1	AC1	-	-	0.000	0.00(0.00)
2	AC2	+	Shoot regeneration	2.020	77.78(61.93)
3	BC7	+	Shoot regeneration	1.310	53.33(46.92)
4	BC8	+	Shoot regeneration	2.597	84.44(66.87)
5	CC7	-	-	0.000	0.00(0.00)
6	CC8	-	-	0.000	0.00(0.00)
7	DC5	+	Shoot regeneration	2.153	82.22(65.15)
8	DC7	+	Shoot regeneration	1.920	68.88(56.12)
9	CC9	-	-	0.000	0.00(0.00)
10	DC2	+	Shoot regeneration	0.973	46.66(43.08)
CD_{0.05}				0.1561	6.555(4.164)
SE±				0.0748	3.142(1.996)

+ = Callus formation; -= No callus formation; -= No shoot regeneration

III. Effect of TDZ (in MS medium) on shoot regeneration

Ten different media (Table 11) were tried for shoot regeneration from cotyledon and hypocotyl, which showed different percentage of shoot regeneration and average number of shoots per explants. During early days of cultures, the explants increased in size about two times. No change in the colour of explants and the media was observed. No callusing was observed. Direct shoot regeneration from cotyledon explants started after 70-75 days of culturing. Maximum per cent shoot regeneration (48.88 %) and average number of shoots per explant (1.063) were obtained from the cotyledon explants on EC2 medium whereas

maximum average number of shoots per explants (1.197) with percent shoot regeneration (46.66) was observed in EC3. Shoot elongation and multiplication was achieved on the same shoot regeneration medium (Table 31).

Table 31: Effect of various concentrations of TDZ (in MS medium) on shoot regeneration from cotyledon explants of cabbage (*Brassica oleracea* L.var. *capitata* cv. *Pride of India*)

S.No	Medium Code	Callus formation	Shoot regeneration	Average number of shoots regenerated per explants	Percent shoot regeneration
1	EC1	-	Shoot regeneration	0.506	26.66(30.97)
2	EC2	-	Shoot regeneration	1.063	48.88(44.36)
3	EC3	-	Shoot regeneration	1.197	46.66(43.09)
4	EC4	-	Shoot regeneration	0.840	35.55(36.59)
5	EC5	-	Shoot regeneration	0.486	26.66(30.97)
6	EC6	-	Shoot regeneration	0.796	35.55(36.59)
7	EC7	-	Shoot regeneration	0.463	22.22(28.07)
8	EC8	-	Shoot regeneration	0.573	26.66(31.09)
9	EC9	-	Shoot regeneration	0.510	24.44(29.47)
10	EC10	-	Shoot regeneration	0.730	33.33(35.19)
CD_{0.05}				0.1965	8.547(5.435)
SE\pm				0.0942	4.097(2.605)

-= No callus formation

IV. Effect of TDZ and Adenine (in MS medium) on adventitious shoot bud regeneration

Ten different media (Table 12) were tried for shoot regeneration, which showed different percentage of shoot regeneration and average number of shoots per explant. During early days of cultures, the explants increased in size about two times. No change in the colour

of explants and the media was observed. Slight callusing was observed around cut surfaces of explants after 28-30days in FC1 to FC6 media whereas no callus was observed in FC7 to FC10 media. Adventitious shoot bud formation/shoot regeneration from cotyledon explants started after 55-60 days of culturing. Maximum per cent shoot regeneration (91.11%) and maximum number of shoots per explant (2.997) were obtained from the cotyledon explants on FC3 medium. Direct shoot regeneration was observed after 35-40 days of culturing. Shoot elongation and multiplication was achieved on the same shoot regeneration medium (Table 32, Fig.5).

Table 32: Effect of various concentrations and combinations of TDZ and Adenine (in MS medium) on shoot regeneration from cotyledon explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India)

S.No	Medium code	Callus formation	Shoot regeneration	Average number of shoots regenerated per explants	Percent shoot regeneration
1	FC1	+	Shoot regeneration	2.930	88.88(70.73)
2	FC2	+	Shoot regeneration	0.773	26.66(31.09)
3	FC3	+	Shoot regeneration	2.997	91.11(72.88)
4	FC4	+	Shoot regeneration	1.373	48.51(44.13)
5	FC5	+	Shoot regeneration	1.530	46.66(43.03)
6	FC6	+	Shoot regeneration	0.773	35.00(36.26)
7	FC7	-	Shoot regeneration	1.910	66.66(54.80)
8	FC8	-	Shoot regeneration	0.530	22.22(28.07)
9	FC9	-	Shoot regeneration	1.350	44.44(41.80)
10	FC10	-	Shoot regeneration	1.197	42.22(40.52)
CD_{0.05}				0.2040	9.779(6.197)
SE±				0.0978	4.668(2.971)

+ = Callus formation; - = No callus formation

V. Effect of TDZ and NAA (in MS medium) on shoot bud regeneration

Ten different media (Table 13) were tried for shoot regeneration, which showed different percentage of shoot regeneration and average number of shoots per explant. During

early days of cultures, the explants increased in size about two times. No change in the colour of explants and the media was observed. No callus formation was observed but very little callus was seen in media GC6 and GC7 Adventitious shoot bud formation from cotyledon explant started after 30-35 days of culturing. Maximum per cent shoot regeneration (84.44 %) and maximum number of shoots per explant (2.57) were obtained from the cotyledon explants on GC9 medium. Shoot elongation and multiplication was achieved on the same shoot regeneration medium (Table 33).

Table 33: Effect of various concentrations and combinations of TDZ and NAA (in MS medium) on shoot regeneration from cotyledon explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. *Pride of India*)

S.No	Medium Code	Callus formation	Shoot regeneration	Average number of shoots regenerated per explants	Percent shoot regeneration
1	GC1	-	Shoot regeneration	1.220	46.66(43.08)
2	GC2	-	Shoot regeneration	0.910	53.33(47.69)
3	GC3	-	Shoot regeneration	2.287	73.33(59.64)
4	GC4	-	Shoot regeneration	1.817	57.78(49.48)
5	GC5	-	Shoot regeneration	1.550	55.55(48.20)
6	GC6	+	Shoot regeneration	1.597	60.00(50.77)
7	GC7	+	Shoot regeneration	2.197	66.66(54.80)
8	GC8	-	Shoot regeneration	2.530	80.00(63.44)
9	GC9	-	Shoot regeneration	2.573	84.44(66.87)
10	GC10	-	Shoot regeneration	1.350	44.44(41.80)
CD_{0.05}				0.1837	19.445(12.215)
SE\pm				0.0880	9.321(5.855)

+ = Callus formation; - = No callus formation

VI. Effect of TDZ and IAA (in MS medium) on shoot regeneration

Ten different media (Table 14) were tried for shoot regeneration, which showed different percentage of shoot regeneration and average number of shoots per explant. No

change in the colour of explants and the media was observed. Only expansion was observed in some of the media (HC1-HC5). Slight callusing was observed around cut surfaces of explants after 15-17 days in HC9 and HC10 media. Shoot regeneration from cotyledon explants started after 27-35 days of culturing. Maximum per cent shoot regeneration (66.66 %) and maximum number of shoots per explant (1.91) were obtained from the cotyledon explants on HC9 medium. Shoot elongation and multiplication was achieved on the same shoot regeneration medium (Table 34).

Table 34: Effect of various concentrations and combinations of TDZ and IAA (in MS medium) on shoot regeneration from cotyledon explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India)

S.No	Medium Code	Callus formation	Shoot regeneration	Average number of shoots regenerated per explants	Percent shoot regeneration
1	HC1	-	-	0.000	0.00(0.00)
2	HC2	-	-	0.000	0.00(0.00)
3	HC3	-	-	0.000	0.00(0.00)
4	HC4	-	-	0.000	0.00(0.00)
5	HC5	-	-	0.000	0.00(0.00)
6	HC6	-	Shoot regeneration	1.153	44.44(41.80)
7	HC7	-	Shoot regeneration	0.950	46.66(43.09)
8	HC8	-	Shoot regeneration	0.863	28.88(32.48)
9	HC9	+	Shoot regeneration	1.910	66.66(54.73)
10	HC10	+	Shoot regeneration	1.087	44.44(41.80)
CD_{0.05}				0.1250	3.588(2.134)
SE±				0.0599	1.720(1.023)

+ = Callus formation; - = No callus formation; - = No shoot regeneration

4.1.3 Plant regeneration studies in hypocotyl explants

I. Inoculation of hypocotyl explants

Hypocotyl explants were excised from seven to nine days old aseptically grown seedlings and cut into small pieces of 0.5–1.0cm in size. These small segments of hypocotyl were inoculated on MS medium supplemented with various concentrations and combinations of BAP+IAA (mg/l), BAP+NAA (mg/l), Kn+IAA (mg/l), Kn+NAA (mg/l), TDZ (mg/l), TDZ+Adenine (mg/l), TDZ+NAA (mg/l) and TDZ+IAA (mg/l). The colour of hypocotyl explants did not change. No change in colour of media was observed.

II. Effect of BAP+IAA, BAP+NAA, Kn+IAA and Kn+NAA (in MS medium) on adventitious shoot bud regeneration

The hypocotyl explants were inoculated on MS medium supplemented with ten different combinations and concentrations of BAP, Kn, IAA and NAA for shoot regeneration (Table 10). During the initial days of culture, the hypocotyls began to expand and swell. No callusing was observed. Direct shoot regeneration was observed in eight media after 22-27 days whereas two media showed no result (CH7 and CH9). The maximum shoot regeneration (87.03%) and average number of shoots per explants (2.62) were observed in AH6 medium. The regenerated shoots were transferred to the same medium for multiplication and elongation (Table 35, Fig. 6).

Table 35: Effect of various concentrations and combinations of BAP+IAA, BAP+NAA, Kn+IAA and Kn+NAA (in MS medium) on shoot regeneration from hypocotyl explants of cabbage (*Brassica oleracea* L.var. *capitata* cv. *Pride of India*)

S.No	Medium code	Callus formation	Shoot regeneration	Average number of shoots regenerated per explants	Percent shoot regeneration
1	AH6	-	Shoot regeneration	1.680	74.07(59.42)
2	AH4	-	Shoot regeneration	2.627	87.03(69.59)
3	BH7	-	Shoot regeneration	2.050	74.07(59.42)
4	BH9	-	Shoot regeneration	2.127	81.48(64.56)
5	CH7	-	-	0.000	0.00(0.00)
6	CH9	-	-	0.000	0.000(0.00)
7	DH2	-	Shoot regeneration	1.960	75.93(60.65)
8	DH7	-	Shoot regeneration	1.770	64.81(53.71)
9	BH4	-	Shoot regeneration	1.923	62.96(52.52)
10	DH7	-	Shoot regeneration	2.350	83.33(66.10)
CD_{0.05}				0.2072	8.103(5.908)
SE±				0.0993	3.884(2.832)

-= No callus formation; -= No shoot regeneration

III. Effect of TDZ (in MS medium) on shoot regeneration

The hypocotyl explants were inoculated on MS medium supplemented with ten different concentrations of TDZ for shoot regeneration (Table 11). During the initial days of culture, the hypocotyls began to expand and swell. No callusing was observed in medium supplemented with low concentrations of TDZ (EH1 to EH5) whereas callus initiation was observed in high concentrations of TDZ (EH6 to EH10) after 15-20 days and shoot bud developed after 33-35 days of culture. The maximum shoot regeneration (90.73%) on EH2 medium and maximum average number of shoots per explants (2.62) was observed on EH3 medium. The regenerated shoots were transferred to the same medium for multiplication and elongation (Table 36).

Table 36: Effect of various concentrations of TDZ (in MS medium) on shoot regeneration from hypocotyl explants of cabbage (*Brassica oleracea* L.var. *capitata* cv. *Pride of India*)

S.No	Medium Code	Callus formation	Shoot regeneration	Average number of shoots regenerated per explants	Percent shoot regeneration
1	EH1	-	Shoot regeneration	1.347	55.55(48.20)
2	EH2	-	Shoot regeneration	2.423	90.73(72.47)
3	EH3	-	Shoot regeneration	2.627	87.03(68.98)
4	EH4	-	Shoot regeneration	1.070	47.03(43.30)
5	EH5	-	Shoot regeneration	2.440	74.07(59.42)
6	EH6	+	Shoot regeneration	2.160	75.92(60.65)
7	EH7	+	Shoot regeneration	2.477	77.77(61.87)
8	EH8	+	Shoot regeneration	2.810	88.89(70.93)
9	EH9	+	Shoot regeneration	1.700	57.40(49.31)
10	EH10	+	Shoot regeneration	1.810	61.11(51.45)
CD_{0.05}				0.9637	7.869(5.470)
SE_±				0.0941	3.772(2.622)

+ = Callus formation; - = No callus formation

IV. Effect of TDZ and Adenine (in MS medium) on adventitious shoot bud regeneration

The hypocotyl explants were inoculated on MS medium supplemented with ten different combinations and concentrations of TDZ and Adenine for shoot regeneration (Table 12). During the initial days of culture, the hypocotyls began to expand and swell. The callus imitation was observed after 15-18 days and shoot buds developed after 32-35 days of culture. The maximum shoot regeneration (92.59%) and average number of shoots per explants (2.94) were observed in FH2 medium. The regenerated shoots were transferred to the same medium for multiplication and elongation (Table 37, Fig. 7).

Table 37: Effect of various concentrations and combinations of TDZ and Adenine (in MS medium) on shoot regeneration from hypocotyl explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India)

S.No	Medium Code	Callus formation	Shoot regeneration	Average number of shoots regenerated per explants	Percent shoot regeneration
1	FH1	+	Shoot regeneration	2.347	87.03(68.98)
2	FH2	+	Shoot regeneration	2.947	92.59(74.42)
3	FH3	+	Shoot regeneration	2.070	75.92(60.56)
4	FH4	+	Shoot regeneration	1.070	35.18(36.37)
5	FH5	+	Shoot regeneration	1.610	62.96(52.52)
6	FH6	+	Shoot regeneration	1.980	64.81(53.68)
7	FH7	+	Shoot regeneration	1.883	64.81(53.63)
8	FH8	+	Shoot regeneration	1.607	59.26(50.39)
9	FH9	+	Shoot regeneration	1.477	57.41(49.28)
10	FH10	+	Shoot regeneration	1.790	61.09(51.48)
CD_{0.05}				0.1762	9.453(5.873)
SE_±				0.0844	4.532(2.815)

+ = Callus formation; - = No callus formation

V. Effect of TDZ and NAA (in MS medium) on shoot bud regeneration

The hypocotyl explants were inoculated on MS medium supplemented with ten different combinations and concentrations of TDZ and IAA for shoot regeneration (Table 13). During the initial days of culture, the hypocotyls began to expand and swell. The callus initiation was observed after 12-15 days and shoot buds developed after 27-35 days of culture. The maximum shoot regeneration (81.48%) and average number of shoots per explants (2.05) were observed in GH8 medium. The regenerated shoots were transferred to the same medium for multiplication and elongation (Table 38).

Table 38: Effect of various concentrations and combinations of TDZ and NAA (in MS medium) on shoot regeneration from hypocotyl explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India)

S.No	Medium code	Callus formation	Shoot regeneration	Average number of shoots regenerated per explants	Percent shoot regeneration
1	GH1	+	Shoot regeneration	1.680	51.85(46.06)
2	GH2	+	Shoot regeneration	1.810	64.81(53.71)
3	GH3	+	Shoot regeneration	1.957	75.89(60.62)
4	GH4	+	Shoot regeneration	1.363	51.85(46.06)
5	GH5	+	Shoot regeneration	1.570	59.26(50.34)
6	GH6	+	Shoot regeneration	1.163	35.18(36.37)
7	GH7	+	Shoot regeneration	1.477	61.11(51.42)
8	GH8	+	Shoot regeneration	2.050	81.48(64.56)
9	GH9	+	Shoot regeneration	0.903	35.18(36.37)
10	GH10	+	Shoot regeneration	1.180	50.00(45.00)
CD_{0.05}				0.1445	6.458(3.929)
SE_±				0.0693	3.096(1.883)

+ = Callus formation

VI. Effect of TDZ and IAA (in MS medium) on shoot regeneration

The hypocotyl explants were inoculated on MS medium supplemented with ten different combinations and concentrations of TDZ and NAA for shoot regeneration (Table

14). During the initial days of culture, the hypocotyls began to expand and swell. No callus was observed on medium supplemented with low concentrations of TDZ and NAA (HH1 to HH5). The callusing was observed in high concentrations of TDZ (HH6 to HH10) after 12-15 days of culturing. After 27-33 days of culture shoot buds regeneration were observed. The maximum shoot regeneration (94.44%) and average number of shoots per explants (2.99) were observed in HH2 medium. The regenerated shoots were transferred to the same medium for multiplication and elongation (Table 39).

Table 39: Effect of various concentrations and combinations of TDZ and IAA (in MS medium) on shoot regeneration from hypocotyl explants of cabbage (*Brassica oleracea* L.var. *capitata* cv. *Pride of India*)

S.No	Medium code	Callus Formation	Shoot regeneration	Average number of shoots regenerated per explants	Percent shoot regeneration
1	HH1	-	Shoot regeneration	2.253	81.46(64.55)
2	HH2	-	Shoot regeneration	2.997	94.44(76.36)
3	HH3	-	Shoot regeneration	2.107	74.07(59.42)
4	HH4	-	Shoot regeneration	2.810	90.73(72.47)
5	HH5	-	Shoot regeneration	2.457	83.51(66.05)
6	HH6	+	Shoot regeneration	1.497	59.40(50.43)
7	HH7	+	Shoot regeneration	1.423	53.70(47.13)
8	HH8	+	Shoot regeneration	1.980	59.26(50.34)
9	HH9	+	Shoot regeneration	0.997	33.33(35.26)
10	HH10	+	Shoot regeneration	1.627	51.85(46.06)
CD_{0.05}				0.1854	4.606(3.207)
SE±				0.0890	2.208(1.537)

+ = Callus formation; - = No callus formation

4.1.4 Plant regeneration studies in leaf explants

I. Inoculation of leaf explants

Young and tender leaves were excised from the plants raised in pots in glasshouse. After surface sterilization, leaves were cut into small segments of 0.5–1.0cm size and

cultured on MS medium supplemented with various concentrations and combinations of BAP+IAA (mg/l), BAP+ NAA (mg/l), Kn+IAA (mg/l), Kn+NAA (mg/l), TDZ (mg/l), TDZ+Adenine (mg/l), TDZ+NAA(mg/l) and TDZ+IAA (mg/l). The colour of leaf explants did not change. No change in colour of media was observed (Fig. 1b).

II. Effect of BAP+IAA, BAP+NAA, Kn+IAA and Kn+NAA (in MS medium) on adventitious shoot bud regeneration

The leaf explants were inoculated on MS medium supplemented with ten different combinations and concentrations of BAP, Kn, IAA and NAA for shoot regeneration (Table 9). Swelling and expansion in the leaf explants were observed at the initial days of culture. Shoot regeneration was observed after 27-33 days of culturing. The maximum percent shoot regeneration (84.44%) and maximum average number of shoots (2.24) was observed on DL5 medium whereas only expansion of explants was observed in some of the media (CL7, CL8 and CL9). The regenerated shoots were transferred to the same medium for multiplication and elongation (Table 40, Fig. 8).

Table 40: Effect of various concentrations and combinations of BAP+IAA, BAP+NAA, Kn+ IAA and Kn+NAA (in MS medium) on shoot regeneration from leaf explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. *Pride of India*)

S.No	Medium Code	Callus Formation	Shoot regeneration	Average number of shoots regenerated per explants	Percent shoot regeneration
1	AL1	-	Shoot regeneration	1.063	53.33(46.92)
2	AL2	-	Shoot regeneration	1.753	64.44(53.41)
3	BL7	-	Shoot regeneration	1.577	53.33(46.92)
4	BL8	-	Shoot regeneration	1.930	71.11(57.52)
5	CL7	-	-	0.000	0.00(0.00)
6	CL8	-	-	0.000	0.00(0.00)
7	DL5	-	Shoot regeneration	2.243	84.44(67.51)
8	DL7	-	Shoot regeneration	1.753	64.44(53.41)
9	CL9	-	-	0.000	0.00(0.00)
10	DL2	-	Shoot regeneration	1.573	60.00(50.80)
CD_{0.05}				0.1461	9.036(6.069)
SE±				0.0070	4.332(2.909)

-= No callus formation; -= No shoot regeneration

III. Effect of TDZ (in MS medium) on shoot regeneration

The leaf explants were inoculated on MS medium supplemented with ten different concentrations of TDZ for shoot regeneration (Table 11). Swelling and expansion in the leaf explants were observed at the initial days of culture. Shoot regeneration was observed after 72-80 days of culturing. The maximum percent shoot regeneration (84.44%) and maximum average number of shoots (1.95) was observed on EL5 medium. Direct shoot regeneration was observed in seven concentrations whereas three concentrations (EL4, EL5 and EL10) showed callusing after 30-35 days of culture. The regenerated shoots were transferred to the same medium for multiplication and elongation (Table 41).

Table 41: Effect of various concentrations of TDZ (in MS medium) on shoot regeneration from leaf explants of cabbage (*Brassica oleracea* var. *capitata* L. var. *capitata* cv. Pride of India)

S. No.	Medium Code	Callus formation	Shoot regeneration	Average number of shoots regenerated per explants	Percent shoot regeneration
1	EL1	-	Shoot regeneration	1.197	46.66(43.08)
2	EL2	-	Shoot regeneration	0.773	24.44(29.58)
3	EL3	-	Shoot regeneration	1.420	51.11(45.64)
4	EL4	+	Shoot regeneration	1.377	53.33(46.91)
5	EL5	+	Shoot regeneration	1.953	84.44(66.87)
6	EL6	-	Shoot regeneration	1.400	62.22(52.09)
7	EL7	-	Shoot regeneration	1.287	64.44(53.41)
8	EL8	-	Shoot regeneration	1.087	42.22(40.52)
9	EL9	-	Shoot regeneration	1.130	46.44(43.08)
10	EL10	+	Shoot regeneration	0.997	44.44(41.80)
CD_{0.05}				0.2281	7.470(4.517)
SE±				0.1093	3.581(2.165)

+ = Callus formation; - = No callus formation

IV. Effect of TDZ and Adenine (in MS medium) on adventitious shoot bud regeneration

The leaf explants were inoculated on MS medium supplemented with ten different combinations and concentrations of TDZ and Adenine for shoot regeneration (Table 12). Swelling and expansion in the leaf explants was observed at the initial days of culture. Shoot regeneration was observed after 62-65 days of culturing. The maximum percent shoot regeneration (68.88%) and maximum average number of shoots (2.107) was observed on FL7 medium. Direct shoot regeneration was observed in seven concentrations whereas three concentrations (FL5, FL6 and FL10) showed callusing after 27-32 days of culture. The regenerated shoots were transferred to the same medium for multiplication and elongation (Table 42).

Table 42: Effect of various concentrations and combinations of TDZ and Adenine (in MS medium) on shoot regeneration from leaf explants of cabbage (*Brassica oleracea* L.var. *capitata* cv. *Pride of India*)

S. No	Medium Code	Callus formation	Shoot regeneration	Average number of shoots regenerated per explants	Percent shoot regeneration
1	FL1	-	Shoot regeneration	1.663	66.66(54.80)
2	FL2	-	Shoot regeneration	0.640	24.44(29.58)
3	FL3	-	Shoot regeneration	1.397	55.55(48.20)
4	FL4	-	Shoot regeneration	0.820	46.66(43.08)
5	FL5	+	Shoot regeneration	1.240	46.66(43.08)
6	FL6	+	Shoot regeneration	0.620	35.55(36.59)
7	FL7	-	Shoot regeneration	2.107	68.88(56.12)
8	FL8	-	Shoot regeneration	1.240	44.44(41.80)
9	FL9	-	Shoot regeneration	1.197	44.44(41.80)
10	FL10	+	Shoot regeneration	1.063	40.00(39.19)
CD_{0.05}				0.2120	8.793(5.224)
SE_±				0.1016	4.215(2.504)

+ = Callus formation; - = No callus formation

V. Effect of TDZ and NAA (in MS medium) on shoot bud regeneration

The leaf explants were inoculated on MS medium supplemented with ten different combinations and concentration of TDZ and NAA for shoot regeneration (Table 13). Swelling and expansion in the leaf explants was observed at the initial days of culture. The callus initiation was observed after 19-24 days. Shoot regeneration was observed after 45-55 days of culturing. The maximum percent shoot regeneration (91.11%) and maximum average number of shoots (3.202) was observed on GL2 medium. The regenerated shoots were transferred to the same medium for multiplication and elongation (Table 43, Fig. 9).

Table 43: Effect of various concentrations and combinations of TDZ and NAA (in MS medium) on shoot regeneration from leaf explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India)

S. No	Medium Code	Callus formation	Shoot regeneration	Average number of shoots regenerated per explants	Percent shoot regeneration
1	GL1	-	Shoot regeneration	0.930	31.11(33.87)
2	GL2	-	Shoot regeneration	3.020	91.11(72.88)
3	GL3	-	Shoot regeneration	1.910	62.22(52.13)
4	GL4	+	Shoot regeneration	2.950	88.88(70.73)
5	GL5	+	Shoot regeneration	2.620	80.00(63.64)
6	GL6	+	Shoot regeneration	2.220	71.11(57.52)
7	GL7	+	Shoot regeneration	1.530	51.11(45.64)
8	GL8	+	Shoot regeneration	2.620	84.44(67.51)
9	GL9	+	Shoot regeneration	2.350	86.66(69.02)
10	GL10	+	Shoot regeneration	2.443	82.22(65.15)
CD _{0.05}				0.1869	9.941(7.551)
SE±				0.0896	4.765(3.620)

+ = Callus formation; - = No callus formation

VI. Effect of TDZ and IAA (in MS medium) on shoot regeneration

The leaf explants were inoculated on MS medium supplemented with ten different combinations and concentration of TDZ and IAA for shoot regeneration (Table 14). Swelling and expansion in the leaf explants were observed at the initial days of culture. The callus initiation was observed after 20-24 days at higher concentration of TDZ (HL8, HL9 and HL10) whereas low concentration of TDZ showed direct shoot regeneration. Shoot regeneration was observed after 45-60 days of culturing. The maximum percent shoot regeneration (33.33%) in HL6 and maximum average number of shoots (0.73) was observed on HL9 medium. No shoot regeneration was observed in media HL 3, HL4 and HL 5. The regenerated shoots were transferred to the same medium for multiplication and elongation (Table 44).

Table 44: Effect of various concentrations and combinations of TDZ and IAA (in MS medium) on shoot regeneration from leaf explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India)

S. No	Medium Code	Callus formation	Shoot regeneration	Average number of shoots regenerated per explants	Percent shoot regeneration
1	HL1	-	Shoot regeneration	0.710	26.66(31.09)
2	HL2	-	Shoot regeneration	0.573	22.22(28.07)
3	HL3	-	-	0.000	0.00(0.00)
4	HL4	-	-	0.000	0.00(0.00)
5	HL5	-	-	0.000	0.00(0.00)
6	HL6	-	Shoot regeneration	0.686	33.33(35.26)
7	HL7	-	Shoot regeneration	0.573	24.44(29.58)
8	HL8	+	Shoot regeneration	0.420	20.00(26.57)
9	HL9	+	Shoot regeneration	0.730	31.11(33.87)
10	HL10	+	Shoot regeneration	0.886	31.11(33.87)
CD _{0.05}				0.1491	4.145(2.706)
SE±				0.0715	1.987(1.297)

+ = Callus formation; - = No callus formation; - = No shoot regeneration

4.1.5 Plant regeneration studies in petiole explants

I. Inoculation of petiole explants

Young and tender petiole explants were taken from the plants grown in pots in glasshouse. After surface sterilization the explants were cut into small pieces of 0.5-1cm in size. These small pieces were inoculated on MS medium supplemented with various concentrations and combinations of BAP+IAA (mg/l), BAP+NAA (mg/l), Kn+IAA (mg/l), Kn+NAA (mg/l), TDZ (mg/l), TDZ+Adenine (mg/l), TDZ+NAA(mg/l) and TDZ+IAA (mg/l). The colour of leaf explants did not change. No change in colour of media was observed (Fig. 1b).

II. Effect of BAP+IAA, BAP+NAA, Kn+IAA and Kn+NAA (in MS medium) on adventitious shoot bud regeneration

The petiole explants were inoculated on MS medium supplemented with ten different combinations and concentrations of BAP, Kn, IAA and NAA for shoot regeneration (Table 10). Swelling and expansion in the petiole explants were observed at the initial days of culture. No callus was observed. Shoot regeneration was observed after 25-35 days of culturing. The maximum percent shoot regeneration (85.18%) and maximum average number of shoots (2.44) was observed on BP7 medium. No shoot regeneration was observed in CP7 and CP9. The regenerated shoots were transferred to the same medium for multiplication and elongation (Table 45, Fig. 10).

III. Effect of TDZ (in MS medium) on shoot regeneration

The petiole explants were inoculated on MS medium supplemented with ten different concentrations of TDZ for shoot regeneration (Table 11). Swelling and expansion in the petiole explants were observed at the initial days of culture. The callus was observed after 18-22 days at low concentrations of TDZ whereas 33-38 days at higher concentrations of TDZ. Shoot regeneration was observed after 35-40 days at low concentration of TDZ and 72-75 days at higher concentration after culturing. The maximum percent shoot regeneration (80.00%) and average number of shoots (2.660) was observed on EP5 medium. No shoot regeneration was observed in EP9. The regenerated shoots were transferred to the same medium for multiplication and elongation (Table 46).

Table 45: Effect of various concentrations and combinations of BAP+IAA, BAP+NAA, Kn+IAA and Kn+NAA (in MS medium) on shoot regeneration from petiole explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India)

S.No	Medium code	Callus Formation	Shoot regeneration	Average number of shoots regenerated per explants	Percent shoot regeneration
1	AP6	-	Shoot regeneration	1.997	61.11(51.45)
2	AP4	-	Shoot regeneration	1.790	74.04(59.49)
3	BP7	-	Shoot regeneration	2.440	85.18(67.64)
4	BP9	-	Shoot regeneration	1.700	64.81(53.63)
5	CP7	-	-	0.000	0.00(0.00)
6	CP9	-	-	0.000	0.00(0.00)
7	DP2	-	Shoot regeneration	1.530	51.85(46.06)
8	DP7	-	Shoot regeneration	1.590	59.26(50.34)
9	BP4	-	Shoot regeneration	1.790	61.11(51.45)
10	DP7	-	Shoot regeneration	1.700	62.96(52.52)
CD_{0.05}				0.1457	7.329(4.742)
SE±				0.0698	3.512(2.276)

-= No callus formation; -= No shoot regeneration

Table 46: Effect of various concentrations of TDZ (in MS medium) on shoots regeneration from petiole explants of cabbage (*Brassica oleracea* L.var. *capitata* cv. Pride of India)

S.No	Medium Code	Callus formation	Shoot regeneration	Average number of shoots regenerated per explants	Percent shoot regeneration
1	EP1	+	Shoot regeneration	0.606	27.77(31.73)
2	EP2	+	Shoot regeneration	1.163	33.33(35.21)
3	EP3	+	Shoot regeneration	0.736	25.92(30.58)
4	EP4	+	Shoot regeneration	1.363	55.55(48.20)
5	EP5	+	Shoot regeneration	2.660	80.00(63.64)
6	EP6	+	Shoot regeneration	0.716	22.22(28.01)
7	EP7	+	Shoot regeneration	0.689	24.07(29.35)
8	EP8	+	Shoot regeneration	0.553	24.07(29.35)
9	EP9	+	-	0.00	0.00(0.00)
10	EP10	+	Shoot regeneration	1.253	48.15(43.94)
CD_{0.05}				0.2252	6.908(4.386)
SE±				0.1079	3.312(2.102)

+ = Callus formation; -= No shoot formation

IV. Effect of TDZ and Adenine (in MS medium) on adventitious shoot bud regeneration

The petiole explants were inoculated on MS medium supplemented with ten different combinations and concentrations of TDZ and Adenine for shoot regeneration (Table 12). Swelling and expansion in the petiole explants were observed at the initial days of culture. The callus was observed after 15-17 days of culture. Shoot regeneration was observed after 35-40 days after culturing. The maximum percent shoot regeneration (85.18%) in medium and maximum average number of shoots (2.90) in medium FP2 was observed. The regenerated shoots were transferred to the same medium for multiplication and elongation (Table 47).

Table 47: Effect of various concentrations and combinations of TDZ and Adenine (in MS medium) on shoot regeneration from petiole explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. *Pride of India*)

S. No	Medium Code	Callus formation	Shoot regeneration	Average number of shoots regenerated per explants	Percent shoot regeneration
1	FP1	+	Shoot regeneration	2.830	77.77(61.99)
2	FP2	+	Shoot regeneration	2.900	85.18(67.44)
3	FP3	+	Shoot regeneration	2.517	83.33(66.10)
4	FP4	+	Shoot regeneration	1.200	37.03(37.47)
5	FP5	+	Shoot regeneration	1.423	53.70(47.13)
6	FP6	+	Shoot regeneration	2.400	71.37(57.69)
7	FP7	+	Shoot regeneration	1.310	37.03(37.47)
8	FP8	+	Shoot regeneration	1.383	44.44(41.80)
9	FP9	+	Shoot regeneration	0.903	35.18(36.37)
10	FP10	+	Shoot regeneration	1.753	70.37(57.04)
CD_{0.05}				0.1818	7.086(4.723)
SE_±				0.0871	3.397(2.264)

+ = Callus formation

V. Effect of TDZ and NAA (in MS medium) on shoot bud regeneration

The petiole explants were inoculated on MS medium supplemented with ten different combinations and concentrations of TDZ and NAA for shoot regeneration (Table 13). Swelling and expansion in the petiole explants were observed at the initial days of culture. The callus was observed after 18- 25 days of culture. Shoot regeneration was observed after 35-50 days after culturing. The maximum percent shoot regeneration (88.88%) and maximum average number of shoots (3.44) was observed in medium GP3. The regenerated shoots were transferred to the same medium for multiplication and elongation (Table 48, Fig. 11).

Table 48: Effect of various concentrations and combinations of TDZ and NAA (in MS medium) on shoot regeneration from petiole explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India)

S. No	Medium Code	Callus formation	Shoot regeneration	Average number of shoots regenerated per explants	Percent shoot regeneration
1	GP1	+	Shoot regeneration	1.180	38.88(38.58)
2	GP2	+	Shoot regeneration	2.310	81.48(64.56)
3	GP3	+	Shoot regeneration	3.440	88.88(70.93)
4	GP4	+	Shoot regeneration	1.053	36.85(37.36)
5	GP5	+	Shoot regeneration	1.403	40.73(39.65)
6	GP6	+	Shoot regeneration	1.253	48.15(43.94)
7	GP7	+	Shoot regeneration	1.053	37.03(37.47)
8	GP8	+	Shoot regeneration	0.923	35.18(36.37)
9	GP9	+	Shoot regeneration	0.660	22.22(28.01)
10	GP10	+	Shoot regeneration	2.033	62.96(52.55)
CD_{0.05}				0.1616	6.890(4.804)
SE±				0.0775	3.303(2.303)

+ = Callus formation

VI. Effect of TDZ and IAA (in MS medium) on shoot regeneration

The petiole explants were inoculated on MS medium supplemented with ten different combinations and concentrations of TDZ and IAA for shoot regeneration (Table14). Swelling

and expansion in the petiole explants were observed at the initial days of culture. No callus was observed at lower concentrations of TDZ whereas callus was observed after 18-23 days of culture at higher concentrations of TDZ. Shoot regeneration was observed after 35-45 days after culturing. The maximum percent shoot regeneration (85.18%) and maximum average number of shoots (2.72) was observed in medium HP2. The regenerated shoots were transferred to the same medium for multiplication and elongation (Table 49).

Table 49: Effect of various concentrations and combinations of TDZ and IAA (in MS medium) on shoot regeneration from petiole explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India)

S. No	Medium code	Callus formation	Shoot regeneration	Average number of shoots regenerated per explants	Percent shoot regeneration
1	HP1	-	Shoot regeneration	1.627	57.40(49.27)
2	HP2	-	Shoot regeneration	2.033	75.92(60.65)
3	HP3	-	Shoot regeneration	1.180	38.88(38.55)
4	HP4	-	Shoot regeneration	2.720	85.18(67.44)
5	HP5	-	Shoot regeneration	1.770	70.37(57.04)
6	HP6	+	Shoot regeneration	1.643	61.11(51.42)
7	HP7	+	Shoot regeneration	0.923	37.03(37.47)
8	HP8	+	Shoot regeneration	0.736	31.48(34.11)
9	HP9	+	Shoot regeneration	0.957	37.03(37.47)
10	HP10	+	Shoot regeneration	1.553	51.85(46.06)
CD_{0.05}				0.1833	5.728(3.592)
SE_±				0.0879	2.746(1.722)

+ = Callus formation; - = No callus formation

4.1.6 Root regeneration in *in vitro* developed shoots and development of complete plantlets

I. Effect of different concentrations of IAA, NAA and IBA on root regeneration from *in vitro* developed shoots

The regenerated shoots (about 2–3cm long) obtained from different explants i.e. cotyledon, hypocotyl, leaf and petiole were then transferred to root regeneration medium supplemented with different kinds of auxins.

A) Effect of IAA on root regeneration from *in vitro* developed shoots

The *in vitro* regenerated shoots were excised from explants/callus and cultured on MS medium containing different concentration of IAA (Table 15). Root regeneration was observed after 9-12 days of inoculation and maximum percent root regeneration was observed 97.20% on I-RR₁ and I-RR₂. After 22-25 days complete root system has been developed. Callusing was observed (Table 50).

B) Effect of NAA on root regeneration from *in vitro* developed shoots

The *in vitro* regenerated shoots were excised from explants/callus and cultured on MS medium containing different concentration of NAA (Table 16). Root regeneration was observed after 7-9 days of inoculation and maximum percent root regeneration was observed 100% on II-RR₂. After 18-22 days complete root system has been developed (Table 50, Fig. 12).

C) Effect of IBA on root regeneration from *in vitro* developed shoots

The *in vitro* regenerated shoots were excised from explants/callus and cultured on MS medium containing different concentration of IBA (Table 17). Root regeneration was observed after 7-9 days of inoculation and maximum percent root regeneration was observed 80% on III-RR₂. After 20-24 days complete root system has been developed (Table 50).

Table 50: Effect of different concentrations of various auxins on per cent root regeneration from *in vitro* developed shoots of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India)

S.No.	Treatment	IAA	NAA	IBA
	Concentration (mg/l)			
1.	0.05	97.20(84.39)	77.78(61.97)	69.44(56.49)
2.	0.10	97.20(84.39)	100(90.00)	80.55(63.94)
3.	0.20	86.09(68.32)	94.40(78.77)	71.66(56.84)
CD _{0.05}			7.796(10.628)	
SE±			3.711(5.058)	

4.1.7 Hardening of the regenerated plantlets of cabbage (*Brassica oleracea* L. var. *capitata* cv. *Pride of India*)

After the complete development of roots i.e. about 20-25 days of rooting, cabbage plantlets were taken out of the culture tubes, taking various precautions to avoid any damage to its delicate root system. The roots were washed gently under running tap water to remove adhering medium and then plants were kept in 0.5 % bavistin solution for 5–10 minutes. Plastic cups were first filled to more than half of their capacity with pre-sterilized cocopeat. The root portion of the plantlets were then placed over the cocopeat and covered with sterilized cocopeat gently to full capacity of the cup. The plantlets were watered and covered with polythene bags to maintain high humidity. Water was sprayed daily. After 13-15 days, when plants show initial sign of establishment in plastic pots with appearance of new leaves, they were uncovered overnight and after 21 days were fully uncovered. After keeping, them in these cups containing cocopeat they were transferred to the pots containing potting mixture (Sand: soil: FYM mixture). The plants were watered adequately daily. Maximum percent survival was 80% percent (Fig. 13).

From these experiments, it was found that hypocotyl was better explants than petiole, cotyledon and leaf as they showed high per cent shoot regeneration and average number of shoots per explant and high multiplication rate. Shoot regeneration and root regeneration were comparatively early in hypocotyls as compared to other three explants.

4.2 Kanamycin sensitivity in cultured tissues of cabbage (*Brassica oleracea* L. var. *capitata* cv. *Pride of India*)

I. Kanamycin sensitivity in leaf tissues of cabbage

The leaf explants were excised from *in vivo* grown seedlings, surface sterilized and cut into small uniform pieces. Fresh weight of these explants were measured under aseptic conditions and then inoculated on MS selective shoot regeneration medium having different concentrations of kanamycin (0, 10, 20, 30, 40, 50 and 60mg/l) (Table 18)

The explants in control medium i.e. MS shoot regeneration medium without kanamycin were very healthy and showed appropriate growth on this medium. But, on the selective media at concentration as low as 10mg/l kanamycin, the colour of explant/tissue had changed to pale greenish yellow. In control experiment, adventitious shoot bud regeneration was observed after 30 days in culture, whereas no shoot regeneration or shoot bud formation

was observed even after 5 weeks on culture in MS selective medium containing 10mg/l kanamycin. A gradual decline in fresh weight of leaf explants were recorded with increased concentration of kanamycin (10 to 60mg/l) from 7–35 days. The maximum decline in fresh weight was observed at 50mg/l kanamycin in leaf explants, whereas in case of control (MS regeneration medium without kanamycin) there was a maximum increase in fresh weight of explants (Table 51, Fig. 14).

Table 51: Effect of different concentrations of kanamycin on the relative growth (fresh weight) of leaf explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. *Pride of India*)

S. No.	Days	Average fresh weight of leaf explants/callus of cabbage(mg)						
		Kanamycin concentrations (mg/l)						
		0	10	20	30	40	50	60
1.	0	45.00±0.58	42.00±0.00	50.00±1.15	43.00±0.29	27.30±0.61	31.00±1.15	30.00±1.15
2.	7	95.00±0.58	87.20±0.12	92.00±1.15	81.00±0.29	62.40±0.61	51.00±1.15	45.00±1.15
3.	14	130.00±1.73	118.20±0.12	120.50±1.15	105.00±0.00	81.40±0.61	63.00±1.44	54.00±0.58
4.	21	201.00±1.73	177.2±0.69	171.00±2.52	150.00±0.00	105.7±0.61	76.00±1.44	64.00±0.58
5.	28	299.00±1.15	263.20±0.69	252.50±1.53	220.00±0.00	125.70±0.61	85.00±1.44	70.00±0.58
6.	35	448.00±1.15	388.20±0.69	350.50±1.53	289.00±0.00	131.20±0.61	81.50±1.44	65.00±0.58
CD _{0.05}		1.434						
SE±		1.014						

Table 52: Coefficient of correlation between concentration of kanamycin (x) and change in average fresh weight of leaf tissue/ callus (y) at different intervals of time

S.No.	Variables	Coefficient of correlation (r)
1.	Concentration of kanamycin and fresh weight of explants/ callus on day 0	1.0000
2.	Concentration of kanamycin and fresh weight of explants/ callus on 7 th day	-0.9548
3.	Concentration of kanamycin and fresh weight of explants/ callus on 14 th day	-0.9713
4.	Concentration of kanamycin and fresh weight of explants/ callus on 21 st day	-0.9787
5.	Concentration of kanamycin and fresh weight of explants/ callus on 28 th day	-0.9688
6.	Concentration of kanamycin and fresh weight of explants/ callus on 35 th day	-0.9699

Statistical analysis of the recorded data showed that there was a significant difference between fresh weights of all six treatments of kanamycin concentrations (0 to 60mg/l). Negative coefficient of correlation was observed between different concentrations of kanamycin and fresh weight of explants/tissues. It indicates that kanamycin has an inhibitory effect on the growth of cultured tissues as it is potent inhibitor of protein synthesis (Table 52).

II. Kanamycin sensitivity in petiole tissues of cabbage

The petiole explants were excised from *in vivo* grown seedlings, surface sterilized and cut into small uniform pieces. Fresh weight of these explants were measured under aseptic conditions and then inoculated on MS selective shoot regeneration medium having different concentrations of kanamycin (0, 10, 20, 30, 40, 50 and 60mg/l) (Table 19).

The explants in control medium i.e. MS shoot regeneration medium without kanamycin were healthy and showed appropriate growth on this medium. But on the selective media at concentration as low as 10mg/l kanamycin, the colour of tissues had changed to pale yellow during initial days. In the control experiment, adventitious shoot bud regeneration was observed after 35 days in culture, whereas the media containing kanamycin even at 10mg/l, there was little callusing at the initial stage but later on it turned into brownish black. A gradual decline in fresh weight of petiole explants were recorded with increased concentrations of kanamycin (10 to 60mg/l) from 7 to 35 days. The maximum decline in fresh weight was observed at 50 and 60mg/l kanamycin in petiole explants, whereas, in case of control (MS medium without kanamycin), there was a maximum increase in fresh weight of explants (Table 53, Fig. 15).

Statistical analysis of the recorded data showed that there was a significant difference between fresh weights of all six treatments of kanamycin concentrations (0 to 60 mg/l). Negative coefficient of correlation was observed between different concentrations of kanamycin and fresh weight of explants/tissue. It indicates that kanamycin has an inhibitory effect on the growth of cultured tissues as it is a potent inhibitor of protein synthesis (Table 54).

Table 53: Effect of different concentrations of kanamycin on the relative growth (fresh weight) of petiole explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. *Pride of India*)

S. No.	Days	Average fresh weight of leaf explants/callus of cabbage(mg)						
		Kanamycin concentrations (mg/l)						
		0	10	20	30	40	50	60
1.	0	5.66±0.41	5.00±0.29	5.60±0.00	5.40±0.12	5.00±0.58	5.80±0.23	5.20±0.12
2.	7	17.17±0.41	11.00±0.29	14.20±0.00	12.46±0.12	11.32±0.58	7.92±0.23	6.68±0.12
3.	14	52.67±0.41	39.10±0.29	32.87±0.20	23.36±0.12	19.32±0.58	14.62±0.23	11.28±0.12
4.	21	125.50±0.87	103.70±0.29	92.30±0.21	65.26±0.12	41.12±0.58	22.62±0.23	16.28±0.12
5.	28	215.50±0.87	158.90±0.22	126.00±0.21	80.36±0.12	50.69±0.79	24.82±0.23	16.08±0.12
6.	35	366.40±0.87	257.50±0.22	188.00±0.21	110.10±0.12	58.32±0.58	24.52±0.23	14.98±0.12
CD _{0.05}		0.5396						
SE±		0.3816						

Table 54: Coefficient of correlation between concentration of kanamycin (x) and change in average fresh weight of petiole tissue/ callus (y) at different intervals of time

S.No.	Variables	Coefficient of correlation (r)
1.	Concentration of kanamycin and fresh weight of explants/ callus on day 0	1.0000
2.	Concentration of kanamycin and fresh weight of explants/ callus on 7 th day	-0.7893
3.	Concentration of kanamycin and fresh weight of explants/ callus on 14 th day	-0.9848
4.	Concentration of kanamycin and fresh weight of explants/ callus on 21 st day	-0.9871
5.	Concentration of kanamycin and fresh weight of explants/ callus on 28 th day	-0.9822
6.	Concentration of kanamycin and fresh weight of explants/ callus on 35 th day	-0.9691

4.3 Effect of antibiotics on regeneration potential of explants in cabbage (*Brassica oleracea* L. var. *capitata* cv. *Pride of India*)

4.3.1 Effect of cefotaxime on the regeneration potential in cotyledon tissues of cabbage (*Brassica oleracea* L. var. *capitata* cv. *Pride of India*) (without co-cultivation)

I. Inoculation of cotyledon explants

The cotyledon explants were excised from seven to nine days old *in vitro* grown seedlings and cut into uniform pieces of same size and cultured on the best shoot regeneration medium containing different concentrations of cefotaxime (0, 100, 200, 300, 400 and 500mg/l) (Table 20).

II. Effect of different concentrations of cefotaxime on shoot regeneration

The maximum per cent (88%) shoot regeneration with average number of shoots (2.66) were obtained on MS shoot regeneration medium with 300mg/l cefotaxime. With the increase in the concentrations of cefotaxime, percent shoot regeneration was same till 300 mg/l cefotaxime but increased at 300mg/l cefotaxime then the percent shoot regeneration decreased. All the observations were recorded after 45 days of inoculation of explants (Table 55, Fig. 16).

Table 55: Effect of different concentrations of cefotaxime on the regeneration potential of cotyledon explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India)(without co-cultivation)

Sr. No.	MS basal+ 2.0mg/l Kn+ 0.50mg/l NAA+ Cefotaxime concentration	Callus Formation	Shoot regeneration	Average number of shoots regenerated per explants	Percent shoot regeneration
1	0 mg/l	+	Shoot regeneration	2.597	84.44(66.87)
2	100 mg/l	+	Shoot regeneration	2.460	84.44(66.87)
3	200 mg/l	+	Shoot regeneration	2.579	84.44(66.87)
4	300 mg/l	+	Shoot regeneration	2.666	88.88(70.73)
5	400 mg/l	+	Shoot regeneration	2.423	80.00(63.44)
6	500 mg/l	+	Shoot regeneration	2.597	84.44(66.87)
CD_{0.05}				0.156	5.66(3.435)
SE±				0.078	2.59(1.632)

+ = Callus formation

4.3.2 Effect of cefotaxime on the regeneration potential in hypocotyl tissues of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India) (without co-cultivation)

I. Inoculation of hypocotyl explants

The hypocotyl explants were excised from seven to nine days old *in vitro* grown seedlings and cut into uniform pieces of same size and cultured on the best shoot regeneration medium containing different concentrations of cefotaxime (0, 100, 200, 300, 400 and 500mg/l) (Table 21).

II. Effect of different concentrations of cefotaxime on shoot regeneration

In this experiment, very interesting and different shoot regeneration was observed on the same medium on different segments of hypocotyl explants. The maximum per cent (90.73%) shoot regeneration with average number of shoots (2.460) were obtained on MS shoot regeneration medium with 0mg/l cefotaxime. Percent shoot regeneration decreased in medium supplemented with 300mg/l cefotaxime whereas remain constant at 100, 200, 400 and 500mg/l cefotaxime but maximum average number of shoots (2.466) were observed in medium supplemented with 400mg/l cefotaxime (Table 56, Fig. 17).

Table 56: Effect of different concentration of cefotaxime on the regeneration potential of hypocotyl explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India) (without co-cultivation)

Sr. No.	MS basal+ 1.5mg/l Kn+ 0.25mg/l IAA+ Cefotaxime concentration	Callus Formation	Shoot regeneration	Average number of shoots regenerated per explants	Percent shoot regeneration
1	0 mg/l	-	Shoot regeneration	2.460	90.73(73.47)
2	100 mg/l	-	Shoot regeneration	2.393	87.03(69.59)
3	200 mg/l	-	Shoot regeneration	2.460	87.03(69.59)
4	300 mg/l	-	Shoot regeneration	2.367	81.49(64.56)
5	400 mg/l	-	Shoot regeneration	2.466	87.03(69.59)
6	500 mg/l	-	Shoot regeneration	2.533	87.03(69.59)
CD_{0.05}				0.086	6.589(4.091)
SE_±				0.039	3.024(1.915)

-= No callus formation

4.3.3 Effect of cefotaxime and kanamycin (50mg/l) on the regeneration potential in cotyledon tissues of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India) (after co-cultivation)

The cotyledon explants were excised from seven to nine days old *in vitro* grown seedlings and cut into uniform pieces of same size and cultured on the best shoot regeneration medium with 50mg/l kanamycin and different concentrations of cefotaxime (0, 100, 200, 300, 400 and 500mg/l) (Table 22).

The effect of varying concentrations of cefotaxime with same concentrations of kanamycin was studied on the regeneration efficiency and their capability to control the overgrowth of agrobacterial cells after co-cultivation. At lower concentrations (0, 100, 200, 300mg/l) of cefotaxime showed overgrowth of agrobacterial cells and at higher concentrations of cefotaxime, the agrobacterial cells growth was completely inhibited. Per cent shoot regeneration (35.55%) and average number of shoots (2.93) per plant was found maximum in 400mg/l cefotaxime with 50mg/l kanamycin. At lower concentrations of cefotaxime, all the explants died due to uncontrolled growth of agrobacterial cells. At the concentration of 300 mg/l cefotaxime the density of agrobacterial cells started decreasing (Table 57, Fig. 18).

Table 57: Effect of various concentrations of cefotaxime and kanamycin (50mg/l) on the regeneration potential of cotyledon explants in cabbage (*Brassica oleracea* L.var. *capitata* cv. Pride of India) (after co-cultivation)

S. No.	Medium composition	Average number of shoots per explants	Per cent shoot regeneration
1.	MS basal+ 2.0mg/l Kn+ 0.50mg/l NAA+ 50mg/l Kanamycin+ 0mg/l Cefotaxime	0.000	0.00 (0.00)
2.	MS basal+ 2.0mg/l Kn+ 0.50mg/l NAA+ 50mg/l Kanamycin+ 100mg/l Cefotaxime	0.000	0.00 (0.00)
3.	MS basal+ 2.0mg/l Kn+ 0.50mg/l NAA+ 50mg/l Kanamycin+ 200mg/l Cefotaxime	0.000	0.00 (0.00)
4.	MS basal+ 2.0mg/l Kn+ 0.50mg/l NAA+ 50mg/l Kanamycin+ 300mg/l Cefotaxime	0.400	16.66(19.16)
5.	MS basal+ 2.0mg/l Kn+ 0.50mg/l NAA+ 50mg/l Kanamycin+ 400mg/l Cefotaxime	2.930	35.55(36.59)
6.	MS basal+ 2.0mg/l Kn+ 0.50mg/l NAA+ 50mg/l Kanamycin+ 500mg/l Cefotaxime	1.35	22.22(28.07)
CD_{0.05}		0.330	3.140(1.998)
SE±		0.150	2.024(1.347)

4.3.4 Effect of cefotaxime and kanamycin (50mg/l) on the regeneration potential in hypocotyl tissues of cabbage (*Brassica oleracea* L.var. *capitata* cv. Pride of India) (after co-cultivation)

The hypocotyl explants were excised from seven to nine days old *in vitro* grown seedlings and cut into uniform pieces of 0.5–1.0cm size and cultured on the best shoot

regeneration medium containing 50mg/l Kanamycin with different concentrations of cefotaxime (0, 100, 200, 300, 400 and 500mg/l) (Table 23).

The effect of varying concentrations of cefotaxime with same concentrations of kanamycin was studied on the regeneration efficiency and their capability to control the overgrowth of agrobacterial cells after co-cultivation. At lower concentrations (0, 100, 200mg/l) of cefotaxime the agrobacterial cells showed overgrowth and at higher concentrations of cefotaxime, agrobacterial cells growth was completely inhibited. Per cent shoot regeneration (48.15%) and average number of shoots (1.770) per plant was found maximum in 400mg/l cefotaxime with 50mg/l kanamycin. At lower concentrations of cefotaxime, all the explants died due to uncontrolled growth of agrobacterial cells. At the concentration of 300mg/l cefotaxime the density of agrobacterial cells started decreasing and it was controlled only by 3 to 4 subcultures of explants on the fresh selective medium (Table 58, Fig. 19).

Table 58: Effect of various concentrations of cefotaxime and kanamycin (50mg/l) on the regeneration potential of hypocotyl explants in cabbage (*Brassica oleracea* L.var. *capitata* cv. *Pride of India*) (after co-cultivation)

S. No.	Medium composition	Average number of shoots per explants	Per cent shoot regeneration
1.	MS basal+ 1.5mg/l Kn+ 0.25mg/l IAA+ 50mg/l Kanamycin+ 0mg/l Cefotaxime	0.000	0.00 (0.00)
2.	MS basal+ 1.5mg/l Kn+ 0.25mg/l IAA+ 50mg/l Kanamycin+ 100mg/l Cefotaxime	0.000	0.00 (0.00)
3.	MS basal+ 1.5mg/l Kn+ 0.25mg/l IAA+ 50mg/l Kanamycin+ 200mg/l Cefotaxime	0.000	0.00 (0.00)
4.	MS basal+ 1.5mg/l Kn+ 0.25mg/l IAA+ 50mg/l Kanamycin+ 300mg/l Cefotaxime	0.700	20.33 (26.38)
5.	MS basal+ 1.5mg/l Kn+ 0.25mg/l IAA+ 50mg/l Kanamycin+ 400mg/l Cefotaxime	1.770	48.15(43.94)
6.	MS basal+ 1.5mg/l Kn+ 0.25mg/l IAA+ 50mg/l Kanamycin+ 500mg/l Cefotaxime	1.250	35.18(36.37)
CD_{0.05}		0.410	4.510(3.098)
SE±		0.190	2.740(1.701)

4.4 Genetic transformation studies in cabbage (*Brassica oleracea* L. var. *capitata* cv. *Pride of India*)

Genetic transformation studies were carried out with cotyledon and hypocotyl explants obtained from aseptically grown seven to nine days old seedlings of cabbage using *Agrobacterium*- mediated gene transfer technique.

4.4.1 Development of putative transgenic plantlets from cotyledon explants of cabbage

I. Preculturing of cotyledon explants

The cotyledon explants were excised from seven to nine days old aseptically grown seedlings of cabbage and cut into uniform size of 0.5–1.0cm and inoculated on media(GTC1 and GTC2), which were found best for shoot regeneration in cotyledon explants (Table 28). The explants were precultured for 24, 48 and 72 hours. During preculturing the cells were in a state of active growth. After 24, 48 and 72 hours of preculturing, co-cultivation experiment was carried out. The 72 hours was observed to be best for the pre-culturing of cotyledon explants (Fig. 20).

II. Co-cultivation of cotyledon explants with *Agrobacterium* strain

Precultured cotyledon explants (for 24, 48 and 72 hours) were co-cultivated with *Agrobacterium tumefaciens* strain containing the binary vector, pBin-1Aa for different time intervals i.e. 48, 72 and 96 hours. Optimum growth of *Agrobacterium* was obtained after 48 hours of co-cultivation whereas excessive growth of agrobacterial cells was occurred after 72 and 96 hours of co-cultivation (Fig. 20).

III. Selection of transformed cells on the selective medium and regeneration of putative transgenic shoots

After co-cultivation, the explants were transferred to fresh selective shoot regeneration media (GTC1 and GTC2) containing antibiotics (cefotaxime 500mg/l and kanamycin 50mg/l). These antibiotics were added into the medium to kill the agrobacterial cells and to select the transformed cabbage cells, respectively. Callus formation was seen after 22-25 days at cut edges of cotyledons and also at wound site where the tissue was damaged during inoculation in GTC1 medium whereas in GTC2 the callus formation was observed after 38-40 days after inoculation. The calli which were obtained on the selective media were maintained on the same selective media for shoot regeneration/organogenesis and

shoot regeneration was observed in GTC1 after 35-40 days and in GTC2 after 65-70 days on the selective media respectively. The cells/tissues which were not transformed turned brown or black on the selective media after 40 to 50 days in culture. The putative transgenic shoots obtained on the selective medium were maintained on the same selective medium for shoot multiplication and elongation.

In GTC1 medium, Maximum percentage of shoot regeneration (3.00%) was obtained when preculturing and co-cultivation were carried out for 72 and 48 hours respectively. The explants which were pre-cultured for 72 hours have survived after co-cultivation with *Agrobacterium* strain than those of 24 and 48 hours of preculturing and co-cultivation was found optimum for 48 hours. At 72 and 96 hours of co-cultivation, overgrowth of agrobacterial cells was observed (Table 59, Fig. 22).

Table 59: Effect of pre-culturing and co-cultivation time on transformation frequency in cotyledon explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India)

S. No.	Hours of pre-culture/incubation (in hrs)	Hours of co-cultivation (in hrs)	Medium code (Selective regeneration medium)	Total Number of explants cultured	Total Number explants forming shoots	Percent shoot regeneration
1.	24	48	GTC1	250	-	0.00(1.00)
		72		250	-	0.00(1.00)
		96		250	-	0.00(1.00)
2.	48	48		250	-	0.00(1.00)
		72		250	-	0.00(1.00)
		96		250	-	0.00(1.00)
3.	72	48		400	13	3.00(1.99)
		72		350	6	1.62(1.62)
		96		250	-	0.00(1.00)
CD_{0.05}						0.0941(0.0252)
SE±						0.0444(0.0193)
Effect of preculturing and co-cultivation CD_{0.05}						0.1630(0.0438)
SE±						0.0543(0.0142)

GTC1= MS basal+ 2.0mg/l Kn+ 0.50mg/l NAA+ 50mg/l Kanamycin+ 400mg/l Cefotaxime

In GTC2 medium, Maximum percentage of shoot regeneration (4.66%) was obtained when preculturing and co-cultivation were carried out for 72 and 48 hours respectively. The explants which were pre-cultured for 72 hours have survived after co-cultivation with *Agrobacterium* strain than those of 24 and 48 hours of preculturing and co-cultivation was

found optimum for 48 hours. At 72 and 96 hours of co-cultivation, overgrowth of agrobacterial cells were observed (Table 60, Fig.23).

Table 60: Effect of preculturing and co-cultivation time on transformation frequency in cotyledon explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India)

S. No.	Hours of pre-culture/ incubation (in hrs)	Hours of co-cultivation (in hrs)	Medium code (Selective regeneration medium)	Total Number of explants cultured	Total Number explants forming shoots	Percent shoot regeneration
1.	24	48	GTC2	250	-	0.00(1.00)
		72		250	-	0.00(1.00)
		96		250	-	0.00(1.00)
2.	48	48		250	-	0.00(1.00)
		72		250	-	0.00(1.00)
		96		250	-	0.00(1.00)
3.	72	48		400	19	4.667(2.38)
		72		350	9	2.573(1.88)
		96		250	-	0.00(1.00)
CD_{0.05}						0.1017(0.0260)
SE±						0.0479(0.0122)
Effect of preculturing and co-cultivation CD_{0.05}						0.1759(0.0451)
SE±						0.0587(0.0150)

GTC2= MS basal+ 0.330mg/l TDZ+ 79.7mg/l Adenine+ 50mg/l Kanamycin+ 400mg/l Cefotaxime

4.4.2 Development of putative transgenic plantlets from hypocotyl explants of cabbage

I. Preculturing of hypocotyl explants

The hypocotyl explants were excised from seven to nine days old aseptically grown seedlings of cabbage and cut into uniform size of 0.5–1.0 cm and inoculated on media (GTH1 and GTH2), which were found best for shoot regeneration in cotyledon explants (Table 28). The explants were precultured for 24, 48 and 72 hours. During preculturing the cells were in a state of active growth. After 24, 48 and 72 hours of preculturing, co-cultivation experiment was carried out. The 72 hours was observed to be best for the preculturing of hypocotyl explants (Fig. 21).

II. Co-cultivation of hypocotyl explants with *Agrobacterium* strain

Precultured hypocotyl explants (for 24, 48 and 72 hours) were co-cultivated with *Agrobacterium tumefaciens* strain containing the binary vector, pBin-1Aa for different time

intervals i.e. 48, 72 and 96 hours. Optimum growth of *Agrobacterium* was obtained after 48 hours of co-cultivation whereas excessive growth of agrobacterial cells was occurred after 72 and 96 hours of co-cultivation (Fig. 21).

III. Selection of transformed cells on the selective medium and regeneration of putative transgenic shoots

After co-cultivation, the explants were transferred to fresh selective shoot regeneration media (GTH1 and GTH2) containing antibiotics (cefotaxime 500mg/l and kanamycin 50mg/l). These antibiotics were added into the medium to kill the agrobacterial cells and to select the transformed cabbage cells, respectively. Little callus formation was seen after 18-22 days at cut edges of hypocotyls and also at wound site where the tissue was damaged during inoculation in GTH1 medium whereas in GTH2 the callus formation was observed after 23-26 days after inoculation. The calli which were obtained on the selective media were maintained on the same selective media for shoot regeneration/organogenesis and shoot regeneration was observed in GTH1 after 32-40 days and in GTH2 after 38-45 days on the selective media respectively. The cells/tissues which were not transformed, turned brown or black on the selective medium after 40 to 50 days in culture. The putative transgenic shoots obtained on the selective medium were maintained on the same selective medium for shoot multiplication and elongation.

In GTH1, Maximum percentage of shoot regeneration (10.25%) was obtained when preculturing and co-cultivation were carried out for 72 hours and 48 hours respectively. The explants which were pre-cultured for 72 hours have shown better survival after co-cultivation with *Agrobacterium* strain than those of 24 and 48 hours of preculturing. At 72 and 96 hours of co-cultivation, overgrowth of agrobacterial cells were observed. The cells/explants which were not transformed did not survive on selective medium (Table 61, Fig. 24).

In GTH2, Maximum percentage of shoot regeneration (14.45%) was obtained when preculturing and co-cultivation were carried out for 72 hours and 48 hours respectively. The explants which were pre-cultured for 72 hours have shown better survival after co-cultivation with *Agrobacterium* strain than those of 24 and 48 hours of preculturing. At 72 and 96 hours of co-cultivation, overgrowth of agrobacterial cells was observed. The cells/explants which were not transformed did not survive on selective medium (Table 62, Fig. 25).

Table 61: Effect of preculturing and co-cultivation time on transformation frequency in hypocotyl explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India)

S. No.	Hours of pre-culture/incubation (in hrs)	Hours of co-cultivation (in hrs)	Medium code (Selective regeneration medium)	Total Number of explants cultured	Total Number explants forming shoots	Percent shoot regeneration
1.	24	48	GTH1	250	-	0.00(1.00)
		72		250	-	0.00(1.00)
		96		250	-	0.00(1.00)
2.	48	48		250	6	2.267(1.80)
		72		250	3	1.600(1.60)
		96		250	-	0.00(1.00)
3.	72	48		400	42	10.25(3.35)
		72		350	30	8.760(3.12)
		96		250	-	0.00(1.00)
CD_{0.05}						0.2161(0.0887)
SE±						0.1019(0.0261)
Effect of preculturing and co-cultivation CD_{0.05}						0.3743(0.0914)
SE±						0.1249(0.0305)

GTH1= MS basal+ 1.5mg/l Kn+ 0.25mg/l IAA+ 50mg/l Kanamycin + 400mg/l Cefotaxime

Table 62: Effect of preculturing and co-cultivation time on transformation frequency in hypocotyl explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India)

S. No.	Hours of pre-culture/incubation (in hrs)	Hours of co-cultivation (in hrs)	Medium code (Selective regeneration medium)	Total Number of explants cultured	Total Number explants forming shoots	Percent shoot regeneration
1.	24	48	GTH2	250	-	0.00(1.00)
		72		250	-	0.00(1.00)
		96		250	-	0.00(1.00)
2.	48	48		250	11	4.40(2.32)
		72		250	7	2.40(1.97)
		96		250	-	0.00(1.00)
3.	72	48		400	58	14.50(3.93)
		72		350	43	12.28(3.64)
		96		250	-	0.00(1.00)
CD_{0.05}						0.0528(0.0567)
SE±						0.0249(0.0096)
Effect of preculturing and co-cultivation CD_{0.05}						0.9779(0.1283)
SE±						0.3262(0.0428)

GTH2= MS basal+ 0.220mg/l TDZ+ 0.088mg/l IAA+ 50mg/l Kanamycin+ 400mg/l Cefotaxime

4.4.3 Effect of different concentrations of acetosyringone on shoot regeneration from cotyledon explants in cabbage (*Brassica oleracea* L. var. *capitata* cv. **Pride of India**) (after co-cultivation)

The cotyledon explants were excised from seven to nine days old aseptically grown seedlings of cabbage and cut into uniform size of 0.5–1.0cm and inoculated on two best shoot regeneration media (BC8 and FC3) which were found best for shoot regeneration in cotyledon explants containing different concentrations of acetosyringone (50, 75, 100, 125, 150µM). The explants were precultured for 72 hours and co-cultivated with agrobacterial cells for 72 hours. After co-cultivation the explants were transferred to the selective shoot regeneration media containing antibiotics and different concentrations of acetosyringone (Table 24 and 25).

In both media, the maximum percent shoot regeneration (16.44 and 18.66) were obtained from those explants which were precultured and cocultivated for 72 hours and 48 hours respectively on the medium containing 100µM acetosyringone. It was also observed that first increase in the concentration of acetosyringone upto 100µM the percent shoot regeneration increased, but later on it declined (Table 63 and 64, Fig.26).

Table 63: Effect of different concentrations of acetosyringone on the transformation frequency of cabbage (*Brassica oleracea* L. var. *capitata* cv. **Pride of India) from cotyledon explants**

S. No.	Medium composition	Percent shoot regeneration
1	MS basal+ 2.0mg/l Kn+ 0.50mg/l NAA+ 50µM Acetosyringone	6.440(2.53)
2.	MS basal+ 2.0mg/l Kn+ 0.50mg/l NAA+ 75µM Acetosyringone	9.330(3.05)
3.	MS basal+ 2.0mg/l Kn+ 0.50mg/l NAA+ 100µM Acetosyringone	16.44(4.05)
4.	MS basal+ 2.0mg/l Kn+ 0.50mg/l NAA+ 125µM Acetosyringone	14.44(3.80)
5.	MS basal+ 2.0mg/l Kn+ 0.50mg/l NAA+ 150µM Acetosyringone	5.330(2.30)
CD_{0.05}		1.213(0.194)
SE±		0.544(0.087)

Table 64: Effect of different concentrations of acetosyringone on the transformation frequency of cabbage (*Brassica oleracea* L. var. *capitata* cv. *Pride of India*) from cotyledon explants

S.No.	Medium code	Percent shoot regeneration
1	MS basal+ 0.330mg/l TDZ+ 79.7mg/l Adenine+ 50µM Acetosyringone	7.997(2.82)
2.	MS basal+ 0.330mg/l TDZ+ 79.7mg/l Adenine+ 75µM Acetosyringone	10.440(3.22)
3.	MS basal+ 0.330mg/l TDZ+ 79.7mg/l Adenine+ 100µM Acetosyringone	18.660(4.32)
4.	MS basal+ 0.330mg/l TDZ+ 79.7mg/l Adenine+ 125µM Acetosyringone	16.000(3.99)
5.	MS basal+ 0.330mg/l TDZ+ 79.7mg/l Adenine+ 150µM Acetosyringone	7.107(2.66)
CD_{0.05}		1.289(0.194)
SE±		0.578(0.087)

4.4.4 Effect of different concentrations of acetosyringone on shoot regeneration from hypocotyl explants in cabbage (*Brassica oleracea* L. var. *capitata* cv. *Pride of India*) (after co-cultivation)

The hypocotyl explants were excised from seven to nine days old aseptically grown seedlings of cabbage and cut into uniform size of 0.5–1.0cm and inoculated on two best shoot regeneration media (AH4 and HH2) which were found best for shoot regeneration in hypocotyl explants containing different concentrations of acetosyringone (50, 75, 100, 125, 150µM). The explants were precultured for 72 hours and co-cultivated with agrobacterial cells for 48 hours. After co-cultivation the explants were transferred to the selective shoot regeneration media containing antibiotics and different concentrations of acetosyringone (Table 26 and 27).

In both media, the maximum percent shoot regeneration (26.44 and 32.00) were obtained from those explants which were precultured and cocultivated for 72 hours and 48 hours respectively on the medium containing 100µM acetosyringone. It was also observed that first increase in the concentration of acetosyringone upto 100µM the percent shoot regeneration increased, but later on it declined (Table 65 and 66, Fig. 27).

Table 65: Effect of different concentrations of acetosyringone on the transformation frequency of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India) from hypocotyl explants

S. No.	Medium composition	Percent shoot regeneration
1	MS basal+ 1.5mg/l Kn+ 0.25mg/l IAA+ 50µM Acetosyringone	11.780(3.43)
2.	MS basal+ 1.5mg/l Kn+ 0.25mg/l IAA+ 75µM Acetosyringone	14.660(3.82)
3.	MS basal+ 1.5mg/l Kn+ 0.25mg/l IAA+ 100µM Acetosyringone	26.440(5.14)
4.	MS basal+ 1.5mg/l Kn+ 0.25mg/l IAA+ 125µM Acetosyringone	21.330(4.61)
5.	MS basal+ 1.5mg/l Kn+ 0.25mg/l IAA+ 150µM Acetosyringone	7.100(2.66)
CD_{0.05}		0.940(0.122)
SE±		0.422(0.055)

Table 66: Effect of different concentrations of acetosyringone on the transformation frequency of cabbage (*Brassica oleracea* L.var. *capitata* cv. Pride of India) from hypocotyl explants

S.No.	Medium code	Percent shoot regeneration
1	MS basal+ 0.220mg/l TDZ+ 0.088mg/l IAA+ 50µM Acetosyringone	16.420(4.05)
2.	MS basal+ 0.220mg/l TDZ+ 0.088mg/l IAA+ 75µM Acetosyringone	21.550(4.64)
3.	MS basal+ 0.220mg/l TDZ+ 0.088mg/l IAA+ 100µM Acetosyringone	32.000(5.65)
4.	MS basal+ 0.220mg/l TDZ+ 0.088mg/l IAA+ 125µM Acetosyringone	28.660(5.35)
5.	MS basal+ 0.220mg/l TDZ+ 0.088mg/l IAA+ 150µM Acetosyringone	14.000(3.74)
CD_{0.05}		1.032(0.112)
SE±		0.463(0.050)

4.4.5 Shoot multiplication, elongation and maintenance of *in vitro* developed putative transgenic shoots

In vitro developed putative transgenic shoots were further maintained and multiplied on the same selective shoot regeneration medium with lower concentration of antibiotics (kanamycin 20mg/l, cefotaxime 300mg/l). After obtaining 2-3 cm height, the shoots were transferred to the selective root regeneration medium.

4.4.6 *In vitro* root regeneration from putative transgenic shoots of cabbage (*Brassica oleracea* L. var. *capitata*) and development of complete plantlets

The putative transgenic shoots (2.5-3.0cm height) obtained on the selective shoot multiplication medium were excised and transferred to the selective root regeneration medium containing 0.10mg/l NAA+ 50mg/l kanamycin + 400mg/l cefotaxime. Roots initiated after 20-23 days of inoculation on the selective medium and within 35-40 days well developed roots were observed (Fig. 28).

4.4.7 Hardening of *in vitro* developed putative transgenic plantlets of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India)

After the complete development of transgenic cabbage plantlets, they were taken out from the culture tubes and washed gently under running tap water to remove adhering medium. The plantlets were kept in 0.5 per cent bavistin solution for 5-10 minutes and placed in sterilized cocopeat. The plantlets were watered and covered with polythene bags to maintain high humidity. Water was sprayed daily. The transgenic plantlets showed initial sign of establishment in plastic pots after one week of hardening. The polythene bags were punctured gradually after one week of hardening and 50-60 percent survival of plants were observed during acclimatization (Fig. 29).

4.5 Molecular analyses of putative transgenic shoots/ plantlets of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India)

A) Presence/integration of transgenes i.e. *cryIAa* and *npt-II*

4.5.1 Confirmation of presence of transgenes i.e. *npt-II* (kanamycin resistance gene) and *cryIAa* (insect resistance gene) into the genome of cabbage using polymerase chain reaction using gene specific designed primers

Polymerase chain reaction was carried out to study the presence and integration of the target genes (*npt-II*) and (*cryIAa*) in the putative transgenic shoots of cabbage and to confirm the transfer of *npt-II* and *cryIAa* genes from *Agrobacterium tumefaciens* into the genome of transgenic shoots of cabbage. Total genomic DNA was isolated from 40 randomly selected putative transgenic plantlets along with control plantlets by the CTAB method. Gene specific primers (Table 29) were used to amplify 500bp fragment of *npt-II* gene and 1.0 kb fragment of *cryIAa* gene by Polymerase chain reaction. Out of 40 randomly selected putative transgenic plantlets, 20 had shown the amplification of *npt-II* and *cryIAa* genes there by indicating the presence/integration of *npt-II* and *cryIAa* genes into the genome of transgenic plantlets of cabbage. As the number of cycle increases the amount of target DNA (gene)

synthesized, increases exponentially. PCR products were visualized after electrophoresis in 1.2 % agarose gel and photographed by Alfa imager gel documentation system (Fig. 30 and 31).

4.5.2 Confirmation of integration of transgene i.e. *cryIAa* (insect resistance gene) into the genome of cabbage using Southern hybridization

This isolated, purified and quantified DNA (plasmid DNA and genomic DNA of putative transgenic shoots/ plantlets and control plantlets) was used for blotting with radio labeled DNA probe. Total genomic DNA was isolated from the leaves of randomly selected 6 PCR +ve shoots/plantlets along with control plantlets by the method of Doyle and Doyle (1990). Gene specific 1.8 kb radioactive labeled DNA fragment was used as a probe. Out of 6 PCR +ve plantlets, 5 shoots have been able to develop the imprints on the X-ray film there by indicating the presence/ integration of *cryIAa* gene into the genome of transgenic cabbage. The data in Figure suggest that plant T0 and T2 had two T-DNA insertions while plant T7 and T33 had three T-DNA insertion whereas plant T39 had single T-DNA insertion (Fig. 32).

B) Expression of *cryIAa* and *npt-II*

4.5.3 Confirmation of expression of the transgenes i.e. *npt-II* (kanamycin resistance gene) and *cryIAa* (insect resistance gene) by using reverse transcriptase polymerase chain reaction (RT-PCR) technique

The expression of the transgene i.e. *cryIAa* (the insect resistance gene) and *npt-II* (the selectable marker gene) was studied at the transcriptional level using reverse transcriptase polymerase chain reaction (RT-PCR) technique. The total RNA was isolated from the PCR positive shoots/ plantlets (20) of cabbage i.e. putative transgenic cabbage . The cDNA was synthesized using the oligo dT primer and then the cDNA was amplified by using the gene specific primer i.e. forward primer and reverse primer of *npt-II* and *cryIAa* genes. The specific designed primers (Table 29) have amplified 500bp fragment of *npt-II* gene and 1.0 kb fragment of *cryIAa* gene in the putative transgenic cabbage. The amplified cDNA was run on 1.2% agarose gel along with molecular weight marker. Out of the 20 samples, only 10 had shown amplification of cDNA thereby indicating that the genes were being expressed at the transcriptional level in these transgenic lines (Fig. 33 and 34).

4.5.4 Confirmation of expression of the transgene i.e. *cryIAa* (insect resistance gene) by using Real Time polymerase chain reaction (Real Time-PCR) technique

The expression of the transgene i.e. *cryIAa* (the insect resistance gene) was studied at the transcriptional level using Real Time- polymerase chain reaction (Real Time-PCR)

technique. The total RNA was isolated from the RT-PCR positive shoots/ plantlets of cabbage i.e. transgenic cabbage. The cDNA was synthesized using the oligo dT primer and then the cDNA was amplified by using the gene specific primer i.e. forward primer and reverse primer of *cryIAa* gene. The SYBR Green system was used to record the expression of different transgenic plantlets. The amplified cDNA had shown the different peak of fluorescence whereas, cDNA of control plantlets (non-transformed) did not show any peak of fluorescence. All the samples had shown good ct value (less than 26) thereby indicating that the gene was being expressed at the transcriptional level in these transgenic lines. Relative quantity of expression was studied by using relative quantity chart. The data in Fig. 36 suggest the comparison of expression of transgene with negative control and the plant T33 showed maximum expression whereas, the plant T7 showed minimum expression. The plants T0, T2 and T39 showed more or less same expression level of transgene i.e. *cryIAa* (Table 67, Fig. 35).

Table 67: Confirmation of presence/expression of the transgene i.e. *cryIAa* (insect resistance gene) by using Real-Time polymerase chain reaction (RealTime-PCR) technique

S. No.	Colour	Name	Type	Ct
1		Negative Control	Standard	38.78
2		T0	Standard	24.29
3		T2	Standard	24.18
4		T7	Standard	25.63
5		T33	Standard	23.81
6		T39	Standard	24.62

4.5.5 Bioassay of transgenic and control plantlets of cabbage (*Brassica oleracea* L. var. *capitata* cv. **Pride of India)**

A high degree of insect protection was evident in our bioassays. The larvae fed on transgenic leaves were severely stunted in growth when compared to larvae fed on control (non-transformed) leaves after one day from start of bioassay. Transgenic plants provided 100 % mortality of larvae at day 3 after the bioassay was started, whereas, the larvae on control plants (non-transformed) were growing healthy. It has been convincingly demonstrated by several groups that the genes encoding δ -endotoxins from *Bt* impart resistance to lepidopteran insects pests, when introduced into different crop plants. High-level

expression was important for our research work of imparting insect-pest resistance to cabbage cultivar 'Pride of India'. Our insect bioassays, although at small scales initially, also provided evidence that the introduction of a insect resistance gene into transgenic crop plants can be efficacious (Fig. 37). However, the basis of this concluding result needs further confirmatory evidence by scanning/recording the different evolutionary stages of insect life cycle.

Fig. 4(A-F): Plant regeneration studies in cotyledon explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. **Pride of India) using Kinetin and NAA in MS basal medium.**

- A. Inoculated cotyledon explants on shoot regeneration medium BC8 (MS basal + 2mg/l Kn + 0.5mg/l NAA) at day 0 in culture.
- B. Cotyledon explants showing swelling and expansion after 5 days in culture on medium BC8 (MS basal + 2mg/l Kn + 0.5mg/l NAA).
- C. Cotyledon explants showing callus initiation after 15 days in culture on medium BC8 (MS basal + 2mg/l Kn + 0.5mg/l NAA).
- D. Callus formation and shoot initiation from cotyledon explants after 28 days in culture on medium BC8 (MS basal + 2mg/l Kn + 0.5mg/l NAA).
- E. Shoot formation for cotyledon explants after 35 days in culture on medium BC8 (MS basal + 2mg/l Kn + 0.5mg/l NAA).
- F. Shoot elongation and multiplication after 48 days in culture on medium BC8 (MS basal + 2mg/l Kn + 0.5mg/l NAA).



Fig. 4(A-F)

Fig. 5(A-F): Plant regeneration studies in cotyledon explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. *Pride of India*) using TDZ and Adenine in MS basal medium.

- A. Inoculated cotyledon explants on shoot regeneration medium FC3 (MS basal + 0.330mg/l TDZ + 79.7mg/l Adenine) at day 0 in culture.
- B. Cotyledon explants showing swelling and expansion after 12 days in culture on medium FC3 (MS basal + 0.330mg/l TDZ + 79.7mg/l Adenine).
- C. Cotyledon explants showing callus initiation after 28 days in culture on medium FC3 (MS basal + 0.330mg/l TDZ + 79.7mg/l Adenine).
- D. Callus formation and Shoot initiation from cotyledon explants after 45 days in culture on medium FC3 (MS basal + 0.330mg/l TDZ + 79.7mg/l Adenine).
- E. Shoot proliferation for cotyledon explants after 63 days in culture on medium FC3 (MS basal + 0.330mg/l TDZ + 79.7mg/l Adenine).
- F. Shoot elongation and multiplication after 80 days in culture on medium FC3 (MS basal + 0.330mg/l TDZ + 79.7mg/l Adenine).

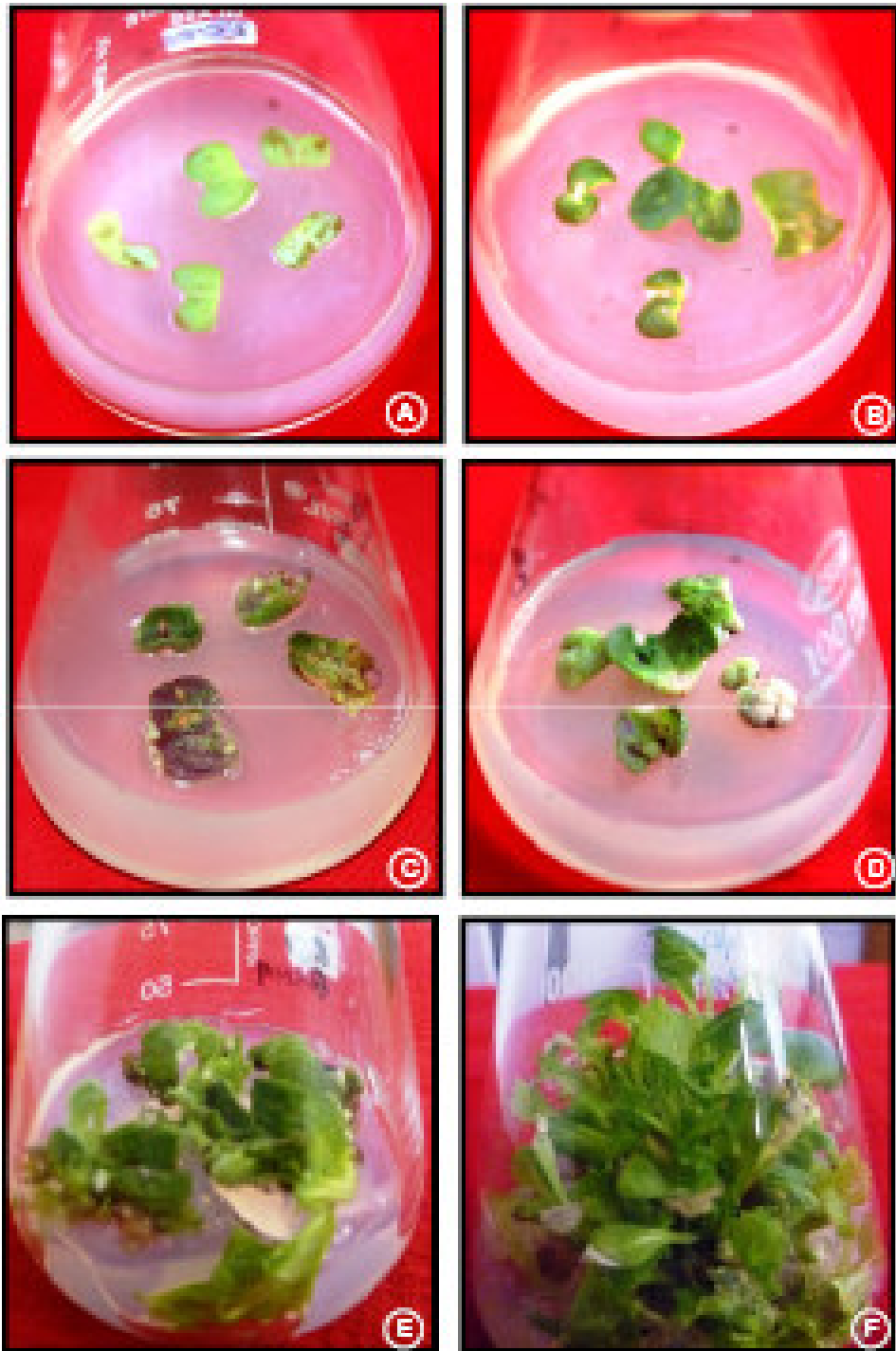


Fig. 5(A-F)

Fig. 6(A-E): Plant regeneration studies in hypocotyl explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India) using Kinetin and IAA in MS basal medium.

- A. Hypocotyl explants cultured on shoot regeneration medium AH4 (MS basal + 1.5mg/l Kn + 0.25mg/l IAA) at 0 day.
- B. Hypocotyl explants showing swelling after 6 days in culture on medium AH4 (MS basal + 1.5mg/l Kn + 0.25mg/l IAA).
- C. Hypocotyl explants showing direct shoot initiation after 25 days in culture on medium AH4 (MS basal + 1.5mg/l Kn + 0.25mg/l IAA).**
- D. Shoot proliferation from hypocotyl explants after 36 days in culture on medium AH4 (MS basal + 1.5mg/l Kn + 0.25mg/l IAA).
- E. Shoot elongation and multiplication after 48 days in culture on medium AH4 (MS basal + 1.5mg/l Kn + 0.25mg/l IAA).

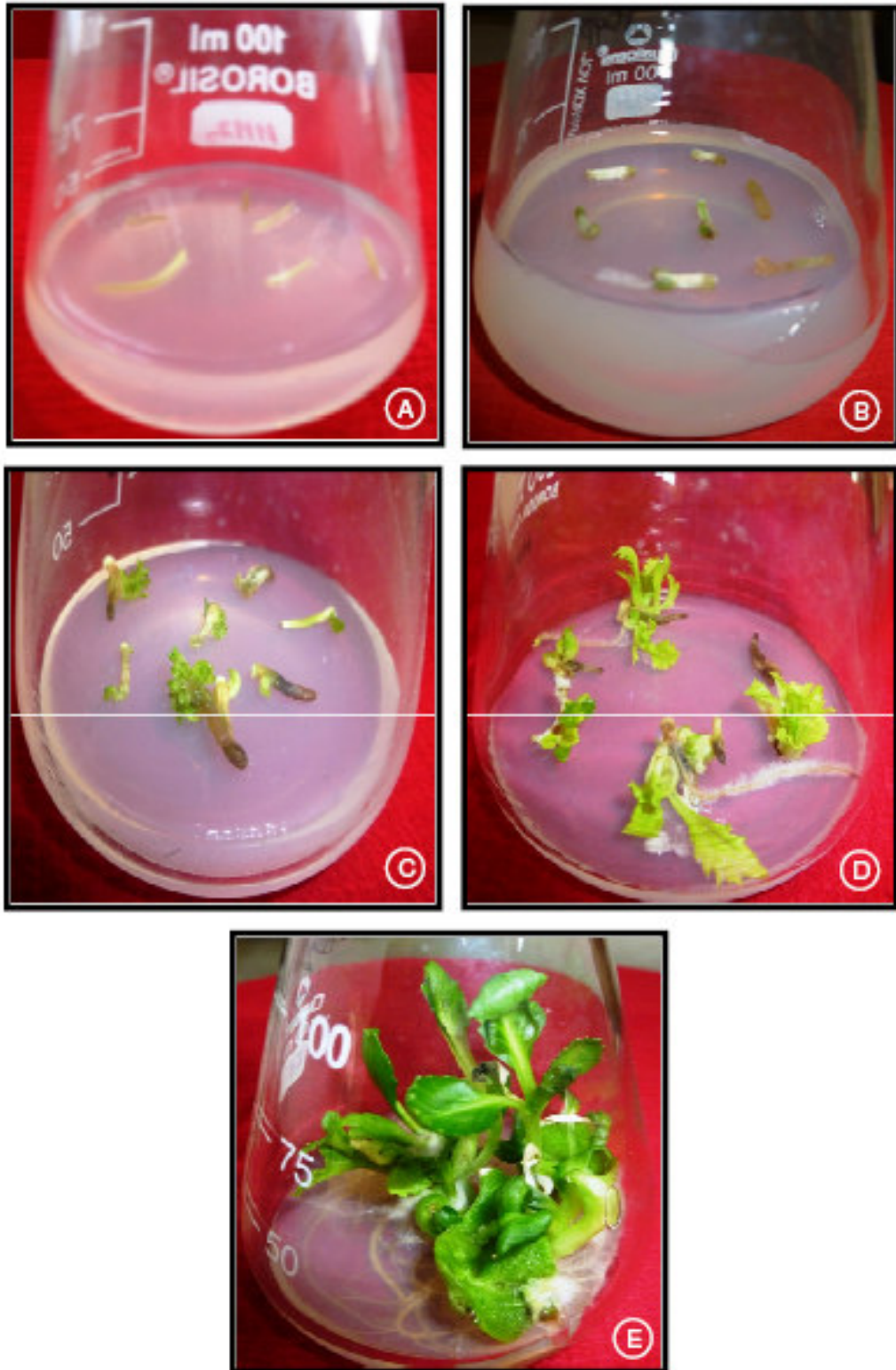


Fig. 6(A-E)

Fig. 7(A-F): Plant regeneration studies in hypocotyl explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. *Pride of India*) using TDZ and Adenine in MS basal medium.

- A. Inoculated hypocotyl explants on shoot regeneration medium FH2 (MS basal + 0.220mg/l TDZ + 79.7mg/l Adenine) at day 0 in culture.
- B. Hypocotyl explants showing callus initiation after 15 days in culture on medium FH2 (MS basal + 0.220mg/l TDZ + 79.7mg/l Adenine).
- C. Hypocotyl explants showing callus formation after 22 days in culture on medium FH2 (MS basal + 0.220mg/l TDZ + 79.7mg/l Adenine).
- D. Shoot initiation from hypocotyl explants after 34 days in culture on medium FH2 (MS basal + 0.220mg/l TDZ + 79.7mg/l Adenine).
- E. Shoot formation for hypocotyl explants after 48 days in culture on medium FH2 (MS basal + 0.220mg/l TDZ + 79.7mg/l Adenine).
- F. Shoot elongation and multiplication after 55 days in culture on medium FH2 (MS basal + 0.220mg/l TDZ + 79.7mg/l Adenine).

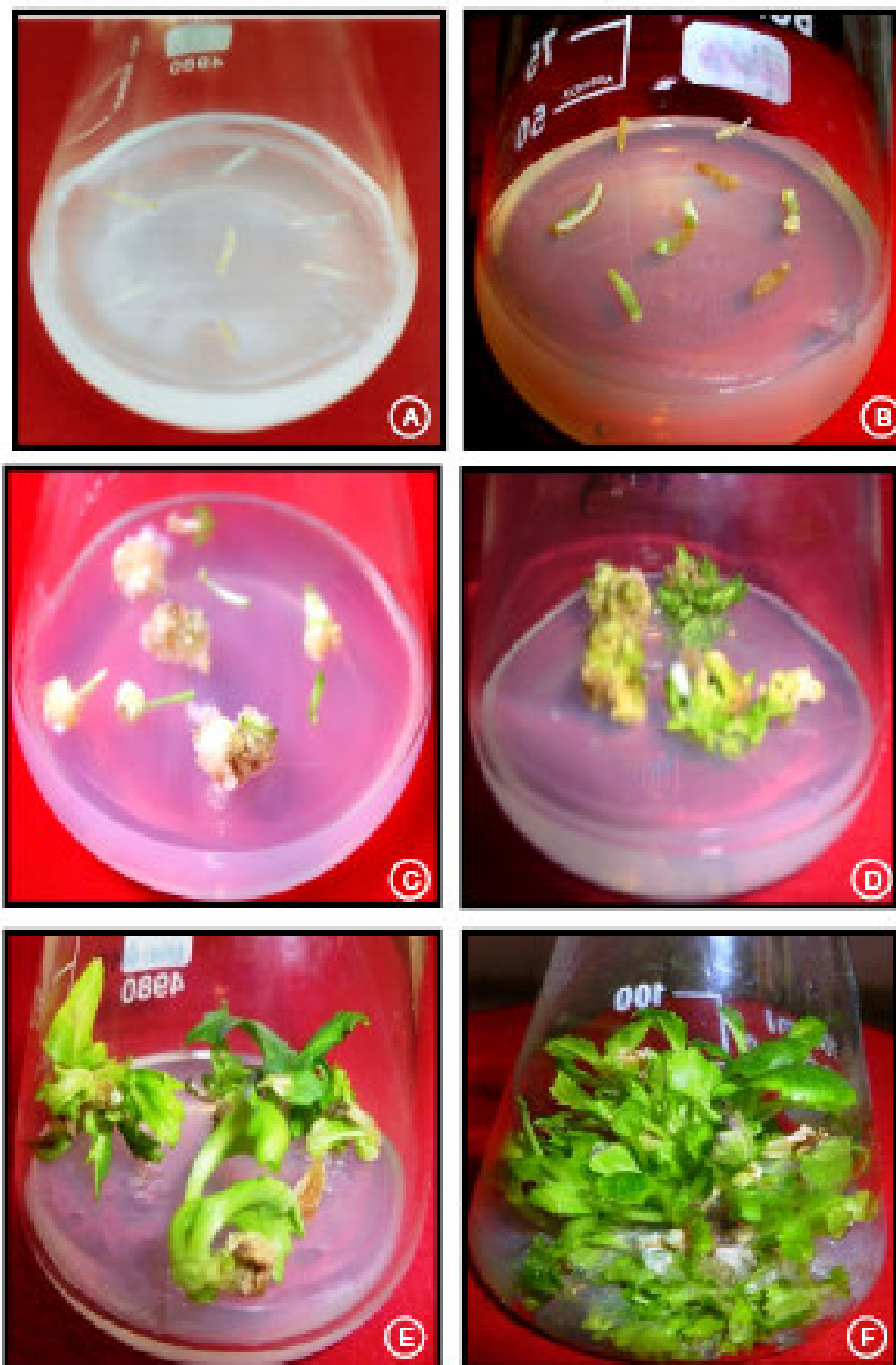


Fig. 7(A-F)

Fig. 8(A-E): Plant regeneration studies in leaf explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India) using BAP and NAA in MS basal medium.

- A. Inoculated leaf explants on shoot regeneration medium DL5 (MS basal + 1.5mg/l BAP + 0.5mg/l NAA) at day 0 in culture.
- B. Leaf explants showing swelling and expansion after 7 days in culture on medium DL5 (MS basal + 1.5mg/l BAP + 0.5mg/l NAA).
- C. Shoot initiation from leaf explants after 30 days in culture on medium DL5 (MS basal medium + 1.5mg/l BAP + 0.5mg/l NAA).**
- D. Shoot formation from leaf explants after 40 days in culture on medium DL5 (MS basal + 1.5mg/l BAP + 0.5mg/l NAA).
- E. Shoot elongation and multiplication after 52 days in culture on medium DL5 (MS basal + 1.5mg/l BAP + 0.5mg/l NAA).



Fig. 8(A-E)

Fig. 9(A-F): Plant regeneration studies in leaf explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. *Pride of India*) using TDZ and NAA in MS basal medium.

- A. Inoculated leaf explants on shoot regeneration medium GL2 (MS basal + 0.220 mg/l TDZ + 0.02mg/l NAA) at day 0 in culture.
- B. Leaf explants showing swelling and expansion after 10 days in culture on medium GL2 (MS basal + 0.220 mg/l TDZ + 0.02mg/l NAA).
- C. Leaf explants showing callus initiation after 22 days in culture on medium GL2 (MS basal + 0.220mg/l TDZ + 0.02mg/l NAA).
- D. Callus proliferation from leaf explants after 30 days in culture on medium GL2 (MS basal + 0.220mg/l TDZ + 0.02mg/l NAA).
- E. Shoot formation for leaf explants after 45 days in culture on medium GL2 (MS basal + 0.220mg/l TDZ + 0.02mg/l NAA).
- F. Shoot elongation and multiplication after 58 days in culture on medium GL2 (MS basal + 0.220mg/l TDZ + 0.02mg/l NAA).



Fig. 9(A-F)

Fig. 10(A-E): Plant regeneration studies in petiole explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. **Pride of India) using Kinetin and NAA in MS basal medium.**

- A. Petiole explants cultured on shoot regeneration medium BP7 (MS basal + 2.0mg/l Kn + 0.25mg/l NAA) at 0 day in culture.
- B. Petiole explants showing swelling after 10 days in culture on medium BP7 (MS basal + 2.0mg/l Kn + 0.25mg/l NAA).
- C. Petiole explants showing direct shoot initiation after 32 days in culture on medium BP7 (MS basal + 2.0mg/l Kn + 0.25mg/l NAA).**
- D. Shoot proliferation from petiole explants after 43 days in culture on medium BP7 (MS basal + 2.0mg/l Kn + 0.25mg/l NAA).
- E. Shoot elongation and multiplication after 55 days in culture on medium BP7 (MS basal + 2.0mg/l Kn + 0.25mg/l NAA).

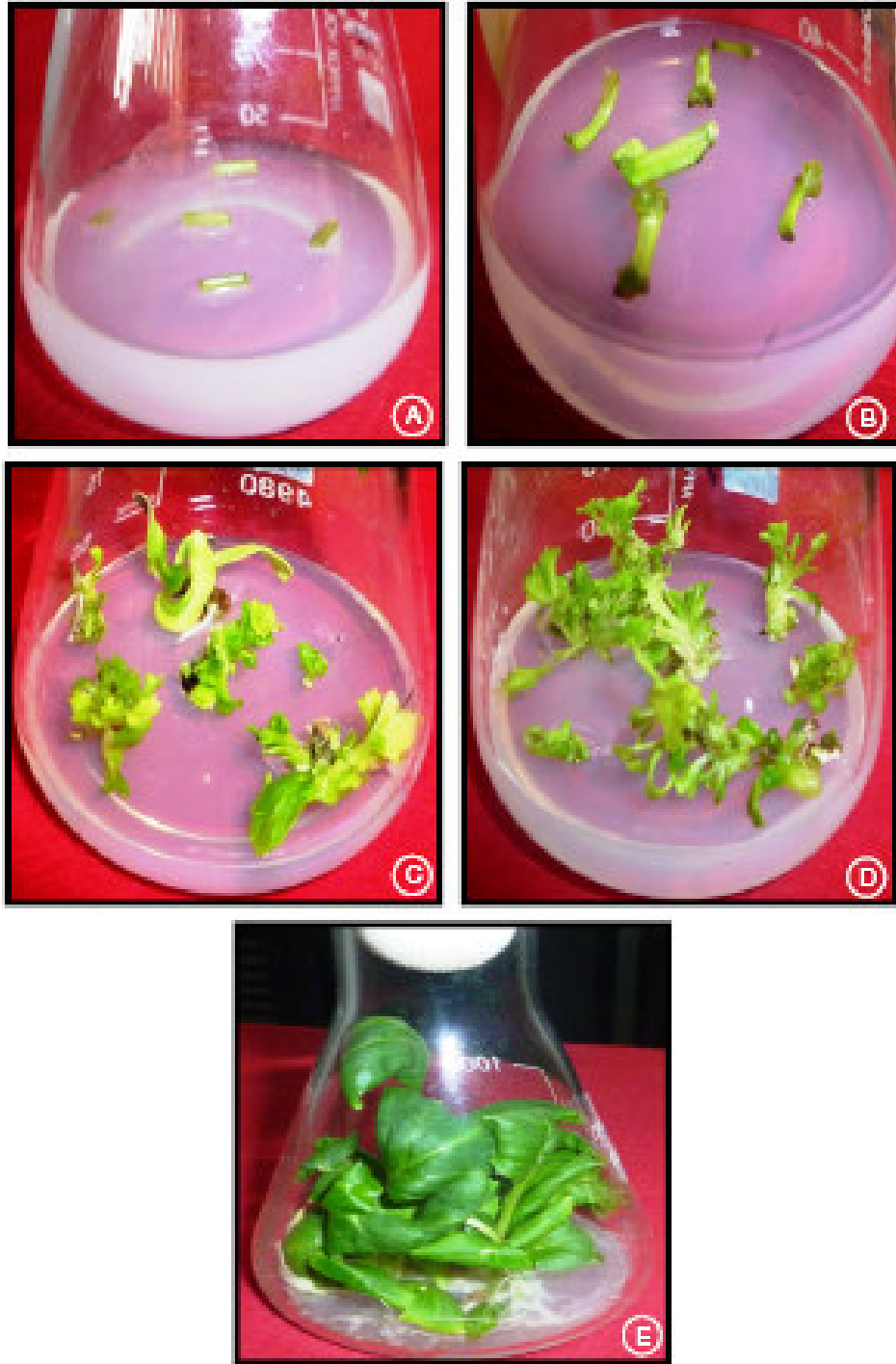


Fig. 10(A-E)

Fig. 11(A-F): Plant regeneration studies in petiole explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. *Pride of India*) using TDZ and NAA in MS basal medium.

- A. Inoculated petiole explants on shoot regeneration medium GP3 (MS basal + 0.330mg/l TDZ + 0.02mg/l NAA) at day 0 in culture.
- B. Petiole explants showing swelling after 9 days in culture on medium GP3 (MS basal + 0.330mg/l + 0.02mg/l NAA).
- C. Petiole explants showing callus initiation after 16 days in culture on medium GP3 (MS basal + 0.330mg/l + 0.02mg/l NAA).
- D. Petiole explants showing callus formation after 28 days in culture on medium GP3 (MS basal + 0.330mg/l + 0.02mg/l NAA).
- E. Shoot formation from petiole explants after 42 days in culture on medium GP3 (MS basal + 0.330mg/l + 0.02mg/l NAA).
- F. Shoot elongation and multiplication after 55 days in culture on medium GP3 (MS basal + 0.330mg/l + 0.02mg/l NAA).

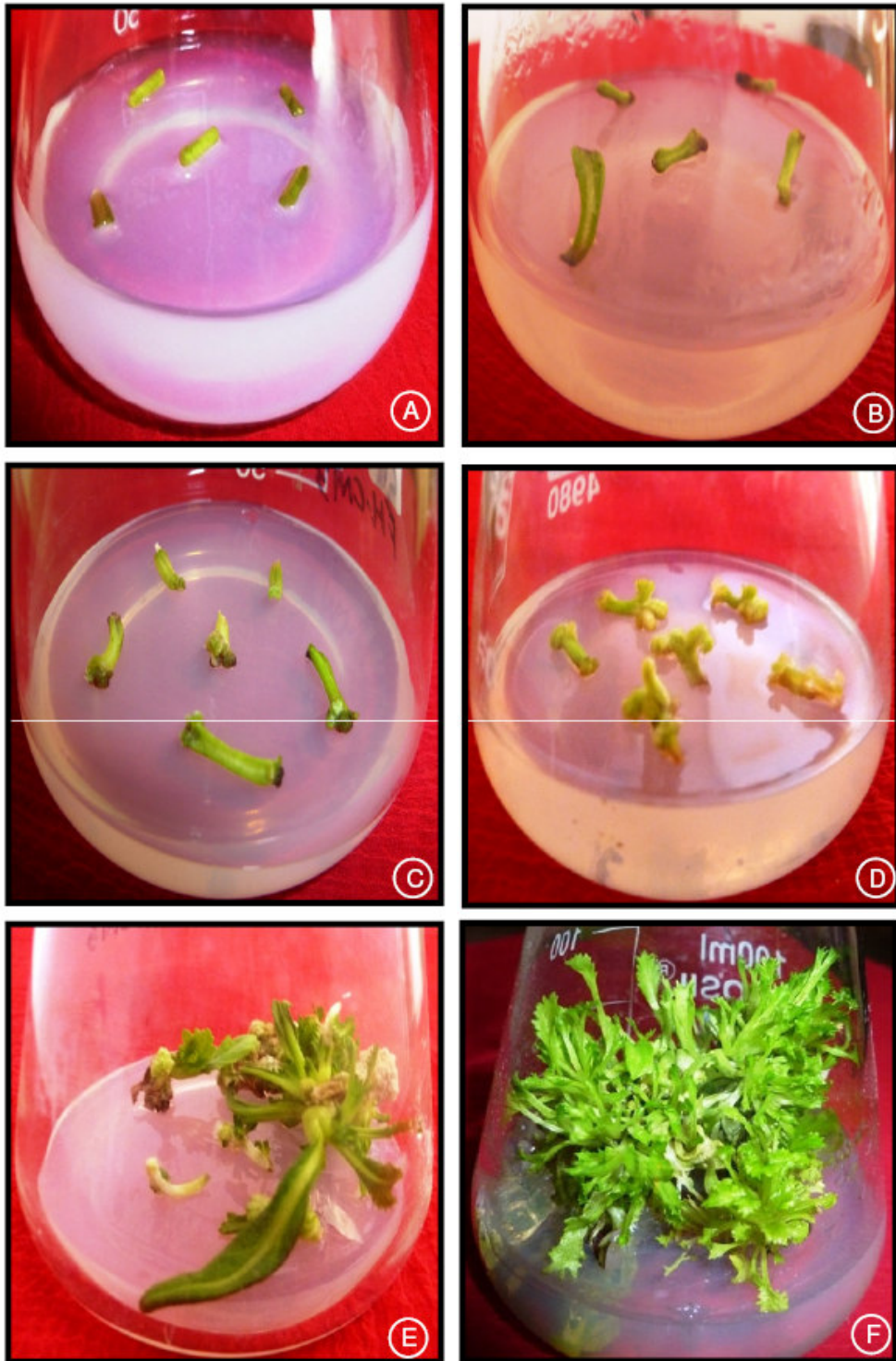


Fig. 11(A-F)

Fig. 12(A-E): Root regeneration in *in vitro* developed shoots of cabbage (*Brassica oleracea* L. var. *capitata* cv. **Pride of India).**

- A. *In vitro* developed shoots transferred to root regeneration medium at day 0 in culture.
- B. Root regeneration in *in vitro* developed shoots after 9 days of transfer to the medium II-RR₂ (MS basal + 0.10mg/l NAA).
- C. Root regeneration in *in vitro* developed shoots after 15 days of transfer to the medium II-RR₂ (MS basal + 0.10mg/l NAA).
- D. Root regeneration in *in vitro* developed shoots after 15 days of transfer to the medium II-RR₂ (MS basal + 0.10mg/l NAA).
- E. Fully developed plantlets of cabbage taken out of medium (after proper washing) after 15 days of culturing showing well developed root system. (Later on transferred to pots containing pre-sterilized cocopeat for acclimalization).

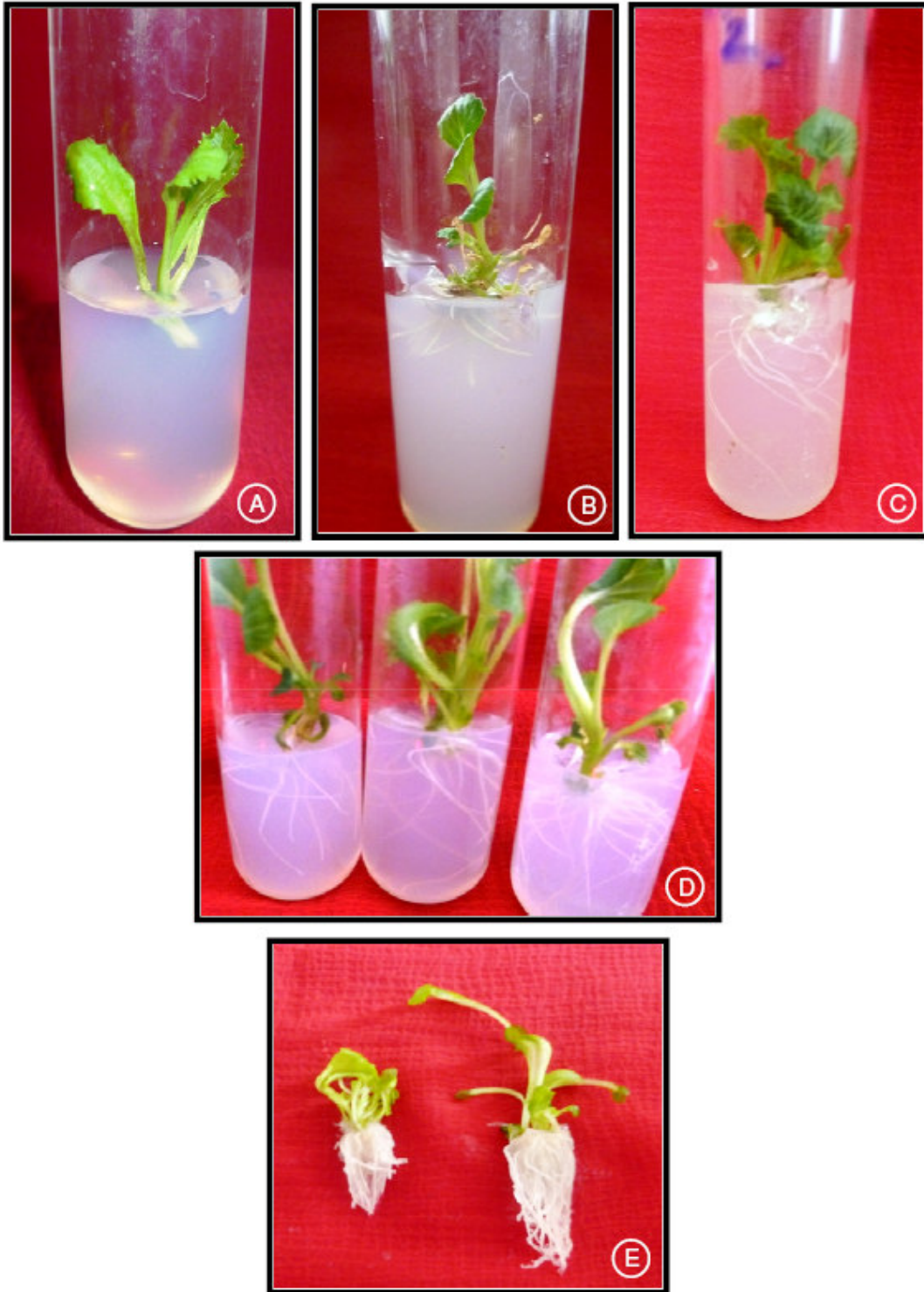


Fig. 12(A-E)

Fig. 13(A-D): Acclimatization of *in vitro* regenerated plantlets of cabbage (*Brassica oleracea* L. var. *capitata* cv. **Pride of India).**

- A. *In vitro* regenerated plantlets kept for hardening in pre-sterilized cocopeat mixture covered with transparent polythene bags to maintain relative humidity at 0 day.
- B. Healthy plantlets uncovered after 2 weeks of hardening on cocopeat mixture.
- C. Plantlets transferred to potting mixture (containing sand + soil + FYM).
- D. Acclimatized plants after 15 days of transfer to potting mixture (containing sand + soil + FYM).



Fig. 13(A-D)

Fig. 14(A-H): Kanamycin sensitivity and its effect on the cultured leaf explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. *Pride of India*).

- A. Leaf explants cultured on selective medium (MS basal + 1.50mg/l BAP + 0.50mg/l NAA + 10mg/l Kanamycin) at day 0 in culture.
- B. Leaf explants cultured on selective medium (MS basal + 1.50mg/l BAP + 0.50mg/l NAA + 50mg/l Kanamycin) at day 0 in culture.
- C. Leaf explants showing expansion on selective medium (MS basal + 1.50mg/l BAP + 0.50mg/l NAA + 10mg/l Kanamycin) at 14 days of culturing.
- D. Leaf explants showing poor growth on selective medium (MS basal + 1.50mg/l BAP + 0.50mg/l NAA + 50mg/l Kanamycin) at 14 days of culturing.
- E. Leaf explants showing callus initiation on selective medium (MS basal + 1.50mg/l BAP + 0.50mg/l NAA + 10mg/l Kanamycin) at 21 days of culturing.
- F. Leaf explants showing poor growth and change in colour from green to brown on selective medium (MS basal + 1.50mg/l BAP + 0.50mg/l NAA + 50mg/l Kanamycin) at 21 days of culturing.
- G. Leaf explants showing callusing on selective medium (MS basal + 1.50mg/l BAP + 0.50mg/l NAA + 10mg/l Kanamycin) at 35 days of culturing.
- H. Leaf explants turned brown (dead) on selective medium (MS basal + 1.50mg/l BAP + 0.50mg/l NAA + 50mg/l Kanamycin) at 35 days of culturing.

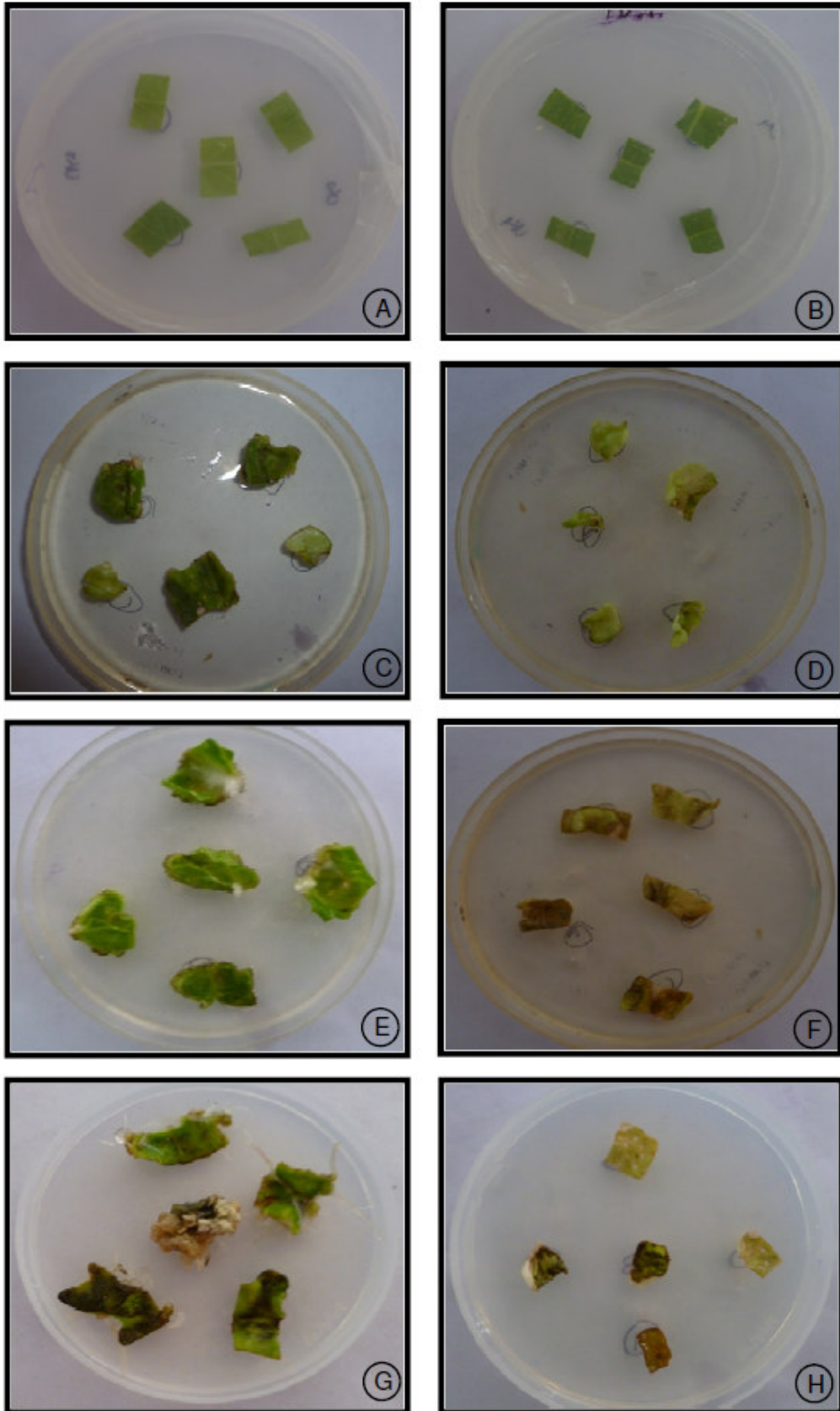


Fig. 14(A-H)

Fig. 15(A-H): Kanamycin sensitivity and its effect on the cultured petiole explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. *Pride of India*).

- A. Petiole explants cultured on selective medium (MS basal + 2.0mg/l Kn + 0.25mg/l NAA + 10mg/l Kanamycin) at day 0 in culture.
- B. Petiole explants cultured on selective medium (MS basal + 2.0mg/l Kn + 0.25mg/l NAA + 50mg/l Kanamycin) at day 0 in culture.
- C. Petiole explants showing swelling on selective medium (MS basal + 2.0mg/l Kn + 0.25mg/l NAA + 10mg/l Kanamycin) at 14 days of culturing.
- D. Petiole explants showing change in colour of explants green to brown on selective medium (MS basal + 2.0mg/l Kn + 0.25mg/l NAA + 50mg/l Kanamycin) at 14 days of culturing.
- E. Petiole explants showing callus initiation on selective medium (MS basal + 2.0mg/l Kn + 0.25mg/l NAA + 10mg/l Kanamycin) at 21 days of culturing.
- F. Petiole explants showing poor growth and change in colour from green to brown on selective medium (MS basal + 2.0mg/l Kn + 0.25mg/l NAA + 50mg/l Kanamycin) at 21 days of culturing.
- G. Petiole explants showing shoot initiation on selective medium (MS basal + 2.0mg/l Kn + 0.25mg/l NAA + 10mg/l Kanamycin) at 35 days of culturing.
- H. Petiole explants turned brown (dead) on selective medium (MS basal + 2.0mg/l Kn + 0.25mg/l NAA + 50mg/l Kanamycin) at 35 days of culturing.

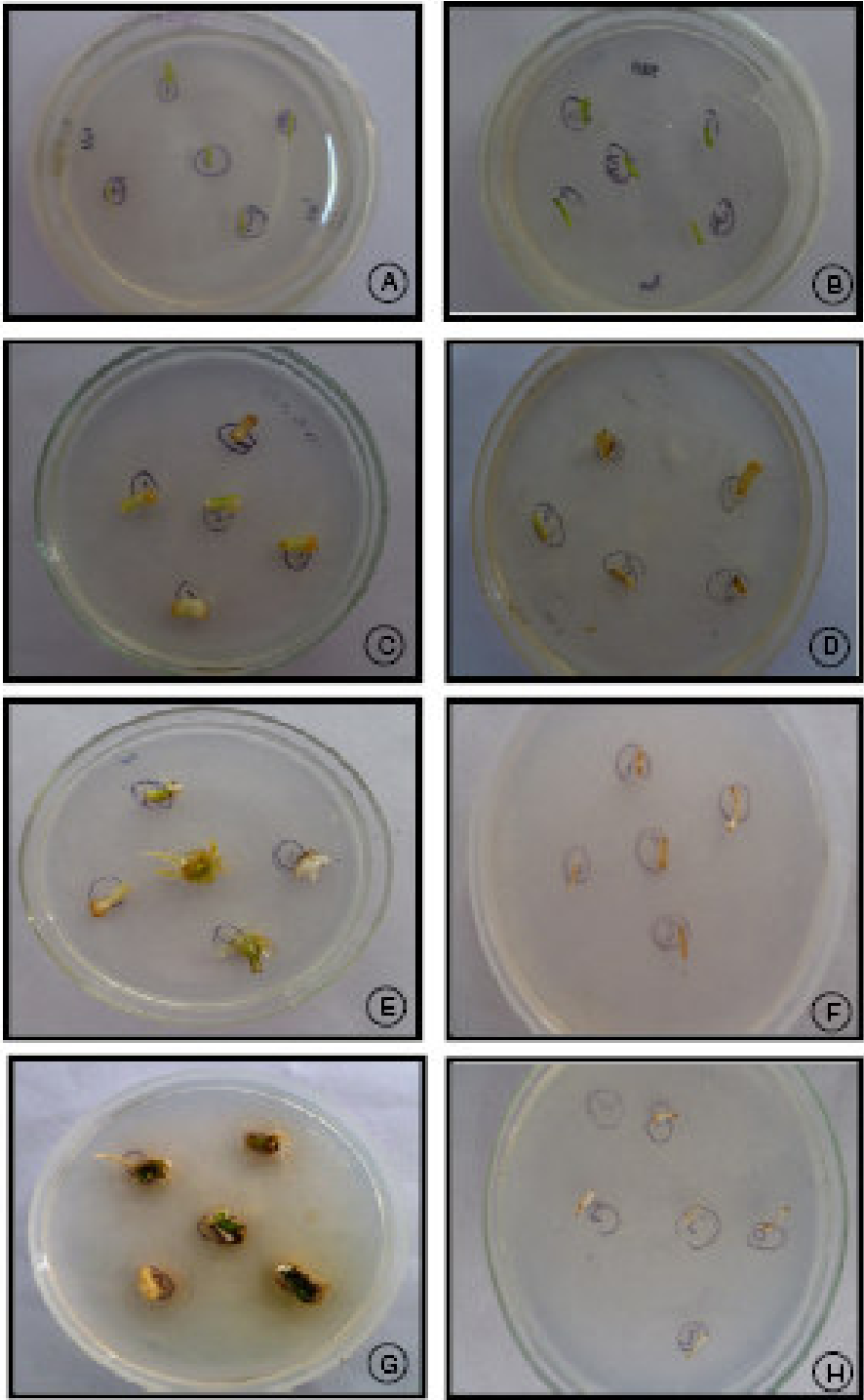


Fig. 15(A-H)

Fig. 16(A-F): Effect of cefotaxime on the regeneration potential of cotyledon explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. *Pride of India*) (without co-cultivation).

- A. Cotyledon explants cultured on selective medium (MS basal + 2.0mg/l Kn + 0.50mg/l NAA + 500mg/l Cefotaxime) at 0 day in culture.
- B. Cotyledon explants showing expansion on selective medium (MS basal + 2.0mg/l Kn + 0.50mg/l NAA + 500mg/l Cefotaxime) after 7 days of culturing.
- C. Cotyledon explants showing callus initiation on selective medium (MS basal + 2.0mg/l Kn + 0.50mg/l NAA + 500mg/l Cefotaxime) after 13 days of culturing.
- D. Cotyledon explants showing callus and rooting on selective medium (MS basal + 2.0mg/l Kn + 0.50mg/l NAA + 500mg/l Cefotaxime) after 20 days of culturing.
- E. Cotyledon explants showing shoot initiation on selective medium (MS basal + 2.0mg/l Kn + 0.50mg/l NAA + 500mg/l Cefotaxime) after 30 days of culturing.
- F. Shoot elongation from cotyledon explants on selective medium (MS basal + 2.0mg/l Kn + 0.50mg/l NAA + 500mg/l Cefotaxime) after 45 days of culturing.



Fig. 18(A-F)

Fig. 17(A-F): Effect of cefotaxime on the regeneration potential of hypocotyl explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. *Pride of India*) (without co-cultivation).

- A. Hypocotyl explants cultured on selective medium (MS basal + 1.5mg/l Kn + 0.25mg/l IAA + 500mg/l Cefotaxime) at 0 day in culture.
- B. Hypocotyl explants showing swelling cultured on selective medium (MS basal + 1.5mg/l Kn + 0.25mg/l IAA + 500mg/l Cefotaxime) after 10 days of culturing.
- C. Hypocotyl explants showing shoot initiation on selective medium (MS basal + 1.5mg/l Kn + 0.25mg/l IAA + 500mg/l Cefotaxime) after 22 days of culturing.
- D. Hypocotyl explants showing shoot regeneration on selective medium (MS basal + 1.5mg/l Kn + 0.25mg/l IAA + 500mg/l Cefotaxime) after 28 days of culturing.
- E. Hypocotyl explants showing shoot proliferation on selective medium (MS basal + 1.5mg/l Kn + 0.25mg/l IAA + 500mg/l Cefotaxime) after 39 days of culturing.
- F. Shoot elongation from hypocotyl explants on selective medium (MS basal + 1.5mg/l Kn + 0.25mg/l IAA + 500mg/l Cefotaxime) after 50 days of culturing.

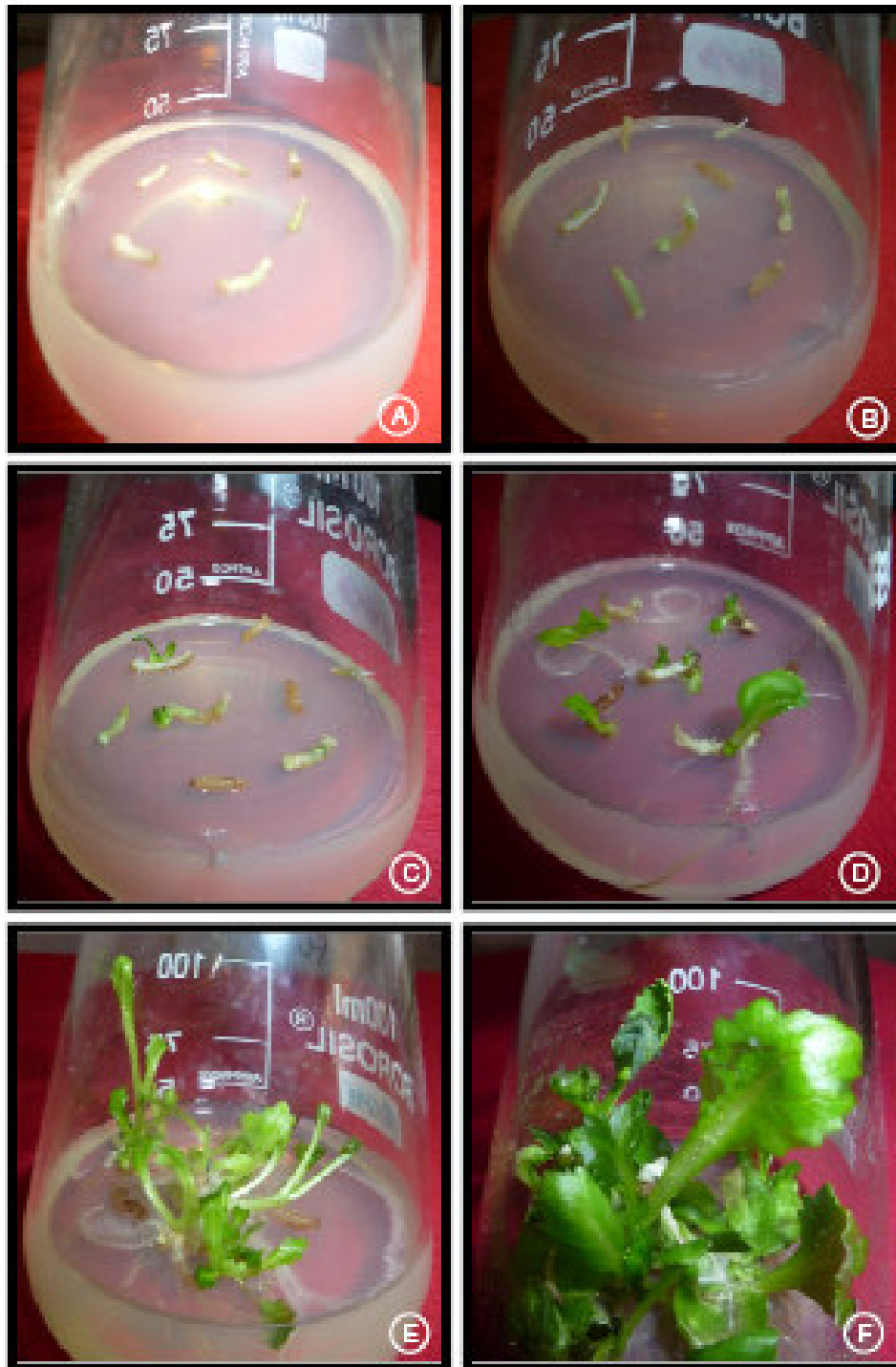


Fig. 17(A-F)

Fig. 18(A-F): Effect of cefotaxime and kanamycin (50 mg/l) on the regeneration potential of cotyledon explants and growth of agrobacterial cells in cabbage (*Brassica oleracea* L. var. *capitata* cv. *Pride of India*) (after co-cultivation).

- A. Cotyledon explants cultured on selective shoot regeneration medium (MS basal + 2.0mg/l Kn + 0.5mg/l NAA + 50mg/l Kanamycin + **0mg/l Cefotaxime**) showing overgrowth of agrobacterial cells.
- B. Cotyledon explants cultured on selective shoot regeneration medium (MS basal + 2.0mg/l Kn + 0.5mg/l NAA + 50mg/l Kanamycin + **100mg/l Cefotaxime**) showing overgrowth of agrobacterial cells.
- C. Cotyledon explants cultured on selective shoot regeneration medium (MS basal + 2.0mg/l Kn + 0.5mg/l NAA + 50mg/l Kanamycin + **200mg/l Cefotaxime**) showing overgrowth of agrobacterial cells.
- D. Cotyledon explants cultured on selective shoot regeneration medium (MS basal + 2.0mg/l Kn + 0.5mg/l NAA + 50mg/l Kanamycin + **300mg/l Cefotaxime**) showing growth of agrobacterial cells and expansion of explants.
- E. Cotyledon explants cultured on selective shoot regeneration medium (MS basal + 2.0mg/l Kn + 0.5mg/l NAA + 50mg/l Kanamycin + **400mg/l Cefotaxime**) showing expansion and callus initiation and very less growth of agrobacterial cells.
- F. Cotyledon explants cultured on selective shoot regeneration medium (MS basal + 2.0mg/l Kn + 0.5mg/l NAA + 50mg/l Kanamycin + **500mg/l Cefotaxime**) showing shoot regeneration and no growth of agrobacterial cells.

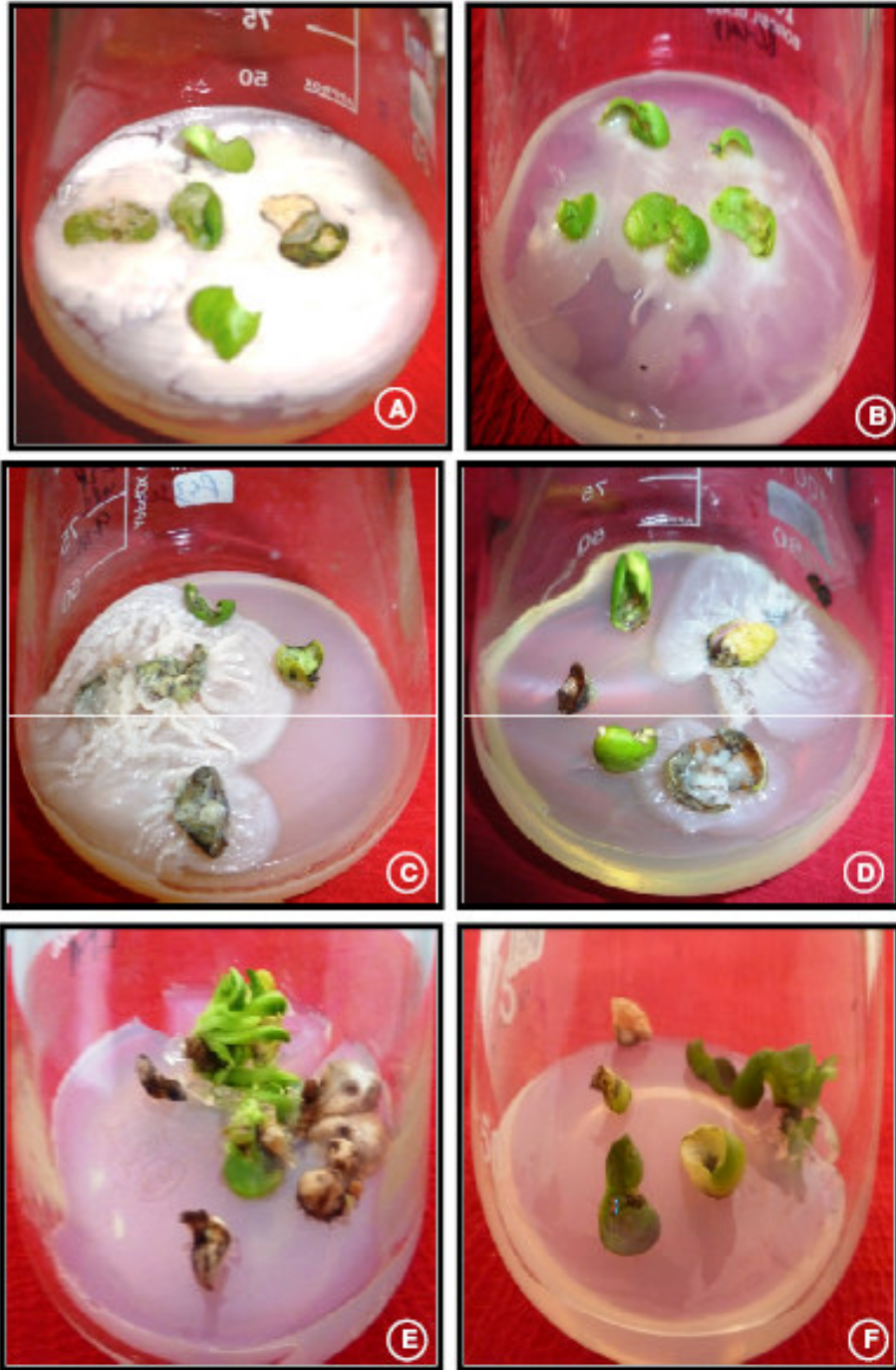


Fig. 18(A-F)

Fig. 19(A-F): Effect of cefotaxime and kanamycin (50mg/l) on the regeneration potential of hypocotyl explants and growth of agrobacterial cells in cabbage (*Brassica oleracea* L. var. *capitata* cv. *Pride of India*) (after co-cultivation).

- A. Hypocotyl explants cultured on selective shoot regeneration medium (MS basal + 1.5mg/l Kn + 0.25mg/l IAA + 50mg/l Kanamycin + **0mg/l Cefotaxime**) showing overgrowth of agrobacterial cells.
- B. Hypocotyl explants cultured on selective shoot regeneration medium (MS basal + 1.5mg/l Kn + 0.25mg/l IAA + 50mg/l Kanamycin + **100mg/l Cefotaxime**) showing overgrowth of agrobacterial cells.
- C. Hypocotyl explants cultured on selective shoot regeneration medium (MS basal + 1.5mg/l Kn + 0.25mg/l IAA + 50mg/l Kanamycin + **200mg/l Cefotaxime**) showing overgrowth of agrobacterial cells.
- D. Hypocotyl explants cultured on selective shoot regeneration medium (MS basal + 1.5mg/l Kn + 0.25mg/l IAA + 50mg/l Kanamycin + **300mg/l Cefotaxime**) showing growth of agrobacterial cells and swelling of explants.
- E. Hypocotyl explants cultured on selective shoot regeneration medium (MS basal + 1.5mg/l Kn + 0.25mg/l IAA + 50mg/l Kanamycin + **400mg/l Cefotaxime**) showing shoot regeneration and no growth of agrobacterial cells.
- F. Hypocotyl explants cultured on selective shoot regeneration medium (MS basal +1.5mg/l Kn + 0.25mg/l IAA + 50mg/l Kanamycin + **500mg/l Cefotaxime**) showing shoot regeneration and no growth of agrobacterial cells.

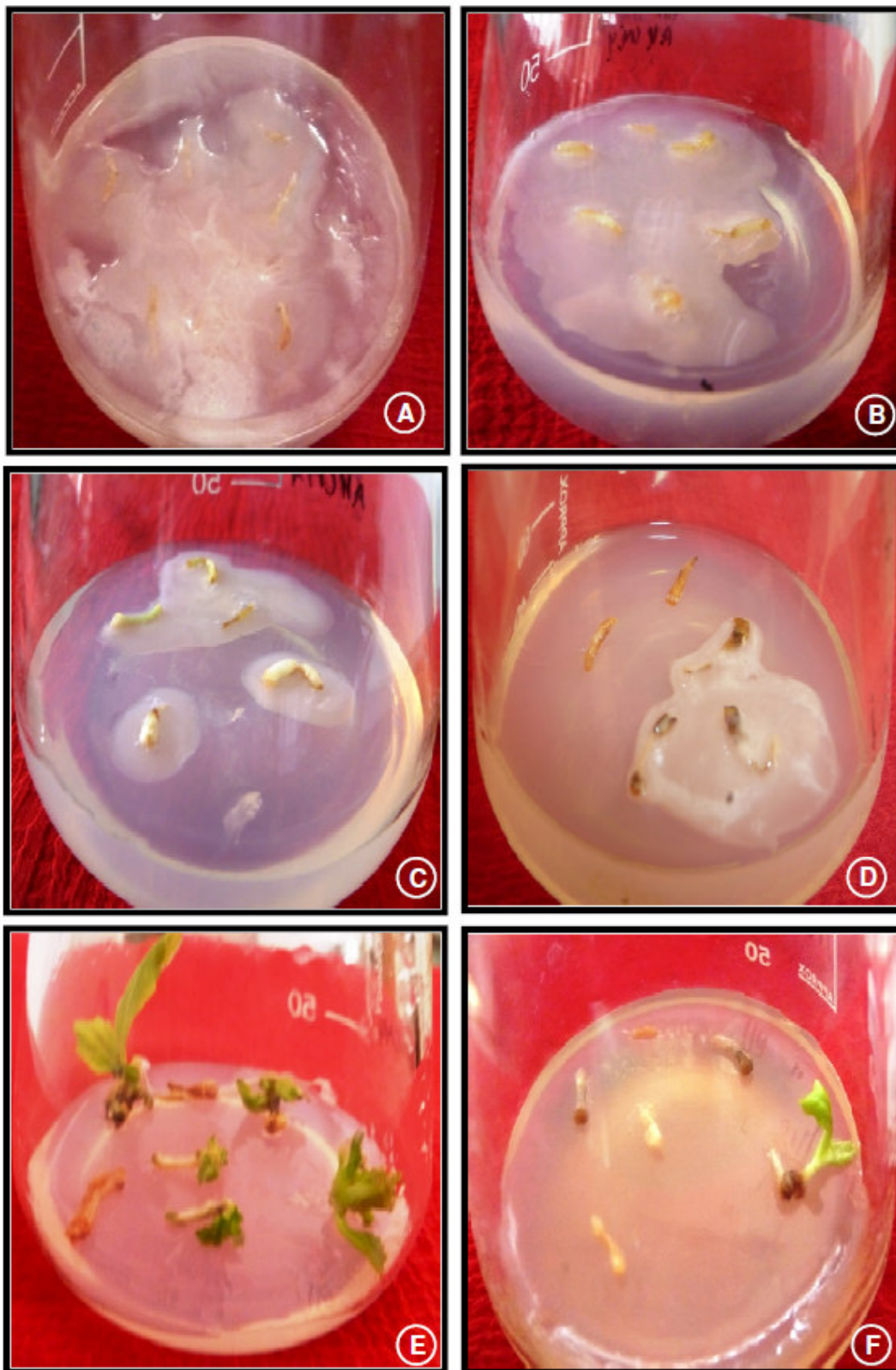


Fig. 19(A-F)

Fig. 20(A-F): Genetic transformation studies in cotyledon explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India).

Preculturing and co-cultivation of cotyledon explants on GTC1 medium (MS basal + 2.0mg/l Kn + 0.50mg/l NAA).

- A. Cotyledon explants cultured on GTC1 medium (after 24 hours of preculturing).
- B. Cotyledon explants cultured on GTC1 medium (after 48 hours of preculturing).
- C. Cotyledon explants cultured on GTC1 medium (after 72 hours of preculturing).
- D. Cotyledon explants cultured on GTC1 medium (after 48 hours of co-cultivation).
- E. Cotyledon explants cultured on GTC1 medium (after 72 hours of co-cultivation).
- F. Cotyledon explants cultured on GTC1 medium (after 96 hours of co-cultivation).

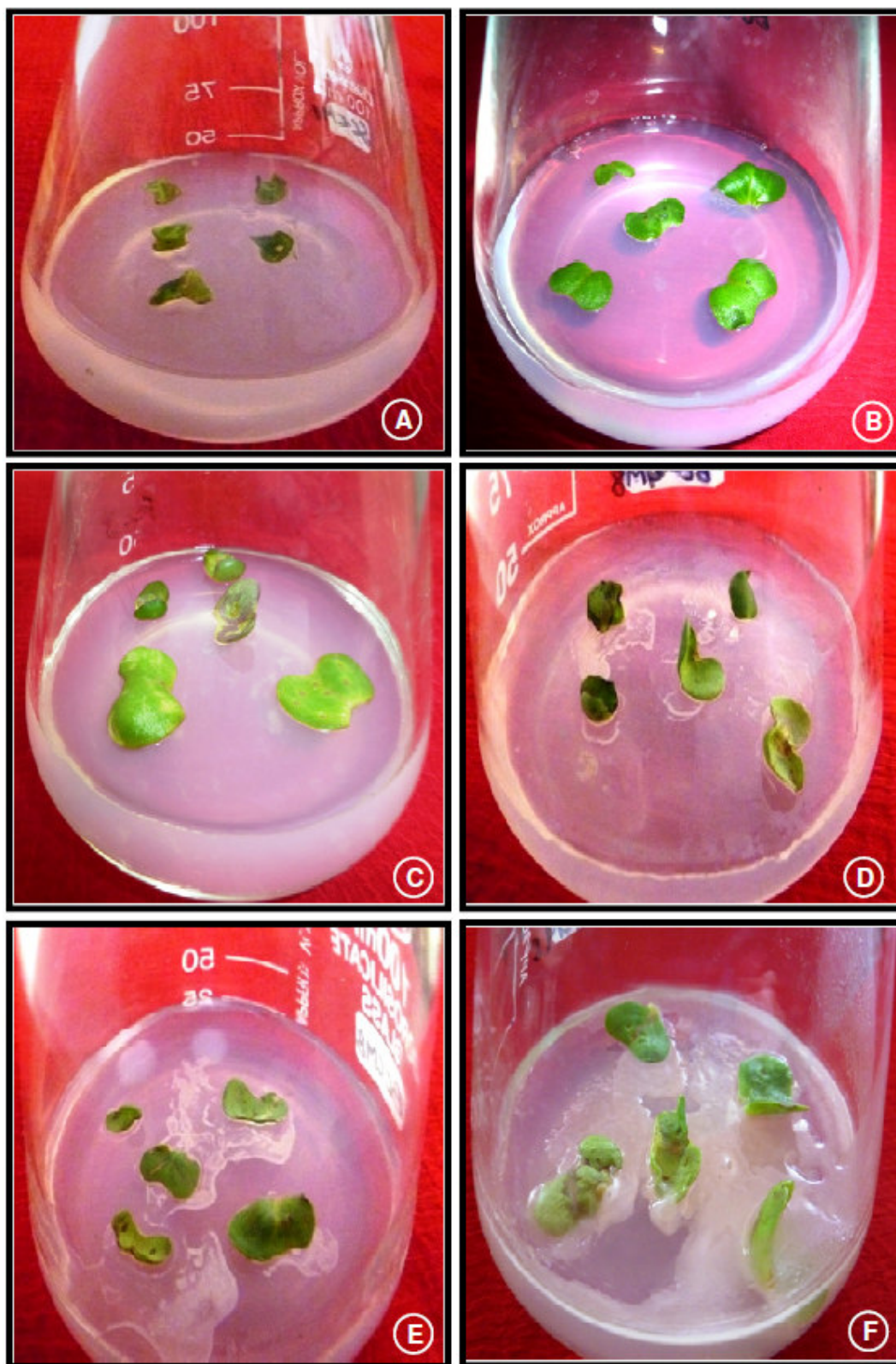


Fig. 20(A-F)

Fig. 21(A-F): Genetic transformation studies in hypocotyl explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India).

Preculturing and co-cultivation of hypocotyl explants on GTH1 medium (MS basal + 1.5mg/l Kn + 0.25mg/l IAA).

- A. Hypocotyl explants cultured on GTH1 medium (after 24 hours of preculturing).
- B. Hypocotyl explants cultured on GTH1 medium (after 48 hours of preculturing).
- C. Hypocotyl explants cultured on GTH1 medium (after 72 hours of preculturing).
- D. Hypocotyl explants cultured on GTH1 medium (after 48 hours of co-cultivation).
- E. Hypocotyl explants cultured on GTH1 medium (after 72 hours of co-cultivation).
- F. Hypocotyl explants cultured on GTH1 medium (after 96 hours of co-cultivation).

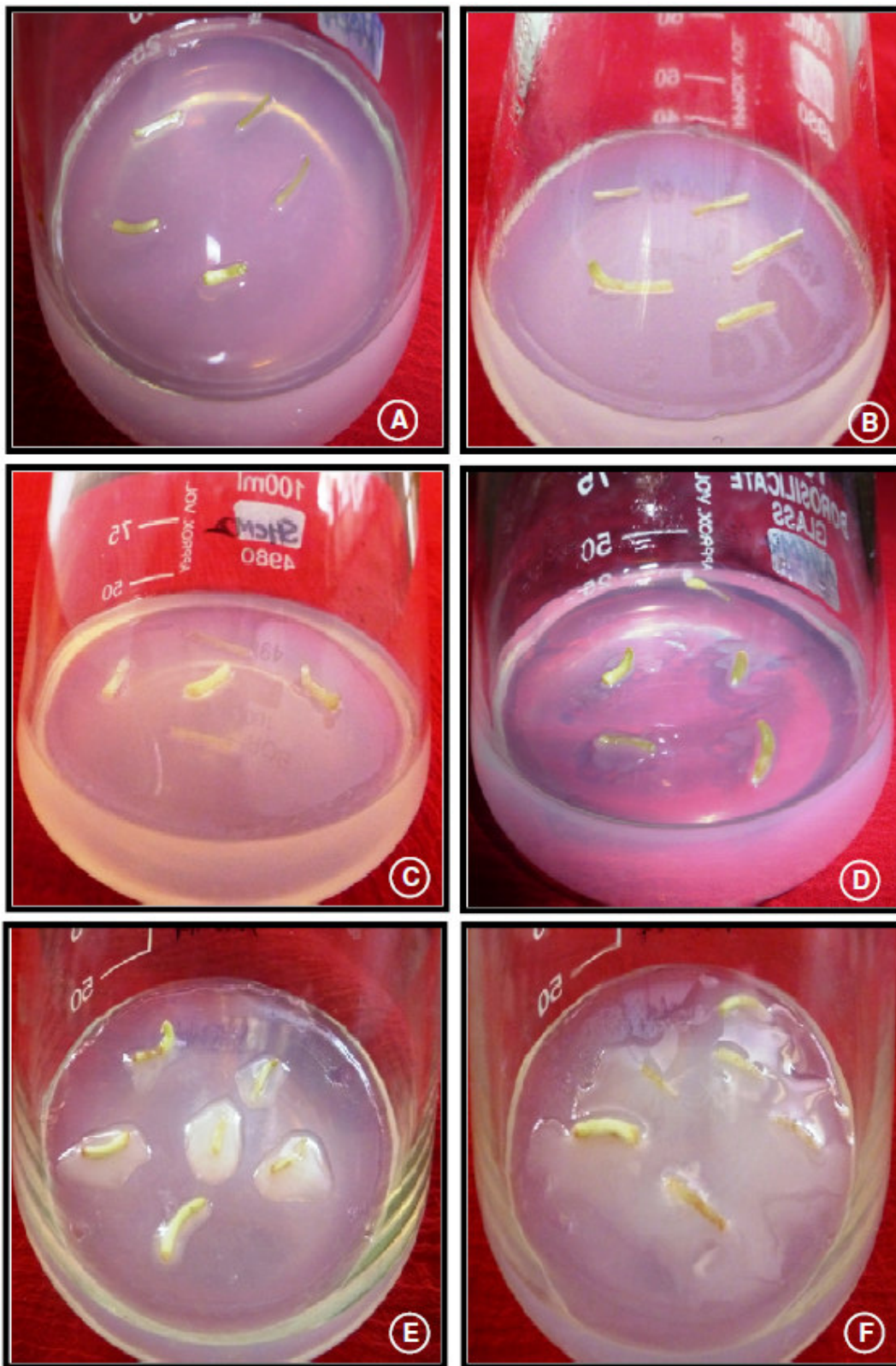


Fig. 21(A-F)

Fig. 22(A-F): Genetic transformation studies in cotyledon explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. **Pride of India).**

- A. Cotyledon explants pre-cultured on shoot regeneration GTC1 medium (MS basal + 2.0mg/l Kn + 0.50mg/l NAA) for 72 hours.
- B. Cotyledon explants co-cultivated with genetically engineered *Agrobacterium tumefaciens* LBA 4404 strain on GTC1 medium (MS basal + 2.0mg/l Kn + 0.50mg/l NAA) for 48 hours.
- C. After co-cultivation the cotyledon explants transferred to selective shoot regeneration GTC1 medium (MS basal + 2.0mg/l Kn + 0.50mg/l NAA + 50mg/l Kanamycin + 400mg/l Cefotaxime) showing callus formation.
- D. Cotyledon explants transferred to selective shoot regeneration GTC1 medium (MS basal + 2.0mg/l Kn + 0.50mg/l NAA + 50mg/l Kanamycin + 400mg/l Cefotaxime) showing shoot initiation after 45 days of culturing.
- E. Cotyledon explants transferred to selective shoot regeneration GTC1 medium (MS basal + 2.0mg/l Kn + 0.50mg/l NAA + 50mg/l Kanamycin + 400mg/l Cefotaxime) showing shoot formation.
- F. Cotyledon explants transferred to selective shoot regeneration GTC1 medium (MS basal + 2.0mg/l Kn + 0.50mg/l NAA + 50mg/l Kanamycin + 400mg/l Cefotaxime) for elongation and multiplication.

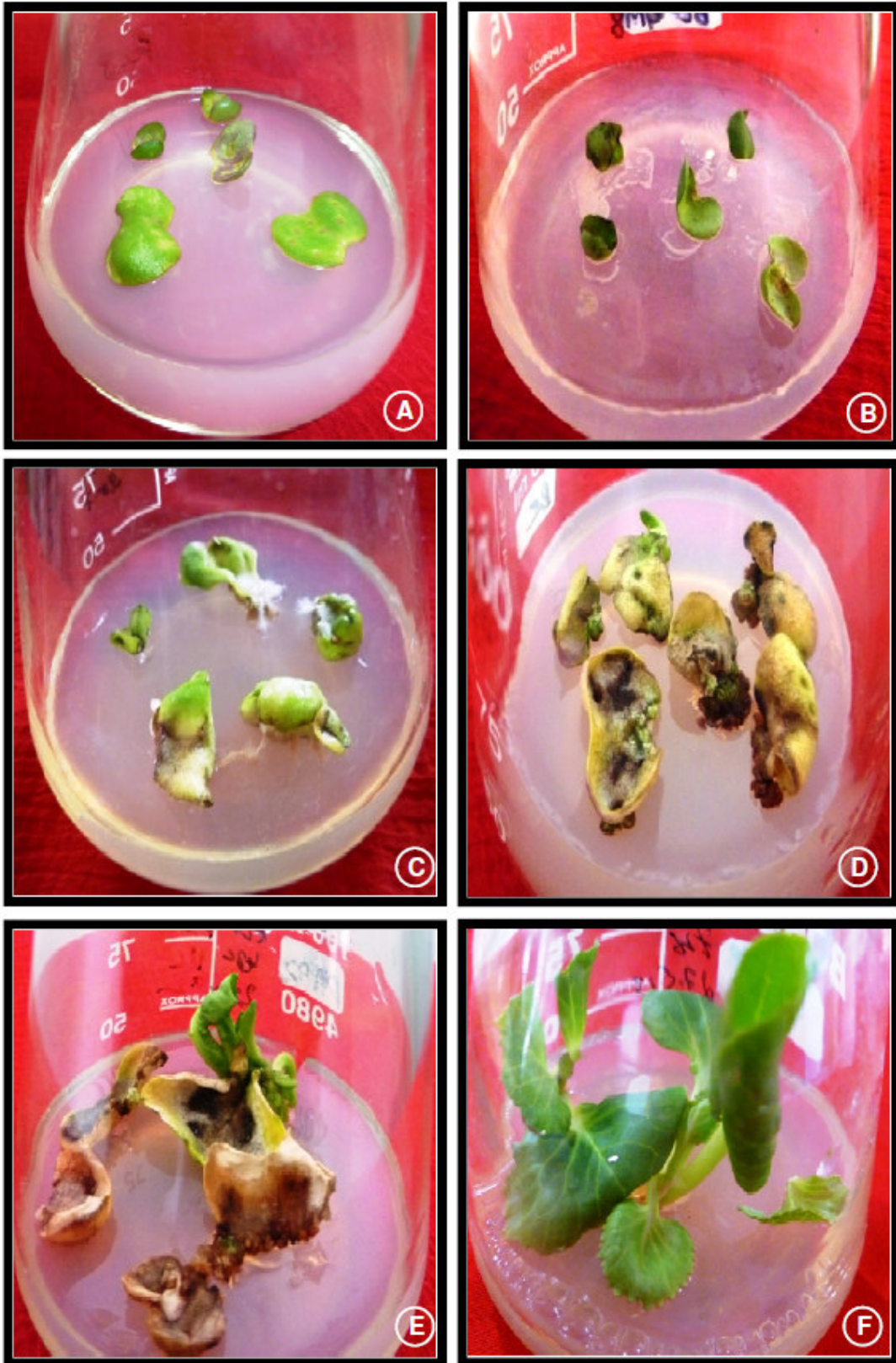


Fig. 22(A-F)

Fig. 23(A-F): Genetic transformation studies in cotyledon explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. **Pride of India).**

- A. Cotyledon explants pre-cultured on shoot regeneration GTC2 medium (MS basal + 0.330mg/l TDZ + 79.7mg/l Adenine) for 72 hours.
- B. Cotyledon explants co-cultivated with genetically engineered *Agrobacterium tumefaciens* LBA 4404 strain on GTC2 medium (MS basal + 0.330mg/l TDZ + 79.7mg/l Adenine) for 48 hours.
- C. After co-cultivation the cotyledon explants transferred to selective shoot regeneration GTC2 medium (MS basal + 0.330mg/l TDZ + 79.7mg/l Adenine + 50mg/l Kanamycin + 400mg/l Cefotaxime) showing expansion and callus initiation.
- D. Cotyledon explants transferred to selective shoot regeneration GTC2 medium (MS basal + 0.330mg/l TDZ + 79.7mg/l Adenine + 50mg/l Kanamycin + 400mg/l Cefotaxime) showing callus formation.
- E. Cotyledon explants transferred to selective shoot regeneration GTC2 medium (MS basal + 0.330mg/l TDZ + 79.7mg/l Adenine + 50mg/l Kanamycin + 400mg/l Cefotaxime) showing shoot regeneration after 77 days in culture.
- F. Cotyledon explants transferred to selective shoot regeneration GTC2 medium (MS basal + 0.330mg/l TDZ + 79.7mg/l Adenine + 50mg/l Kanamycin + 400mg/l Cefotaxime) for elongation and multiplication.

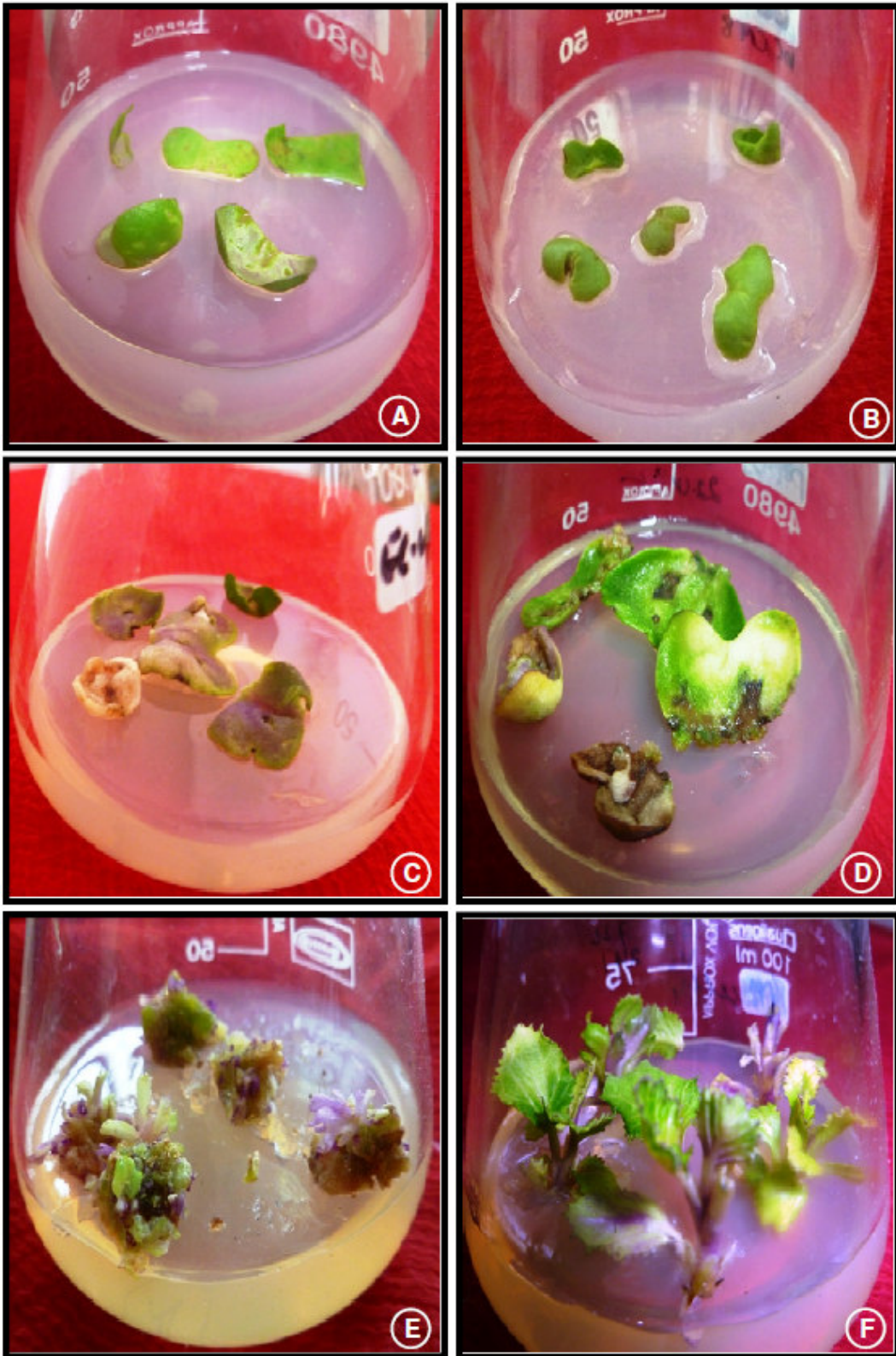


Fig. 23(A-F)

Fig. 24(A-F): Genetic transformation studies in hypocotyl explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. **Pride of India).**

- A. Hypocotyl explants pre-cultured on shoot regeneration GTH1 medium (MS basal + 1.5mg/l Kn + 0.25mg/l IAA) for 72 hours.
- B. Hypocotyl explants co-cultivated with genetically engineered *Agrobacterium tumefaciens* LBA 4404 strain on GTH1 medium (MS basal + 1.5mg/l Kn + 0.25mg/l IAA) for 48 hours.
- C. Hypocotyl explants co-cultivated with genetically engineered *Agrobacterium tumefaciens* LBA 4404 strain on GTH1 medium (MS basal + 1.5mg/l Kn + 0.25mg/l IAA) for 48 hours.
- D. Hypocotyl explants transferred to selective shoot regeneration GTH1 medium (MS basal + 1.5mg/l Kn + 0.25mg/l IAA + 50mg/l Kanamycin + 400mg/l Cefotaxime) showing shoot initiation after 44 days in culture.
- E. Hypocotyl explants transferred to selective shoot regeneration GTH1 medium (MS basal + 1.5mg/l Kn + 0.25mg/l IAA + 50mg/l Kanamycin + 400mg/l Cefotaxime) showing shoot formation.
- F. Hypocotyl explants transferred to selective shoot regeneration GTH1 medium (MS basal + 1.5mg/l Kn + 0.25mg/l IAA + 50mg/l Kanamycin + 400mg/l Cefotaxime) for elongation and multiplication.

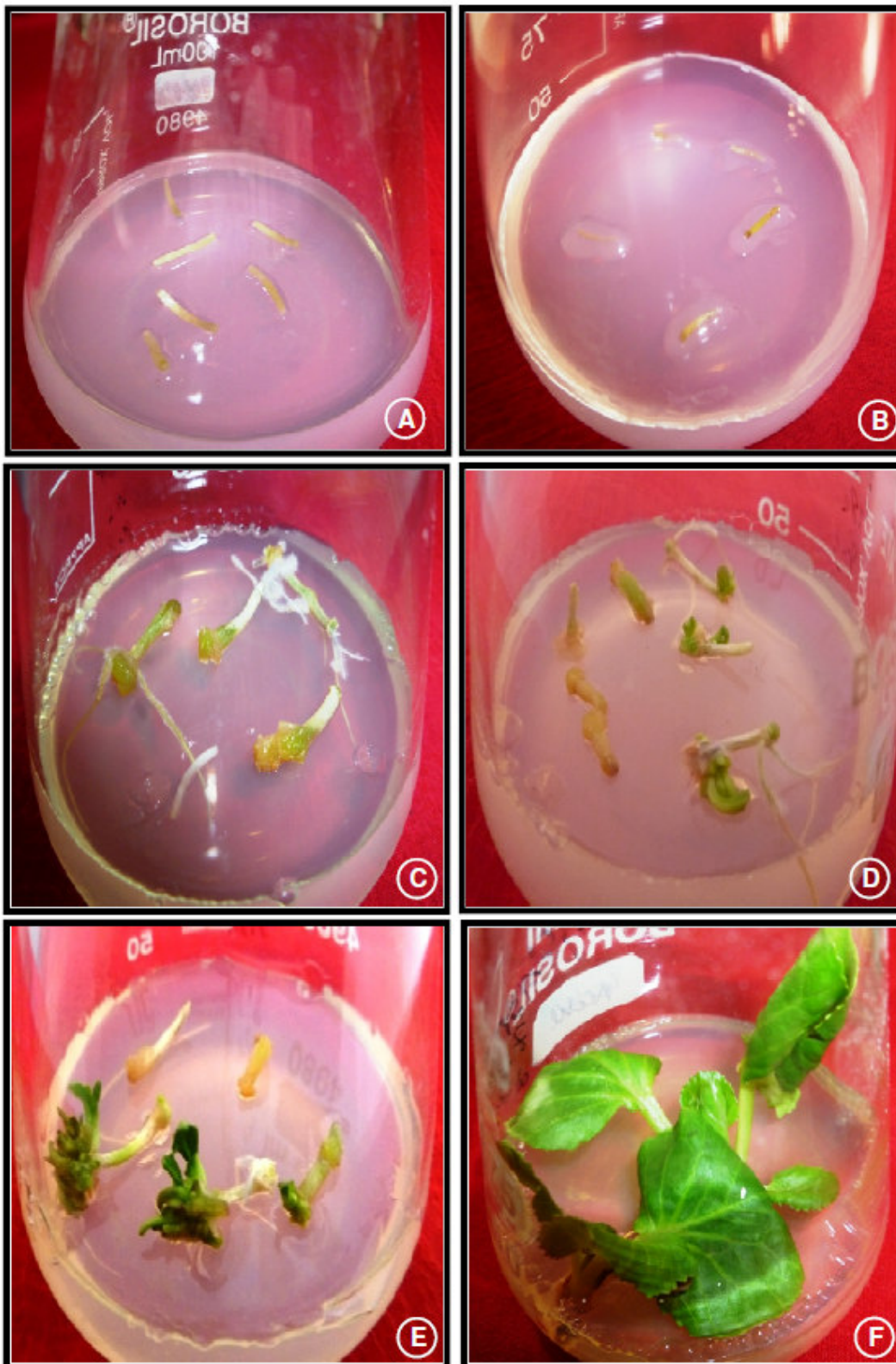


Fig. 24(A-F)

Fig. 25(A-F): Genetic transformation studies in hypocotyl explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. **Pride of India).**

- A. Hypocotyl explants pre-cultured on shoot regeneration GTH2 medium (MS basal + 0.220mg/l TDZ + 0.088mg/l IAA) for 72 hours.
- B. Hypocotyl explants co-cultivated with genetically engineered *Agrobacterium tumefaciens* LBA 4404 strain on GTH2 medium (MS basal + 0.220mg/l TDZ + 0.088mg/l IAA) for 48 hours.
- C. After co-cultivation the hypocotyl explants transferred to selective shoot regeneration GTH2 medium (MS basal + 0.220mg/l TDZ + 0.088mg/l IAA + 50mg/l Kanamycin + 400mg/l Cefotaxime) showing callus formation.
- D. Hypocotyl explants transferred to selective shoot regeneration GTH2 medium (MS basal + 0.220mg/l TDZ + 0.088mg/l IAA + 50mg/l Kanamycin + 400mg/l Cefotaxime) showing shoot initiation after 48 days in culture.
- E. Hypocotyl explants transferred to selective shoot multiplication GTH2 medium (MS basal + 0.220mg/l TDZ + 0.088mg/l IAA + 50mg/l Kanamycin + 400mg/l Cefotaxime) showing shoot formation.
- F. Hypocotyl explants transferred to selective shoot regeneration GTH2 medium (MS basal + 0.220mg/l TDZ + 0.088mg/l IAA + 50mg/l Kanamycin + 400mg/l Cefotaxime) for elongation and multiplication.

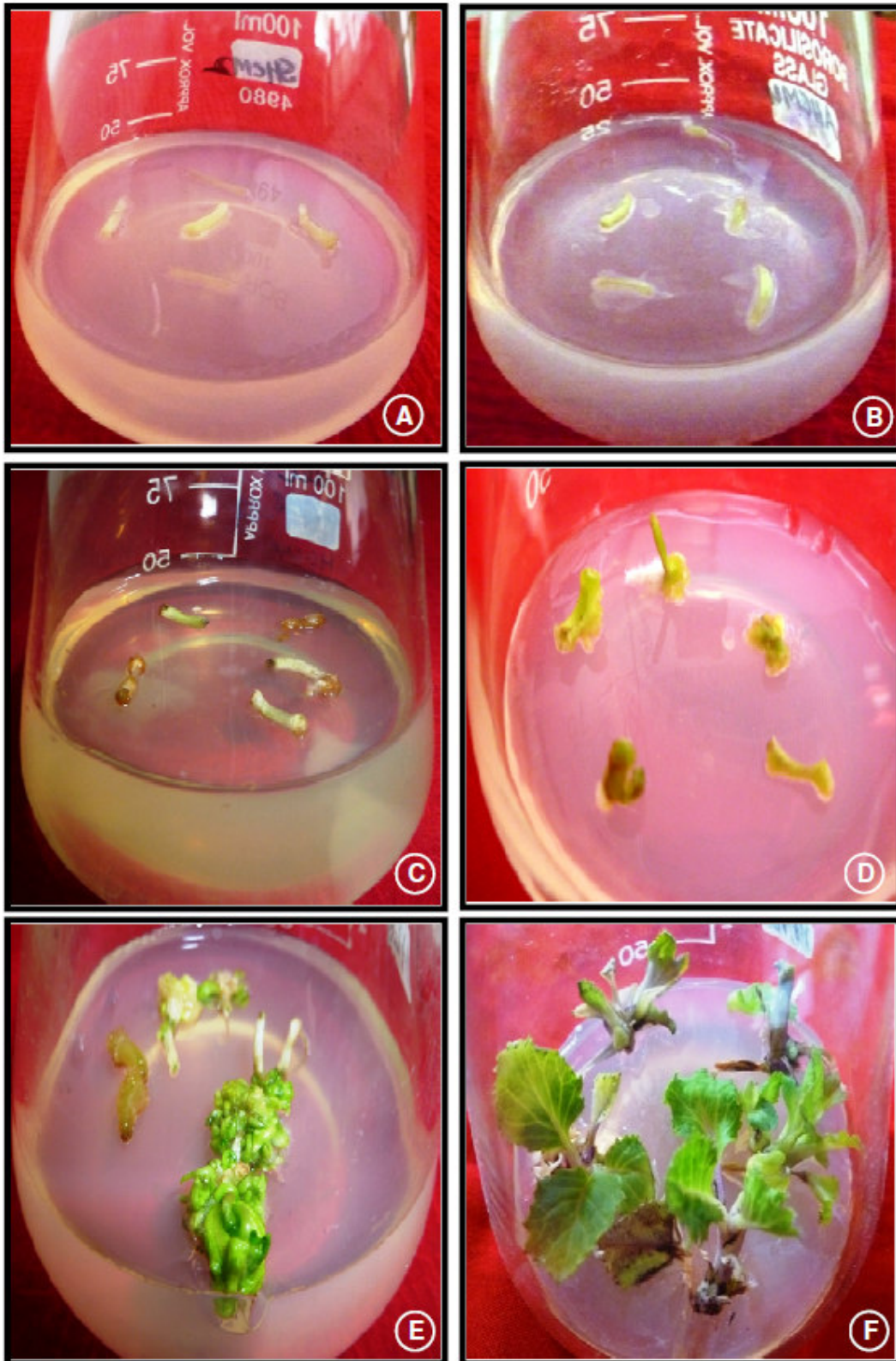


Fig. 25(A-F)

Fig. 26(A-E): Effect of different concentrations of acetosyringone on the transformation frequency of cotyledon explants in cabbage (*Brassica oleracea* L. var. *capitata* cv. **Pride of India**) (after co-cultivation).

Best Shoot regeneration medium: MS basal + 2.0mg/l Kn + 0.50mg/l NAA

- A. Cotyledon explants cultured on best shoot regeneration containing **50 μ M acetosyringone** showing shoot regeneration.
- B. Cotyledon explants cultured on best shoot regeneration medium containing **75 μ M acetosyringone** showing shoot regeneration.
- C. Cotyledon explants cultured on best shoot regeneration medium containing **100 μ M acetosyringone** showing shoot regeneration.
- D. Cotyledon explants cultured on best shoot regeneration medium containing **125 μ M acetosyringone** showing shoot regeneration.
- E. Cotyledon explants cultured on best shoot regeneration medium containing **150 μ M acetosyringone** showing shoot regeneration.



Fig. 26(A-E)

Fig. 27(A-E): Effect of different concentrations of acetosyringone on the transformation frequency of hypocotyl explants in cabbage (*Brassica oleracea* L. var. *capitata* cv. *Pride of India*) (after co-cultivation).

Best shoot regeneration medium: MS basal + 1.5mg/l Kn + 0.25mg/l IAA

- A. Hypocotyl explants cultured on best shoot regeneration medium containing **50 μ M acetosyringone** showing shoot regeneration.
- B. Hypocotyl explants cultured on best shoot regeneration medium containing **75 μ M acetosyringone** showing shoot regeneration.
- C. Hypocotyl explants cultured on best shoot regeneration medium containing **100 μ M acetosyringone** showing shoot regeneration.
- D. Hypocotyl explants cultured on best shoot regeneration medium containing **125 μ M acetosyringone** showing shoot regeneration.
- E. Hypocotyl explants cultured on best shoot regeneration medium containing **150 μ M acetosyringone** showing shoot regeneration.

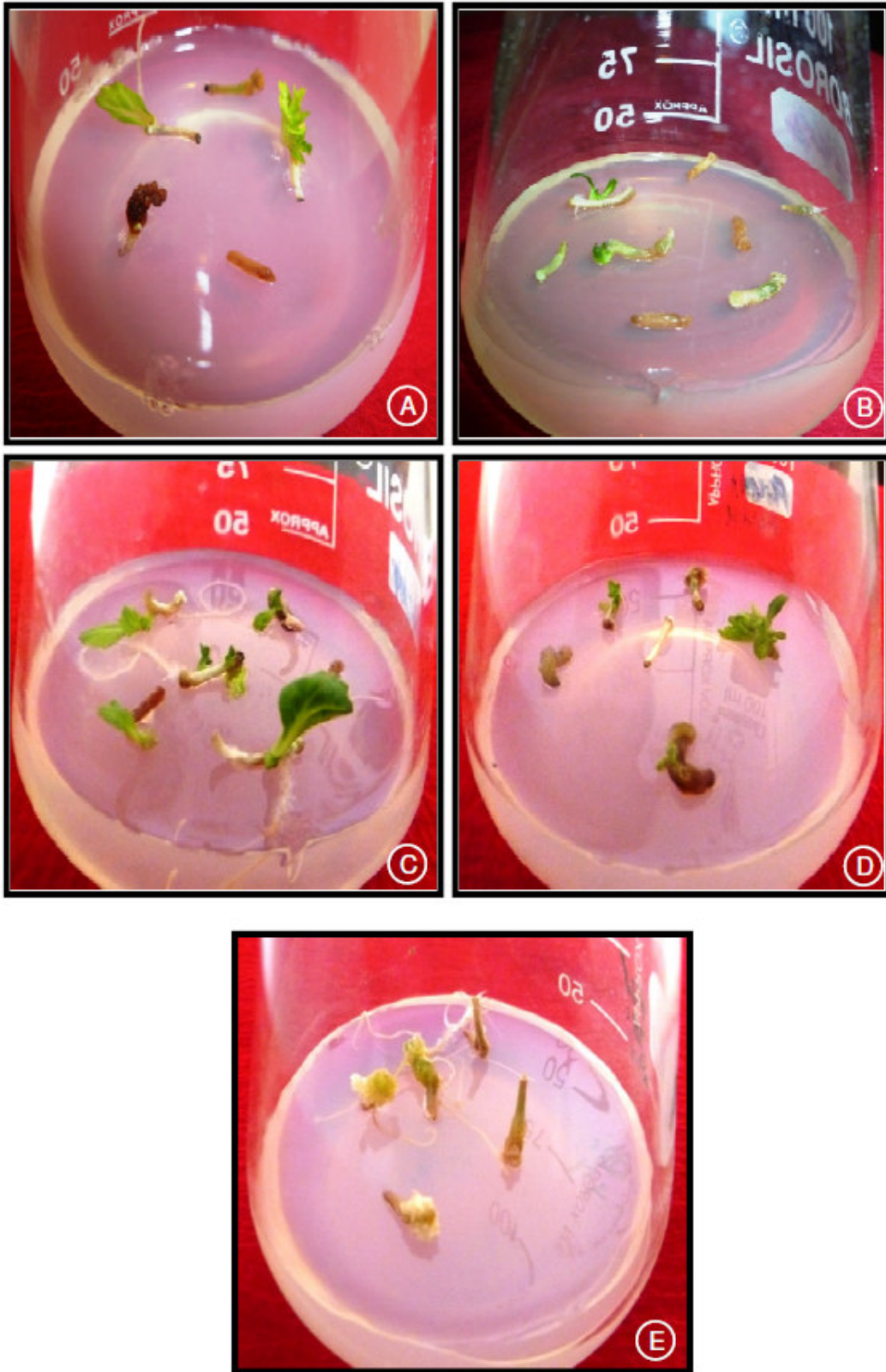


Fig. 27(A-E)

Fig. 28(A-C): Root regeneration from *in vitro* developed putative transgenic shoots of cabbage (*Brassica olerace*L. var. *capitata* cv. Pride of India).

Selective root regeneration medium: MS basal + 0.10mg/l NAA + 50mg/l Kanamycin + 400mg/l Cefotaxime

- A. *In vitro* developed putative transgenic shoots transferred to selective root regeneration medium at 0 day in culture.
- B. Root regeneration in *in vitro* developed transgenic shoots after 22 days of transfer to the selective root regeneration medium.
- C. Root regeneration in *in vitro* developed shoots after 30 days of transfer to the selective root regeneration medium. Well developed plantlets of cabbage taken out of medium (after proper washing) after 30 days of culturing showing well developed root system. (Later on transferred to pots containing pre-sterilized cocopeat for acclimatization).

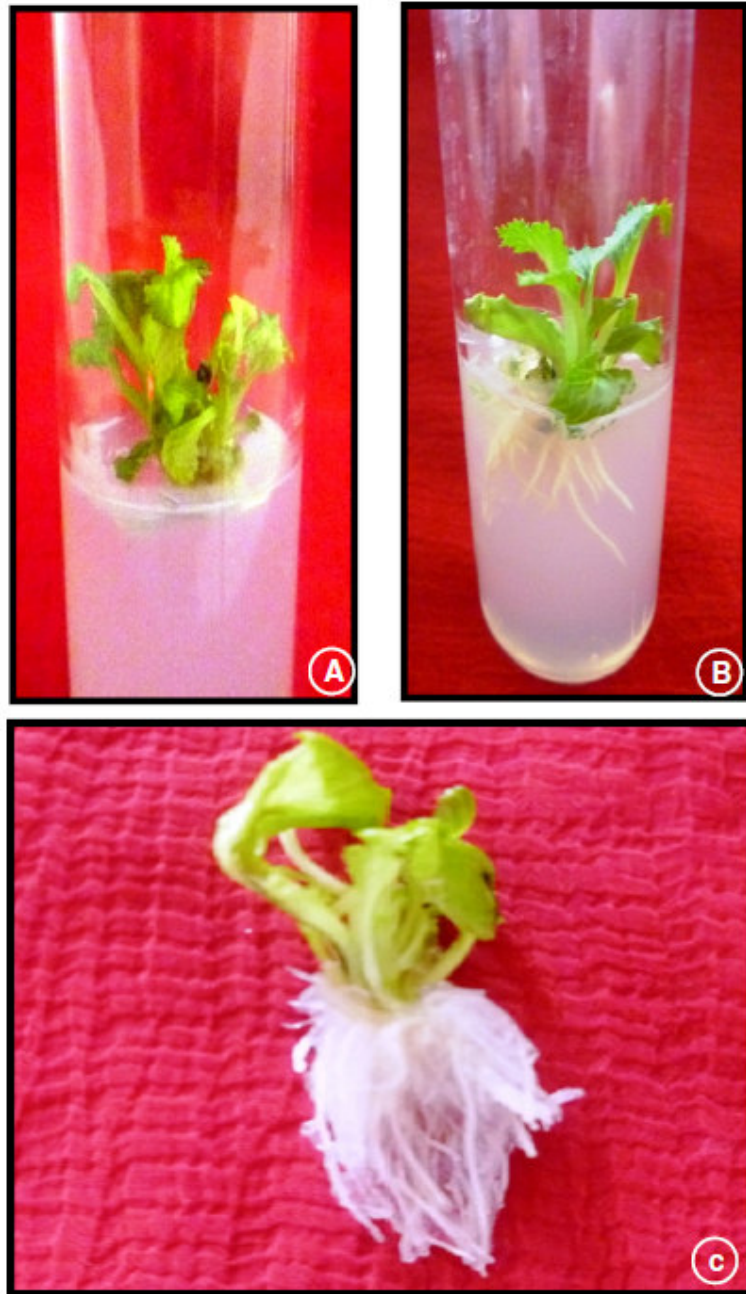


Fig. 28(A-C)

Fig. 29(A-B): Acclimatization of *in vitro* regenerated putative transgenic plantlets of cabbage (*Brassica oleracea* L. var. *capitata* cv. **Pride of India).**

- A. *In vitro* regenerated putative transgenic plantlets kept for hardening in pre-sterilized cocopeat mixture covered with transparent polythene bags to maintain relative humidity at 0 day.
- B. Healthy plantlets uncovered after 3 weeks of hardening on cocopeat mixture.



Fig. 29(A-B)

Fig. 30: PCR analysis showing amplification of 500bp DNA fragment of *npt-II* gene in regenerated transgenic plantlets of cabbage (*Brassica oleracea* L. var. *capitata* cv. **Pride of India**).

- M** : High range DNA ruler (GENEI)
- P** : Positive control (Plasmid DNA of *Agrobacterium* strain)
- C** : Negative control [Genomic DNA of control plantlets (non-transformed) of cabbage]
- T0-T40** : Putative transgenic plantlets of cabbage

Out of 40 randomly selected putative transgenic plantlets, 20 have shown the amplification of *npt-II* gene there by indicating the presence/integration of *npt-II* gene into the genome of transgenic cabbage (*Brassica oleracea* L. var. *capitata* cv. **Pride Of India**).

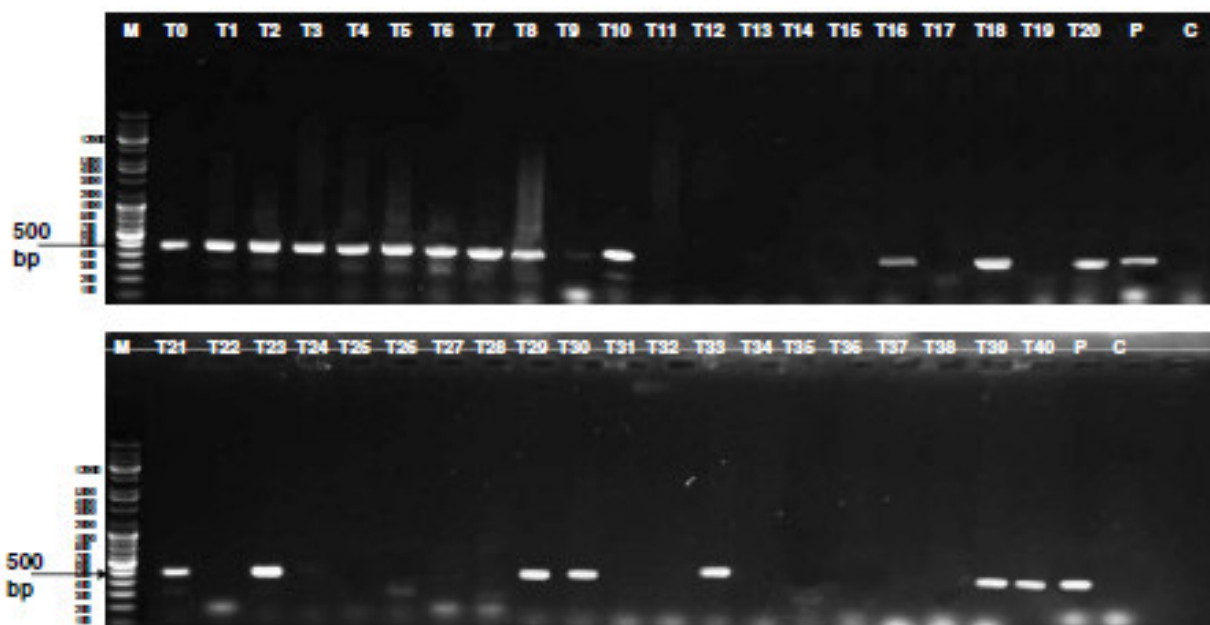


Fig. 30

Fig. 31: PCR analysis showing amplification of 1Kb DNA fragment of *cryIAa* gene in regenerated transgenic plantlets of cabbage (*Brassica oleracea* L. var. *capitata* cv. **Pride of India**).

M : High range DNA ruler (GENEI)

P : Positive control (Plasmid DNA of *Agrobacterium* strain)

C : Negative control [Genomic DNA of control plantlets (non-transformed) of cabbage]

T0-T40 : Putative transgenic plantlets of cabbage

Out of 40 randomly selected putative transgenic plantlets, 20 have shown the amplification of *cryIAa* gene there by indicating the presence/integration of *cryIAa* gene into the genome of transgenic cabbage (*Brassica oleracea* L. var. *capitata* cv. **Pride of India**).

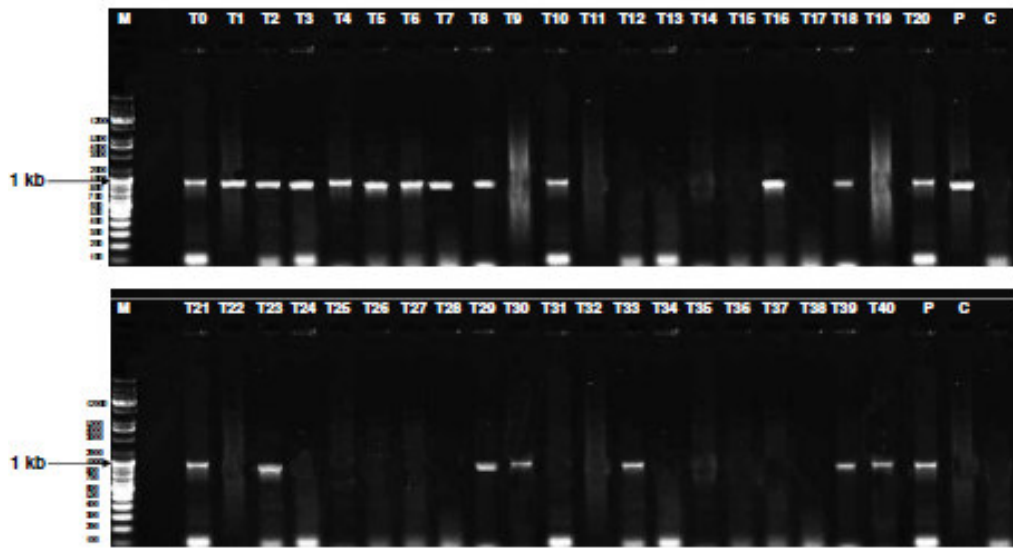


Fig. 31

Fig. 32: Southern hybridization of *cryIAa* gene in regenerated transgenic plantlets of cabbage (*Brassica oleracea* L. var. *capitata* cv. **Pride of India**).

Out of randomly selected six PCR +ve plantlets, 5 RT-PCR +ve have been able to developed the imprints on the autoradiograph thereby indicating the integration of *cryIAa* gene into the genome of transgenic cabbage. The plant T0 and T2 had two copies of insertions while plant T7 and T33 had three copies insertion whereas, plant T39 had single copy insertion into the genome of transgenic cabbage.

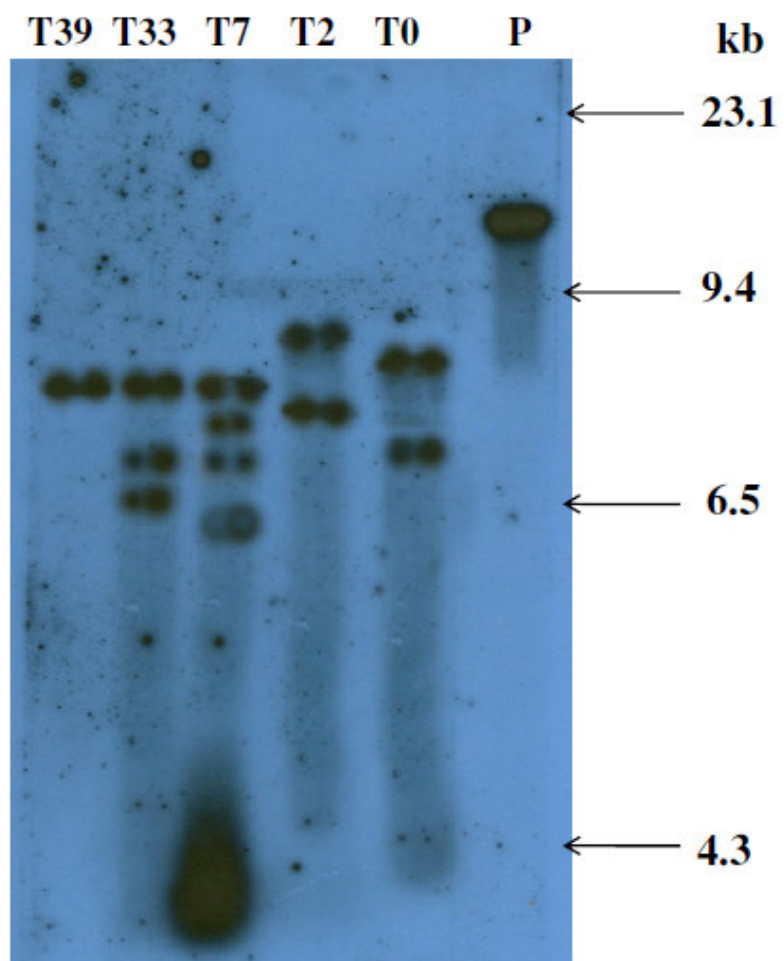


Fig. 32

Fig. 33: RT-PCR analysis showing amplification of 500bp DNA fragment of *npt-II* gene in regenerated transgenic plantlets of cabbage (*Brassica oleracea* L. var. *capitata* cv. **Pride of India**).

M : High range DNA ruler (GENEI)

T0-T40 : Putative transgenic plantlets of cabbage

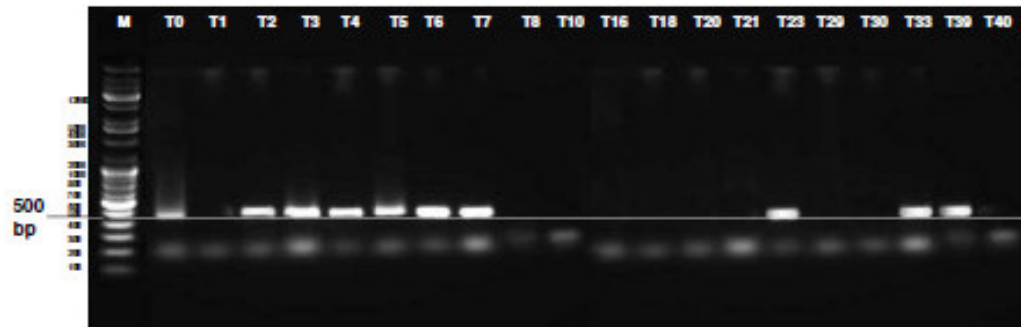


Fig. 33

Fig. 34: RT-PCR analysis showing amplification of 1Kb DNA fragment of *cryIAa* gene in regenerated transgenic plantlets of cabbage (*Brassica oleracea* L. var. *capitata* cv. **Pride of India**).

M : High range DNA ruler (GENEI)

T0-T40 : Putative transgenic plantlets of cabbage

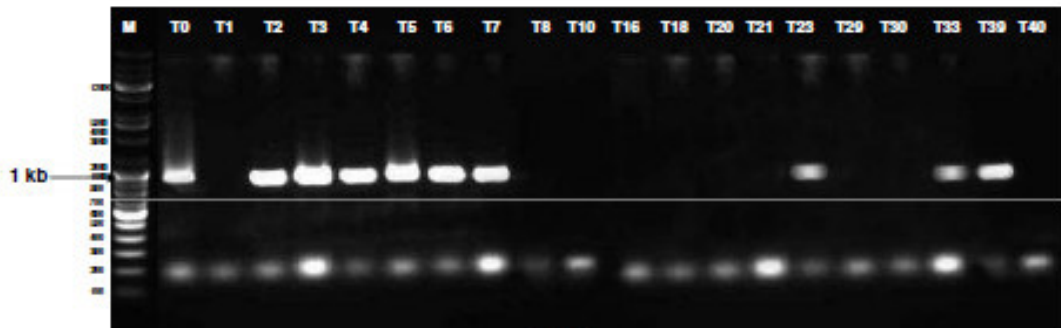


Fig. 34

Fig. 35: Real Time- PCR analysis of *cryIAa* gene in regenerated transgenic plantlets of cabbage (*Brassica oleracea* L. var. *capitata* cv. *Pride of India*).

All samples (T0, T2, T7, T33 and T39) have shown ct value (less than 26) thereby indicating that the gene is expressed at the transcriptional level in these transgenic lines.

S. No.	Colour	Name
1	Dark Blue	Negative Control
2	Light Blue	T0
3	Yellow	T2
4	Grey	T7
5	Red	T33
6	Dark Green	T39

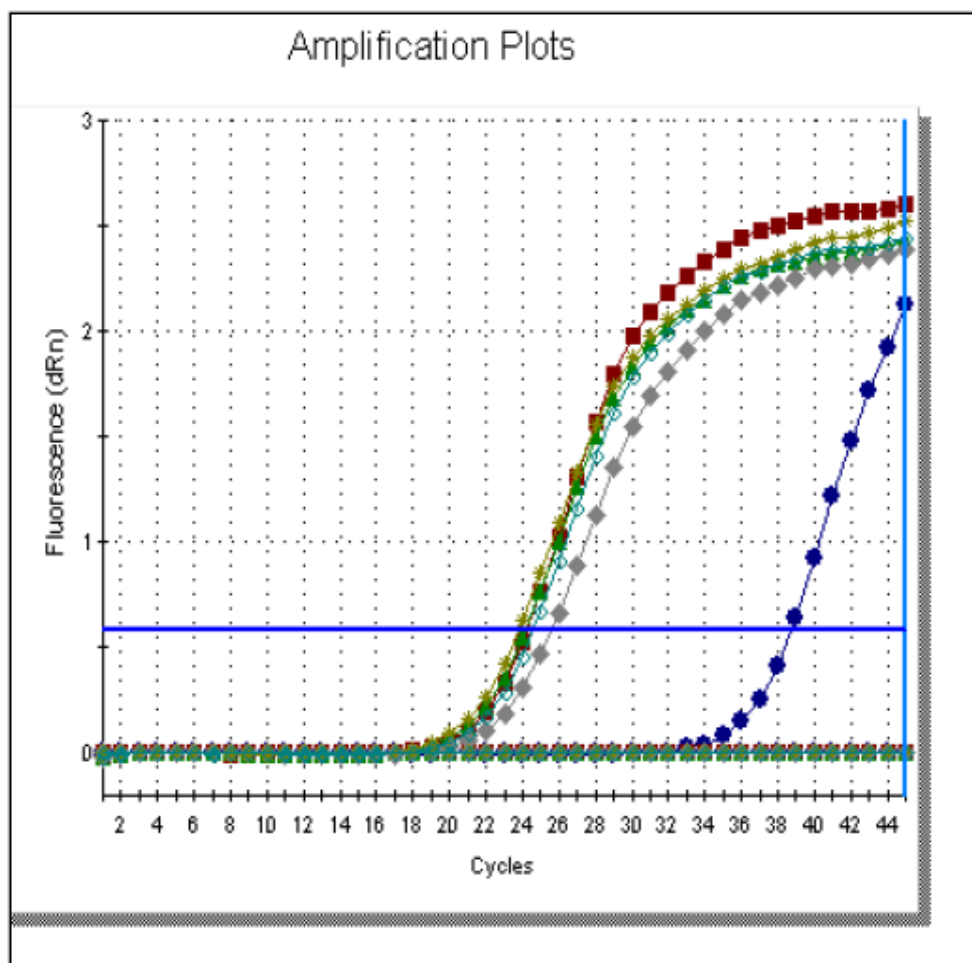


Fig. 35

Fig. 36: Real Time- PCR comparison of *cryIAa* gene in regenerated transgenic plantlets of cabbage (*Brassica oleracea* L. var. *capitata* cv. **Pride of India**).

NC : Negative control (Non-transformed)

T0, T2, T7, T33 and T39 : Transformed plantlets of cabbage

The comparison of expression of transgene of transgenic lines with negative control (non-transformed): Plant T33 showed maximum expression whereas, T7 showed minimum expression of the transgene (*cryIAa*).

Relative Quantity Chart

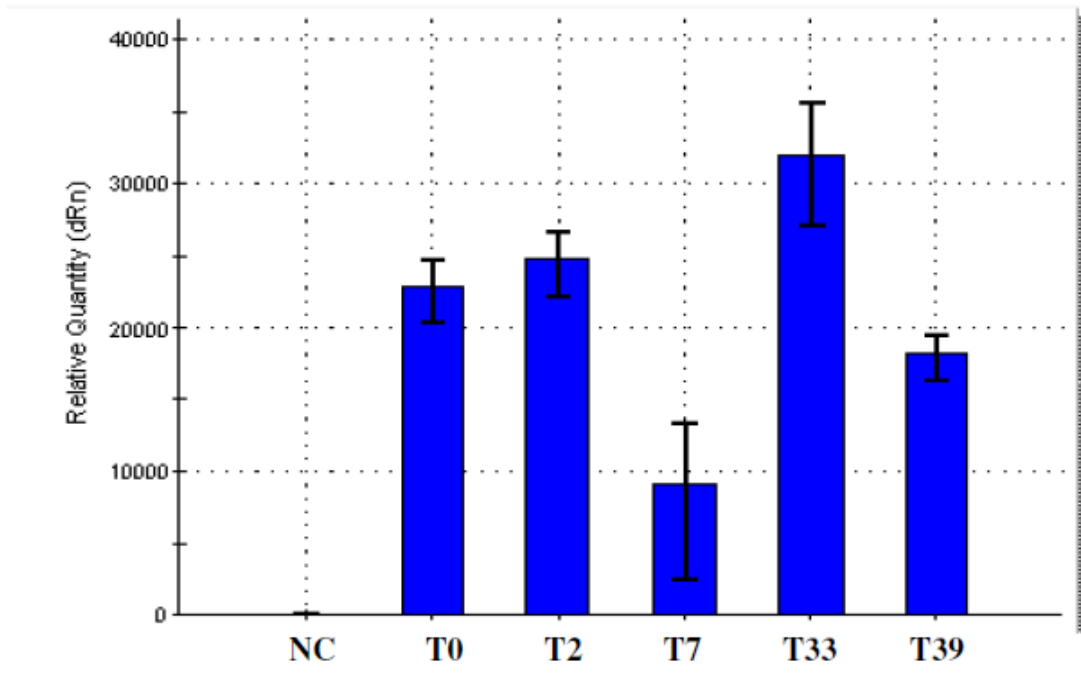


Fig. 36

Fig. 37 (A-F): *In vitro* bioassay of non-transgenic (control) and transgenic cabbage (*Brassica oleracea* L. var. *capitata* cv. *Pride of India*) with diamondback moth (DBM).

- A. Second instar larvae of diamondback moth (DBM) on non-transformed (control) cabbage leaves at 0 day.
- B. Second instar larvae of diamondback moth (DBM) on transgenic cabbage leaves at 0 day.
- C. Live larvae of diamondback moth (DBM) on damaged non-transformed (control) leaves after 72 hours.
- D. Damaged leaves of transgenic cabbage after 72 hours.

All larvae died from feeding on these plants within 72 hours.



Fig. 37(A-D)

Chapter-5

DISCUSSION

Biotechnological techniques have become available for creating genetic variability in plants. One of the most potent approach is the transfer of specifically constructed gene assemblies through various transformation techniques is genetic engineering. Genetically altered crops, into which new DNA sequences of gene(s) have been introduced for resistance to insect-pests and diseases has remained elusive because of the non-availability of resistance sources. The tools of molecular biology and genetic engineering allow the transfer of a gene(s) into vegetable crops, in particular cabbage, thus providing an opportunity to improve cabbage in many aspects. However, successful genetic transformation in cabbage depends on the availability of genotypes with better horticultural traits and high frequency of plant regeneration. Efficient gene transfer and plant regeneration systems are necessary for the development of transgenic plants. Various explants have been successfully used for *Agrobacterium* – mediated gene transfer studies in *Brassica* species for the production of transgenic plants (Paul *et al.*, 2002).

Plants are subjects of predation by virtually all types of organisms, viz, viruses, bacteria, fungi and animals, but in agricultural practices, the greatest problems are caused by insects. The ideal insecticide must be toxic to the insect against which it is targeted, biodegradable and it should be possible to apply in such a way that all parts of the crops are protected against insect attack. Looking at the above mentioned criteria, the ideal insecticide has not yet been discovered but the closest one are the δ -endotoxins produced by the soil bacterium *Bacillus thuringiensis*. Resistance to important insect-pests such as diamondback moth has been achieved by the introduction of insecticidal protein genes of *Bacillus thuringiensis* (Bt). Among the insecticidal genes, *Bacillus thuringiensis* (Bt) crystal protein (or δ -endotoxin) genes have been proven effective in controlling insect larvae in many crop plants (Perlak *et al.*, 1990, 1993; Koziel *et al.*, 1993; Peferoen, 1997; Cheng *et al.*, 1998a; Schuler *et al.*, 1998; Xiang *et al.*, 2000; Sharma and Ortiz, 2000; Srivastava, 2003, 2012a&b, 2013; Charkabarty *et al.*, 2002; Wang *et al.*, 2003; Paul *et al.*, 2005; Liu *et al.*, 2008; Lei *et al.*, 2009; Deng-Xia *et al.*, 2011; Aggarwal, 2012). The larvae of cabbage looper, cabbage worm and diamondback moth cause damage to cabbage by chewing holes in the leaves and

heads. Among these diamondback moth causes more than 50 % loss in marketable yield of cabbage. The transgenic cabbage plants will serve as a good system to study the role of gene pyramiding in resistance management strategies intended to prevent evolution of resistance in diamondback moth (Paul *et al.*, 2005; Awasthi and Srivastava, 2013).

The present investigations were undertaken to standardize the protocols for high frequency plant regeneration and genetic transformation via *Agrobacterium*-mediated insect resistance (*cryIAa*) gene transfer in cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India). For all genetic manipulation works, the development of a high frequency shoot regeneration protocol is the utmost important pre-requisite. A high frequency shoot regeneration protocol was developed in cabbage. To get high frequency transformation in cabbage different parameters viz, effect of varying concentrations of antibiotics, effect of preculturing time, effect of co-cultivation time and effect of different concentrations of acetosyringone were studied.

5.1 Plant regeneration studies in cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India)

In the present investigation, seven to nine days old aseptically grown seedlings (for cotyledon and hypocotyl explants) and 20-25 days old glasshouse grown seedlings (for leaf and petiole explants) for plant regeneration and genetic transformation studies. Among earlier findings, the effects of age of donor seedlings (source of explants) on shoot induction from cotyledon, hypocotyl and leaf have been studied in different species (Kathal *et al.*, 1998; Neidz *et al.*, 1989; Chi and Pua, 1989; Srivastava *et al.*, 1988, 1989, 1991a&b). Similarly in *Brassica* species also the effect of age of donor explants have been reported (David and Tempe, 1988; Eisner *et al.*, 1992; Arora *et al.*, 1996; Dixit *et al.*, 1998; Charkrabarty *et al.*, 2002; Baloda *et al.*, 2003; Sharma and Srivastava, 2003; Singh and Srivastava, 2003; Fan *et al.*, 2005; Zhao *et al.*, 2006c; Chen and Hou, 2008; Deng-Xia *et al.*, 2011; Park *et al.*, 2012; Sharma and Srivastava, 2013; Sharma *et al.*, 2013). Dong and Jia (1991) have reported that shoot differentiation frequencies of cotyledonary explants from seedlings older than seven days dropped sharply, while about five days old cotyledons were the most sensitive to shoot induction. Eisner *et al.* (1992) obtained best result with five to seven days old hypocotyls. Choi *et al.* (1996) reported explants became more competent as the age of the source seedlings increased up to eight days and cotyledon explants from 10-days-old seedlings were not responsive. A possible explanation is that plasticity of cell at younger age is,

physiologically and biochemically more active as well as they have less rigid cell wall and easily affected by the environmental factors, such as exogenous plant growth regulators. Cotyledons from five days old seedlings are still expanding in size and changing colour from yellow to green. Cotyledons from seven to nine days old seedlings, were however almost fully expanded and turned completely green. Hypocotyls of seven to nine days old seedlings were greenish in colour and turgid nature whereas over-aged seedlings were slender and pale green in colour.

In this study, cotyledon, hypocotyl, leaf and petiole were used as explants but hypocotyl was found best for shoot regeneration and multiplication as compared to other explants. Petrova and Antonova (1996); Sharma and Srivastava (2003); Sharma *et al.* (2013) reported hypocotyls were more responsive than cotyledons, whereas Arora *et al.* (1996) found that cotyledons produced shoots in much closer time than hypocotyls, similarly Bhalla and Smith (1998a) also reported cotyledon as best explants as compared to hypocotyl in cauliflower. Advantages of using explants from vegetative tissues include year-round availability, generation from seedlings and no requirement for vernalisation or curd formation; use of vegetative explants results in a reduction in labour and maintenance cost of the explants source (Bhalla and Smith, 1998b). Chen and Hou (2008) reported that the regeneration frequency of cotyledon with petiole was the highest among different explants types. Cheng *et al.* (2001a) observed that among the various explants tested, hypocotyls and young internodal segments from *in vitro* shoots were the most regenerative. According to Lim *et al.* (1998) hypocotyl explants showed better shoot regeneration as compared to cotyledon and petiole explants. Pavlovic *et al.* (2010) have observed highest percentage of shoot formation in hypocotyl explants as compared to cotyledon and root explants in all *Brassica oleracea* L. varieties. Seedling hypocotyls were preferred for regeneration of plants by Lazzeri and Dunwell, 1986; Yang *et al.*, 1991; Fuller *et al.*, 1994 and Cardoza and Stewart Jr., 2004.

In the present studies, various cytokinins viz, BAP, Kinetin and TDZ were used, which were effective in shoot induction from cotyledon, hypocotyl, leaf and petiole explants. In cotyledon and petiole explants high percent shoot regeneration and average number of shoots per explants was obtained from combination of Kinetin+NAA whereas in hypocotyl explants high percent shoot regeneration and average number of shoots per explants was obtained from combination of Kinetin+IAA, whereas, leaf explants showed highest percent

shoot regeneration average number of shoots per explants on MS medium supplemented with BAP + NAA. Yang *et al.* (1994) reported use of 1.5mg/l zeatin, 0.5mg/l BAP and 0.1mg/l IAA for regeneration of adventitious shoots from cotyledon and hypocotyl explants. Similarly, Arora *et al.* (1997) found that MS medium fortified with BAP 1.0mg/l in combination with IAA 0.1mg/l is best for shoot regeneration. Zhang *et al.* (1998) observed maximum shoot regeneration in the presence of 5.0mg/l BA and 0.5mg/l NAA. In present studies no shoot regeneration was observed from cotyledon, hypocotyl, leaf and petiole explants on MS medium supplemented with BAP and IAA. Whereas, Bhalla and Weerd (1999) have also reported that, there was no shoot regeneration from leaf explants on MS basal supplemented with BAP and NAA. Farzinebrahimi *et al.* (2012) reported leaf explants showed best result on MS medium supplemented with 1.5mg/l BAP and 1.0mg/l IBA. The results observed in the present study were in agreement with the previous reports by Chong *et al.* (2006), Maciej *et al.* (2006), Ravanfar *et al.* (2009) and Rafat *et al.* (2010). Pavlovic *et al.* (2012) used fourteen genotypes of cabbage to test their ability to regenerate shoots *in vitro* on medium supplemented with with 1.0 and 2.0mg/l of benzyladenine (BA) or 1.0mg/l 6-furfurylaminopurine (KIN) in combination with 0, 0.5 and 1.0 mg/l indole-3-butyric acid (IBA) and they found the lowest index of multiplication was observed on media supplemented with KIN (without or in combination with IBA).

In the present study, TDZ (thidiazuron) was used in MS medium for shoot regeneration studies. TDZ is a synthetic phenylurea cytokinin like compound that has been proven to be highly effective regulator of shoot morphogenesis (Huetteman and Preece 1993; Murthy *et al.*, 1998; Hui-mei *et al.*, 2008). It is also effective in terms of shoot regeneration in many recalcitrant species (Pelah *et al.*, 2002; Schween and Schwenkel 2002; Liu *et al.*, 2003; Mithila *et al.*, 2003). The effect of various concentrations of TDZ alone and in combination with Adenine, NAA and IAA were studied for enhancing the shoot regeneration frequency from cotyledon, hypocotyl, leaf and petiole explants in cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India). Different explants responded with different intensity on MS medium supplemented with TDZ in combination with Adenine and different auxins. Many published protocols for *Brassica* species regeneration whether they include transformation step or not are based on TDZ (Christey *et al.*, 1997; Henzi *et al.* 1999; Cheng *et al.*, 2001a; Lu *et al.*, 2003; Jonoubi *et al.*, 2005; Chen and Hou, 2008; Song *et al.*, 2012) and all the workers reported that the TDZ based media found to be very efficient for enhancing the frequency of shoot regeneration in *Brassica* species.

During the present investigation, TDZ was found to be superior over the various cytokinin (BAP and Kinetin) in promoting shoot regeneration from cotyledon, hypocotyl, leaf and petiole explants in cabbage. In present studies, the maximum percent shoot regeneration 94.44 % from hypocotyl explants was obtained on TDZ supplemented medium. Similar result reported by Cheng *et al.* (2001a) that hypocotyl explants showed better result on MS medium supplemented with TDZ as compare to MS medium supplemented with BA. In present studies, cotyledon explants showed less or no shoot regeneration on medium supplemented with TDZ or TDZ and IAA. Whereas, Cheng *et al.* (2001a) have reported higher shoot regeneration in cotyledon explants cultured on media containing TDZ or TDZ and IAA. Lu *et al.* (2003) reported that TDZ was more effective than BA. In the medium supplemented with TDZ, it was more efficient and rapid for shoot regeneration and regeneration frequency reached 98.8 % on MS basal medium supplemented with 0.25mg/l TDZ+ 0.5mg/l NAA and 5.0mg/l AgNO₃ in cotyledon explants. Chen and Hou (2008) reported the regeneration rate of petiole with cotyledon was the highest in the medium containing 0.5mg/l TDZ+ 0.5mg/l NAA+ 7.5mg/l AgNO₃.

Fan *et al.* (2005) compared the effect of BA and TDZ and found TDZ was more effective in inducing shoot regeneration in cotyledon explants of Chinese cabbage. Memon *et al.* (2009) reported that low concentration TDZ, NAA and AgNO₃ is more effective in shoot regeneration per leaf explants than the higher concentration in Chinese cabbage. Whereas, Ravanfar *et al.* (2009) reported very different results that BAP in combination with NAA and 2,4-D are more effective hormones compared with Kn and TDZ in broccoli (*Brassica oleracea* L. var. *italica*).

Among all the explants (cotyledon, leaf, petiole) hypocotyl explants had showed highest shoot regeneration frequency on MS medium containing various plant growth regulators i.e. BAP+NAA, BAP+ IAA, Kn+NAA, Kn+ IAA, TDZ alone, TDZ+ NAA, TDZ+Adenine and TDZ+ IAA. Similar results were reported by Pavlovic *et al.* (2010) that among all the *B. oleracea* varieties, the hypocotyl explants showed the highest percentage of shoot regeneration.

Different auxins i.e. IAA, IBA and NAA were used for root regeneration from *in vitro* developed shoots. Root regeneration was observed after 7-9 days in culture on MS media containing NAA and IBA whereas in medium supplemented with IAA root regeneration was

observed after 9-12 days with callusing. In the present studies, 100 per cent root regeneration was achieved from explants derived shoots in the medium containing 0.10mg/l NAA. Chen *et al.* (2004) used half strength MS medium containing 0.1mg/l NAA for regeneration of roots. Memon *et al.* (2009) also used different concentration of NAA for rooting and found 88 % root regeneration on medium containing 0.3mg/l NAA. High percentage (97.20 %) of root regeneration was also observed on MS medium containing IAA, but MS media containing NAA was most effective. Whereas, Yang *et al.* (1994) used medium containing 0.5 mg/l IBA for rooting and Kieffer *et al.* (1995) used 1.0 – 3.0mg/l IBA and obtained root regeneration in 80 per cent shoots. Bhalla and Weerd (1999) used 0.2mg/l IBA for root regeneration. Cheng *et al.* (2001a) reported significant increase in the number of roots formed per explant, i.e. about three-fold on the medium containing IAA. Ravanfar *et al.* (2009) found that medium supplemented with 0.20mg/l IBA was most suitable for root regeneration. Pavlovic *et al.* (2010) reported that the use of IBA in rooting medium increased the number of roots produced per shoot, while at the same time the average root length was decreased.

5.2 Effect of antibiotics on regeneration potential of cabbage from different explants

Kanamycin resistance gene is most widely used selectable marker for plant cell transformation and sensitivity of a particular species to kanamycin is a key element in the development of any new transformation system in which a kanamycin resistance gene will be employed. The genetically engineered *Agrobacterium* strain which we have used in the present study has two genes i.e. *cryIAa* and *npt-II*. Kanamycin sensitivity of cultured tissue of leaf and petiole explants of cabbage had shown similar results, i.e. both explants are highly sensitive to kanamycin even as low as 10 mg/l concentration of kanamycin. The non-transformed tissues did not survive on the selective medium containing Kanamycin during transformation experiment. Srivastava (1997) reported that cells which are not transformed get killed on selective media in such a manner that they become toxic to adjacent transformed cells, resulting in inhibition of the whole callus. Similar results were also reported by Eimert and Siegemund (1992). The alternating culture and repeated selection seem to be necessary for differentiation of transformed shoots against the inhibitory effect of kanamycin and elimination of escapes. The explants in control medium were very healthy and showed appropriate growth on the medium, but on selective medium at concentration as low as 30mg/l kanamycin, the color of the explants/tissues had changed to greenish yellow and finally turned brown after 35 days of culture. Only 35mg/l of kanamycin totally inhibit shoot differentiation from co-cultivated thin cell layer explants of *Brassica napus* (Charest *et al.*,

1988), 20mg/l inhibits shoot induction from stem segments (Pua *et al.*, 1987) and 50 mg/l inhibits shoot regeneration of *Brassica oleracea* (Bhalla and Smith, 1998b; Dixit *et al.*, 1998; Bhattacharya *et al.*, 2002; Sharma and Srivastava, 2003a & b; Singh and Srivastava, 2003; Cao *et al.*, 2008; Deng-Xia *et al.*, 2011). Kang *et al.* (2002) reported that for cotyledon explants of Chinese cabbage, shoot induction was not significantly affected by kanamycin at 1.0mg/l but the number of shoots formed was significantly reduced at 2.0mg/l and no shoot were regenerated from any explants at 6.0mg/l or higher and similar results were obtained in case of hypocotyl explants. Paul *et al.* (2005) reported that hypocotyl explants of cabbage showed inhibition in growth on medium containing 20mg/l Kanamycin. Bhalla and Smith (1998b) also reported that exposure of regenerated green shoots to a higher kanamycin concentration on medium containing low sucrose was used to eliminate non-transformed shoots. Bhau and Wakhlu (2001) reported callus of *Coryphantha elephantidens* showed less or no inhibition in callus growth at lower concentration of kanamycin whereas at higher concentration (10, 15, 20mg/l) observed inhibited callus growth. In contrast some of the monocotyledons indicates a high level of natural resistance to kanamycin. More than 800mg/l is required to inhibit growth of cell suspension cultures of several species of Graminae (Hauptman *et al.*, 1988). Oz *et al.* (2009) used higher concentration of kanamycin (200mg/l) for inhibition of non-transformed tissues of chickpea. Kanwar and Kumar (2011) used higher concentrations of kanamycin (100mg/l) for selection of transformed callus/ tissues of *Dianthus caryophyllus* L.

The effect of different concentrations of cefotaxime has been studied separately on the regeneration potential of cabbage. In the present investigations, maximum per cent shoot regeneration was obtained on the best shoot regeneration medium with 300mg/l cefotaxime in cotyledon explants. It has been observed that the increase in the concentration of cefotaxime showed no much effect on regeneration potential. Cefotaxime has potential to increase the growth, regeneration and embryogenesis *in vitro*. Cefotaxime promoted growth and morphogenesis in callus cultures of wheat and barley (Mathias and Boyd, 1986; Mathias and Mukasa, 1987). Yepes and Aldwinekle (1994) evaluated the effect of antibiotics on morphogenesis of apple. Similar studies were also carried out by Humara *et al.* (1999) and they observed that 250µg/ml cefotaxime enhanced the shoot regeneration capacity. Danilova and Dolgikh (2004) reported stimulatory effect of the antibiotic cefotaxime on plant regeneration in maize tissue culture which enhanced its morphogenesis. The highest increase in the number of regenerated shoots was observed at the antibiotic concentration of 150mg/l.

Kaur *et al.* (2008) obtained enhanced *in vitro* shoot multiplication and elongation in sugarcane used at the rate of 250 and 500mg/l cefotaxime in the medium.

In hypocotyl explants, no increase in shoot regeneration potential was observed on medium supplemented with different concentration of cefotaxime. Borrelli *et al.* (1999) reported similar results that cefotaxime did not affect callus growth in durum wheat. Whereas, Ahmad *et al.* (2012) observed that with the increase of cefotaxime concentration the transformation frequency was lowered and most of the explants of *Solanum tuberosum* L. were dead.

5.3 Genetic transformation studies in cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India)

For introducing foreign genes into a plant cell, *Agrobacterium*-mediated gene transfer, and direct gene transfer techniques are applied and then subsequently the transformed cells are regenerated into transgenic plants. The *Agrobacterium*-mediated gene transfer method is preferred for many plants including cabbage. During present investigation, genetically engineered disarmed *Agrobacterium tumefaciens* strain containing binary vector pBin-1Aa with *cryIAa* (insect resistance gene) and *npt-II* (neomycin phosphotransferase-II) genes for selection in both bacteria and plant was used for co-cultivation experiment to transfer *cryIAa* and *npt-II* genes into cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India). The strain showed satisfactory results including its handling and response to cotyledon and hypocotyl explants. The strain of *Agrobacterium* used in transformation studies plays a significant role in the frequencies of transformation achieved. *Agrobacterium tumefaciens* LBA 4404 strain has routinely been used in *Brassica* sps. (Shahin and Yashar, 1986; Yu and Shao, 1988; Bai *et al.*, 1992; Berthomieu *et al.*, 1994; Bhalla and Smith, 1998b; Metz *et al.*, 1995; Pius and Achar 2000; Bhattacharya *et al.*, 2002; Chakrabarty *et al.*, 2002; Sharma and Srivastava 2003; Gribova *et al.*, 2005; Paul *et al.*, 2005; Zhao *et al.*, 2006b; Lei *et al.*, 2009; Deng-Xia *et al.*, 2011; Sharma *et al.*, 2013).

Pre-culture of the explants in adequate conditions prior to *Agrobacterium* infection can improve transformation frequency by increasing the number of cells competent for regeneration and transgene integration (Birch, 1997). During present investigation, effect of pre-incubation time on the frequency of shoot regeneration was observed from both cotyledon and hypocotyl explants. 72 hours of pre-culturing was found best for both explants of cabbage. The effect of pre-incubation on shoot regeneration was also studied by Srivastava

et al. (1989), De Block *et al.* (1989), Metz *et al.* (1995), Dixit *et al.* (1998), Gupta *et al.* (2001), Singh and Srivastava (2003) and Awasthi and Srivastava (2013). Similar results were observed by Jin *et al.* (2000) and Tsukazaki *et al.* (2002) that 72 hours of preculturing was best for hypocotyl explants of cabbage. Rafat *et al.* (2010) in hypocotyl and shoot tip explants of cabbage observed same result. Lingling *et al.* (2005) have also reported that 72 hours of preculturing was best in cauliflower. Maheshwar *et al.* (2011) standardize 72 hours as best for preculturing time for hypocotyl explants of *Brassica napus*. Whereas, Bhalla and Smith, (1998b) reported that no pre-culture/pre-incubation is required for the genetic transformation of cauliflower. Chakrabarty *et al.* (2002) reported that the pre-incubation period of 48 hours was best for the genetic transformation of cauliflower. Bhattacharya *et al.* (2002) precultured hypocotyl explants of cabbage for 24 hrs. Whereas, Park *et al.* (2005) found 48 hours were best for preculturing of hypocotyl and cotyledon explants of Chinese cabbage for transformation.

The effect of co-cultivation time on transformation frequency (shoot regeneration) from cotyledon and hypocotyl explants was also studied and 48 hours of co-cultivation time was found to be best for genetic transformation of cabbage. The effect of co-cultivation was also studied by Bai *et al.* (1992) ; Li *et al.* (1995) ; Dixit *et al.* (1998) ; Zhang *et al.* (2000) ; Gupta *et al.* (2001); Baloda *et al.* (2003); Singh and Srivastava (2003). Bhalla and Smith (1998b) and Awasthi and Srivastava (2013) reported that in cauliflower, a co-cultivation period of two days (48 hours) was optimal for cotyledon and hypocotyl transformation. A longer (4, 5, 7 days) co-cultivation period also resulted in the explants turning necrotic devoid of shoot regeneration. Lingling *et al.* (2005) and Liu *et al.* (2011a) reported 48 hrs was best for co-cultivation in cauliflower and *Brassica napus*, respectively. In contrast, Metz *et al.* (1995) obtained high frequency of genetic transformation of broccoli and cabbage with 3 days (72 hours) of co-cultivation of hypocotyls and petiole explants, whereas higher peduncle transformation frequency was obtained with a day (24 hours) of co-cultivation. Jin *et al.* (2000) reported 72 hours of co-cultivation was best for genetic transformation of cabbage. Chakrabarty *et al.* (2002); Tsukazaki *et al.*, (2002); Park *et al.* (2005) and Rafat *et al.* (2010) reported 72 hours of co-cultivation was best for genetic transformation of cauliflower, cabbage and Chinese cabbage, respectively.

During the present investigation, the high putative transformation frequency was obtained in hypocotyl (14.50%) and cotyledon (4.66%) explants of cabbage. Hypocotyl showed better putative transformation frequency than cotyledon explants in present studies

and similar results were obtained by Mukopadhyay *et al.* (1992) in *Brassica campestris* and Takasaki *et al.* (1997) with *Brassica rapa* L. that hypocotyl showed better transformation frequency as compared to cotyledon explants. Mukopadhyay *et al.* (1992) and Takasaki *et al.* (1997) failed to obtain any transgenic plant from cotyledon explants. Metz *et al.* (1995) obtained high frequency of genetic transformation from hypocotyl and petiole explants of cabbage and hypocotyl explants of broccoli as compared to any other explants. Kuvshinov *et al.* (1999) obtained higher regeneration capacity of hypocotyls, stem and petiole segments after co-cultivation than any other explants in *Brassica rapa*. Jin *et al.* (2000) reported 10% transformation frequency in hypocotyl explants of cabbage. Kuginuki and Tsukazaki (2001); Min *et al.*, (2007) have also reported that in *Brassica* species, in hypocotyl explants showed high frequency of shoot regeneration. Gribova *et al.* (2005) reported that hypocotyl explants performed better than other explants in cabbage. Cao *et al.* (2008) reported 3% transformation frequency in *Brassica juncea*. Liu *et al.* (2011a) reported low transformation frequency (5.33%) in leaf explants of *Brassica napus*. Song *et al.* (2012) reported higher transformation frequency in hypocotyl explants (4 %) as compared to cotyledon explants (0.7 %).

In contrast, cotyledon explants showed high frequency of shoot regeneration in *Brassica* species (Dai *et al.*, 1994; Choi *et al.*, 1996; Zhang *et al.*, 1997; Dixit *et al.*, 1998; Bhalla and Smith, 1998b). Bhalla and Smith (1998b) also reported similar result that cotyledon showed better result than hypocotyl in cauliflower. Rafat *et al.* (2010) reported 45% transformation frequency in shoot tip explants of cabbage. Bhuiyan *et al.* (2011) reported higher transformation frequency (16.2 %) from cotyledon explants of *B.juncea*.

In cotyledon and hypocotyl explants different concentrations of acetosyringone were tried at standardized 72 hours of preculturing and 48 hours of cocultivation time interval to enhance transformation frequency. At concentration of 100 μ M acetosyringone, the transformation frequency was enhanced to 18 and 32 percent respectively. Similar results were reported in *Brassica* species (Dutta *et al.*, 2008; Rafat *et al.*, 2010; Bhuiyan *et al.*, 2011; Song *et al.*, 2012). Many workers also studied different concentrations of acetosyringone to enhance transformation frequency in various crops (Cervera *et al.*, 1998; Costa *et al.*, 2006; Husaini, 2010) and they reported that the addition of acetosyringone during preculture and co-cultivation increased the number of transformed cells by increasing the virulence of *Agrobacterium*.

5.4 Molecular analyses of putative transgenic shoots/plantlets of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India)

During the present investigation, the confirmation of presence/integration of transgenes (*npt-II* and *cryIAa*) into the genome of cabbage was confirmed by PCR using gene specific primers and Southern blot analysis using radiolabelled DNA probe. The Southern blot analysis has also been used to confirm copy number of transgene (*cryIAa*) into the genome of cabbage. The regenerated putative transgenic shoots of cabbage were maintained on the medium containing 50 mg/l kanamycin and were tested for the integration of *npt-II* and *cryIAa* genes by PCR analysis. The protocol developed eventually lead to the recovery of large number of transgenic cabbage plants. Most of these plants were morphologically indistinguishable from control plants under similar conditions. For PCR analysis, 40 putative transgenic shoots of cabbage were randomly selected and out of 40 putative transgenic shoots/plantlets, 20 shoots were found to be +ve for the integration of transgenes i.e. *npt-II* and *cry IAa* into the genome of cabbage. For Southern blot analysis six RT-PCR +ve shoots were randomly selected out of 10 RT-PCR +ve shoots. Out of the six RT-PCR +ve shoots, five shoots were confirmed +ve for integration of transgene *cryIAa* into the genome of cabbage by Southern hybridization. The copy number of transgene into the genome of cabbage ranges from 1 to 3 copies.

Similarly, Fry *et al.* (1987) suggested that multiple insertion during *Agrobacterium*-mediated gene transfer has to be feature of *Brassicaceae*. Bhalla and Smith (1998b) have also used PCR and Southren blot analysis to confirm integration of transgene into the genome of cauliflower. They reported single to two copies of transgene insertion per genome. Jin *et al.* (2000) transformed cabbage with *cryIAb3* gene and found four copies of transgene by Southern hybridization. Xiang *et al.* (2000) confirmed *cryIAb* and *cryIAc* genes in transformed Chinese cabbage by PCR using specific primer. They used specific probe for Southern hybridization and found one to three copies of transgene. Chen *et al.* (2001) analyzed *ipt* gene along with *npt-II* gene in transformed broccoli by polymerase chain reaction using *npt-II* specific primers and Southern hybridization and found one to four copies of transgene insertion. Bhattacharya *et al.* (2004) analyzed antisense *bet* gene along with *npt-II* gene in transformed cabbage by polymerase chain reaction using *betA* specific primers and did Southern hybridization to demonstrate the gene integration into the genome of cabbage plants. The number of the *betA* gene insertions in transgenic lines varied from one

to four. Park *et al.* (2005) used similar techniques to confirm integration of *LEA* gene into genome of Chinese cabbage and found that out of 126 kanamycin-resistant calli, nine plant lines regenerated were confirmed to be transgenic and suggested that perhaps one to three integrations of the *LEA* gene had occurred in the plant genome. Lingling *et al.* (2005) transformed cauliflower with *CpTi* gene and confirmed its integration by PCR and Southern hybridization.

Cao *et al.* (2008) used PCR and Southern blot analysis to confirm integration of *cryIAc* or *cryIC* gene by specific primer and probe, respectively, in genome of *Brassica juncea*. Liu *et al.* (2011a) analyzed the integration of *sporamin* and *chitinasePjChi-1* genes in genome of *Brassica napus* by PCR and Southern Hybridization. They had found single to two copies of transgene insertion per genome. Vanjildorj *et al.* (2009) used Southern blot technique for confirmation of integration of transgene into the genome of Chinese cabbage. Deng-Xia *et al.* (2011) confirmed integration of *cryIBa3* gene into the genome of cabbage by PCR and Southern hybridization.

The confirmation of expression of the transgene *cryIAa* into the genome of cabbage at transcriptional level was confirmed by Reverse Transcriptase-PCR and Real Time-PCR. For confirmation of expression of transgene at transcriptional level first mRNA was isolated and then cDNA was synthesised from isolated mRNA. Further cDNA was amplified using gene specific primers. During the present investigation, 20 PCR +ve shoots were selected for studying expression of transgene at transcriptional level. Out of 20 PCR +ve shoots 10 shoots have shown expression of transgene at transcriptional level. Chen *et al.* (2001) conducted RT-PCR to confirm the *ipt* gene expression into the genome of broccoli. Bhattacharya *et al.* (2004) analyzed through RT-PCR for the presence of *betA* transcripts into the genome of cabbage. Rafat *et al.* (2010) confirmed the AtHSP101 gene expression in transgenic lines of cabbage by RT-PCR. Deng-Xia *et al.* (2011) reported expression of *cryIBa3* gene into the genome of cabbage by RT-PCR.

In a Real Time-PCR assay a positive reaction was detected by accumulation of a fluorescent signal. The Ct (cycle threshold) was defined as the number of cycles required for the fluorescent signal to cross the threshold (i.e. exceeds background level). Ct levels were inversely proportional to the amount of target nucleic acid in the sample (ie the lower the Ct level the greater the amount of target nucleic acid in the sample). Cts < 26 was strong positive reactions indicative of abundant target nucleic acid in the sample. During the present

investigation, the ct value for all the transgenic plantlets used for Real Time-PCR analysis were below 26 and hence it indicated the greater amount of target nucleic acid (*cry IAa* gene) in the cabbage transgenic plantlets. Bhuiyan *et al.* (2011) analyzed the selected transcripts exhibiting altered transcript profiles in response heavy metals in *Brassica juncea* by real-time quantitative PCR.

The confirmation of expression of the transgene *cryIAa* into genome of cabbage at translational level was confirmed by bioassay. The young leaves of transformed and non-transformed cabbage were cut and placed in small petridishes containing moistened filter paper. On each leaf disc, five late second- instar (6-days-old) larvae of diamondback moth (*Plutella xylostella* L.) were released and reared at 26–28°C with 60 % relative humidity and 100 % mortality was observed after 3 days of the bioassay. Deng-Xia *et al.*(2011) observed similar result. They studied 100 % mortality of larvae within 2-3 days after infestation of transgenic leaves of cabbage. Liu *et al.* (2008) observed control leaves were completely damaged by larvae as compared to transgenic leaves. Bhattacharya *et al.* (2002) showed significant larva mortality from 51.84 to 74.06 % in cabbage. Jin *et al.* (2000) observed one out of 15 larvae survived on transformed leaves of cabbage after 6 days of bioassay.

The present investigations have shown that it is possible to regenerate, transform and obtain transgenic plantlets of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India) using *Agrobacterium*-mediated gene transfer technique. These transgenic plantlets were confirmed for the integration and expression of transgene (*cryIAa*) using various molecular techniques and bioassay.

Chapter-6

SUMMARY AND CONCLUSION

The present research work was undertaken with a view to standardize a protocol for *Agrobacterium*-mediated insect resistance gene (*cryIAa*) transfer in cabbage (*Brassica oleracea* L.var. *capitata* cv. Pride of India) and to develop transgenic plants. Further the presence/integration of the transferred gene was studied through polymerase chain reaction using synthetic designed primers and Southern blot analysis. Finally, the expression of the transgene was studied at transcriptional level by reverse transcriptase-polymerase chain reaction and Real Time- PCR and at translational level by bioassay.

1. Four types of explants viz. cotyledon, hypocotyl, leaf and petiole were used for plant regeneration and genetic transformation studies. Cotyledon and hypocotyl explants were used from seven to nine days old aseptically grown seedlings and leaf and petiole explants were used from *in vivo* 20-25 days old grown seedlings of cabbage cv. Pride of India.
2. Before carrying out genetic transformation studies the best high frequency shoot regeneration medium for cotyledon, hypocotyl, leaf and petiole explants was screened.
3. High efficiency shoot regeneration was obtained in cotyledon (91.11 %), hypocotyl (94.4 %), leaf (91.11 %) and petiole (88.88%) explants on MS medium supplemented with 0.33mg/l TDZ + 79.7 mg/l Adenine, 0.22mg/l TDZ + 0.088mg/l IAA, 0.22mg/l TDZ + 0.02mg/l NAA and 0.33mg/l TDZ + 0.02mg/l NAA, respectively.
4. Three different types of auxins were tried in MS medium for root induction from *in vitro* developed shoots. The presence of 0.10 mg/l NAA in nutrient medium promoted the early root regeneration and high frequency (100%) root regeneration from *in vitro* developed shoots.
5. The regenerated complete plantlets were acclimatized on cocopeat and 80% survival of plants was observed during acclimatization.

6. A protocol for plant regeneration from cotyledon, hypocotyl, leaf and petiole explants has been standardized in cabbage cv. Pride of India.
7. Kanamycin sensitivity in the cultured leaf and petiole explants was studied. Kanamycin concentration as low as 10mg/l was toxic to the leaf and petiole explants on the selective medium. The kanamycin sensitivity affects the recovery of transformed plantlets and varies widely among tissues and species. Kanamycin sensitivity should be determined in the initial stages for developing a plant genetic transformation system.
8. Effect of different concentrations of cefotaxime was studied on the regeneration potential of cotyledon and hypocotyl explants. In cotyledon explants maximum per cent (88.88 %) shoot regeneration with average number of shoots per explant (2.66) were obtained on shoot regeneration medium with 300mg/l cefotaxime. Whereas, in hypocotyl explants maximum per cent (90.73 %) shoot regeneration with average number of shoots per explant (2.46) were obtained on shoot regeneration medium with 0mg/l cefotaxime concentration.
9. Effect of different concentrations of cefotaxime and kanamycin (50mg/l) were studied on the growth of agrobacterial cells and regeneration potential of cotyledon and hypocotyl tissues after co-cultivation. In cotyledon and hypocotyl explants the growth of agrobacterial cells were controlled at concentration of 400mg/l cefotaxime and maximum per cent shoot regeneration in cotyledon (35.55 %) and hypocotyl (48.15 %) was obtained on MS medium supplemented with 400mg/l cefotaxime, respectively.
10. Genetically engineered disarmed *Agrobacterium tumefaciens* strain containing binary vector pBin-1Aa with *cryIAa* (insect resistance gene) and *npt-II* (neomycin phosphotransferase-II) genes for selection in both bacteria and plant was used for co-cultivation experiment to transfer *cryIAa* and *npt-II* genes in cabbage.
11. Agrobacterial suspension having 10^8 cells/ ml had OD 0.521 at 560 nm which was fixed and used for genetic transformation experiment.
12. Preculturing of cotyledon explants for 72 hours and co-cultivation with agrobacterial cells for 48 hours. worked out to be the best treatment as it gave the highest putative transformation frequency (4.66%) in cotyledon explants on MS medium + 0.330mg/l TDZ + 79.7mg/l Adenine + 50mg/l Kanamycin + 400mg/l Cefotaxime.

Whereas in hypocotyl explants 72 hours preculturing and 48 hours co-cultivation was worked out to be the best treatment as it has given the highest transformation frequency (14.50%) on MS medium + 0.220mg/l TDZ + 0.088mg/l IAA + 50mg/l Kanamycin + 400mg/l Cefotaxime.

13. Effect of different concentrations of acetosyringone was studied to increase transformation frequency in cabbage from cotyledon and hypocotyl explants. The maximum percent shoot regeneration (18.66 %) and (32.00 %) was obtained from those explants, respectively, that were cultured on medium containing 100 μ M acetosyringone at standardized preculturing (72 hours) and co-cultivation (48 hours) time.
14. After co-cultivation, only the explants (transformed cells) were able to grow on the selective medium containing 400mg/l cefotaxime and 50mg/l kanamycin, whereas the non-transformed ones could not survive on the selective shoot regeneration medium and turned brown and black.
15. Explants were transferred to fresh selective shoot regeneration medium after every 4-5 weeks and were only discarded when a shoot was excised for root regeneration.
16. The *in vitro* developed putative transgenic shoots rooted on the selective root regeneration medium after 4-5 weeks.
17. Most of the putative transgenic plantlets regenerated from cotyledon and hypocotyl explants appeared phenotypically/ morphologically normal.
18. Integration of transgenes i.e. *npt-II* and *cryIAa* into genome cabbage was confirmed by PCR using specifically designed primers and Southern blot analysis using radiolabelled DNA probe.
19. The expression of transgenes i.e. *npt-II* and *cryIAa* was studied at the transcriptional level using reverse transcriptase-polymerase chain reaction (RT-PCR) technique and Real Time-PCR analyses.

20. The expression of transgene (*cryIAa*) at the translational level was studied by bioassay using second instar larvae of diamondback moth (*Plutella xylostella*)

21. A protocol for *Agrobacterium*-mediated insect resistance gene (*cryIAa*) transfer in cotyledon and hypocotyl explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India) has been standardized. The transgenic plants of cabbage have shown the integration and expression of the transgene.

In the present studies, high frequency plant regeneration and *Agrobacterium*-mediated insect resistance gene (*cryIAa*) transfer protocol from cotyledon and hypocotyl explants in cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India) has been standardized. Although, the frequency of transformation was low as compared to model species, the protocol is reliable and can be used to mobilize genes of agronomic importance in the elite cultivar. The transgenic cabbage cv. Pride of India generated from the present investigation showed promise of practical utility in insect-pest management.

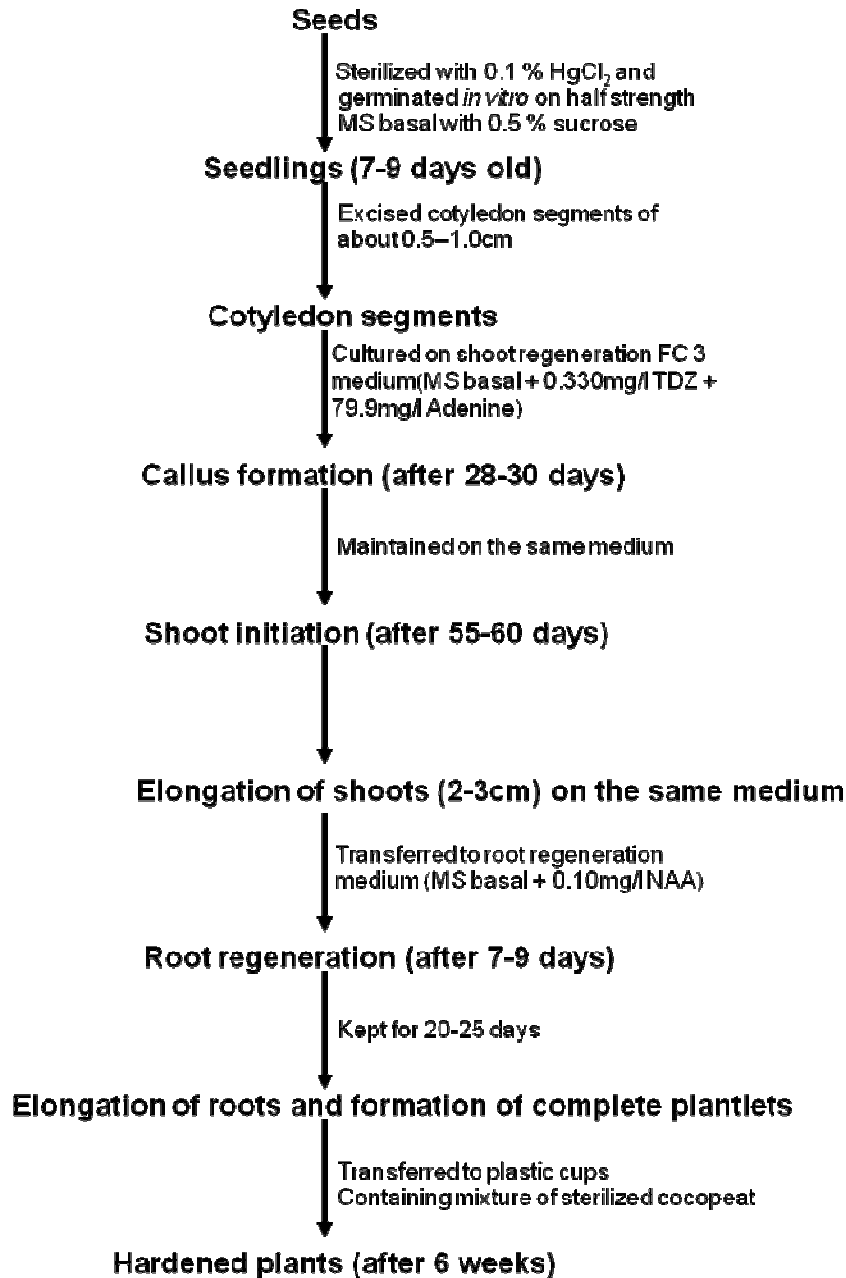


Fig. 38: Flowchart depicting a protocol for plant regeneration from cotyledon explants in cabbage (*Brassica oleracea* L. var. *capitata* cv. *Pride of India*).

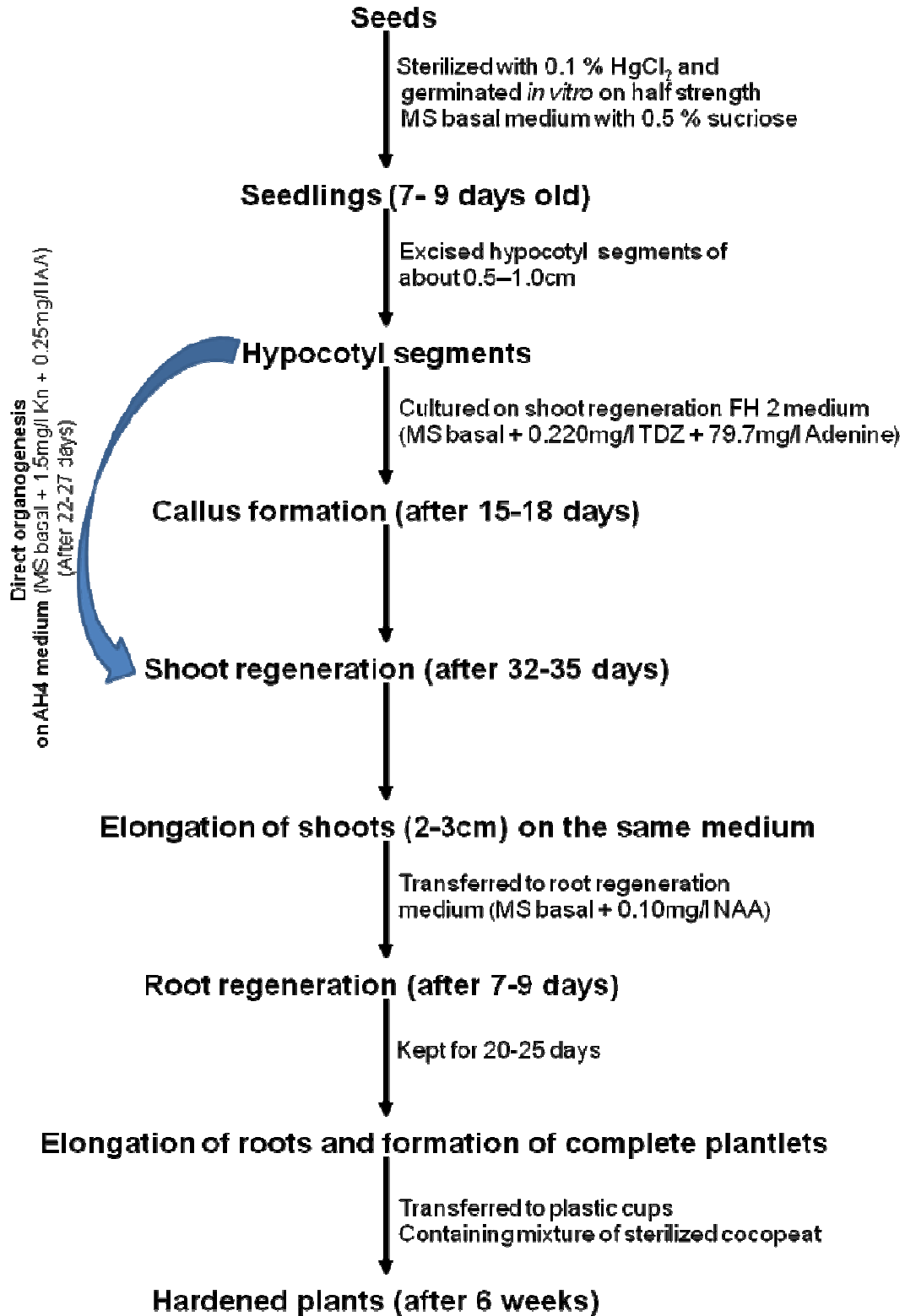


Fig. 39: Flowchart depicting a protocol for plant regeneration from hypocotyl explants in cabbage (*Brassica oleracea* L. var. *capitata* cv. *Pride of India*).

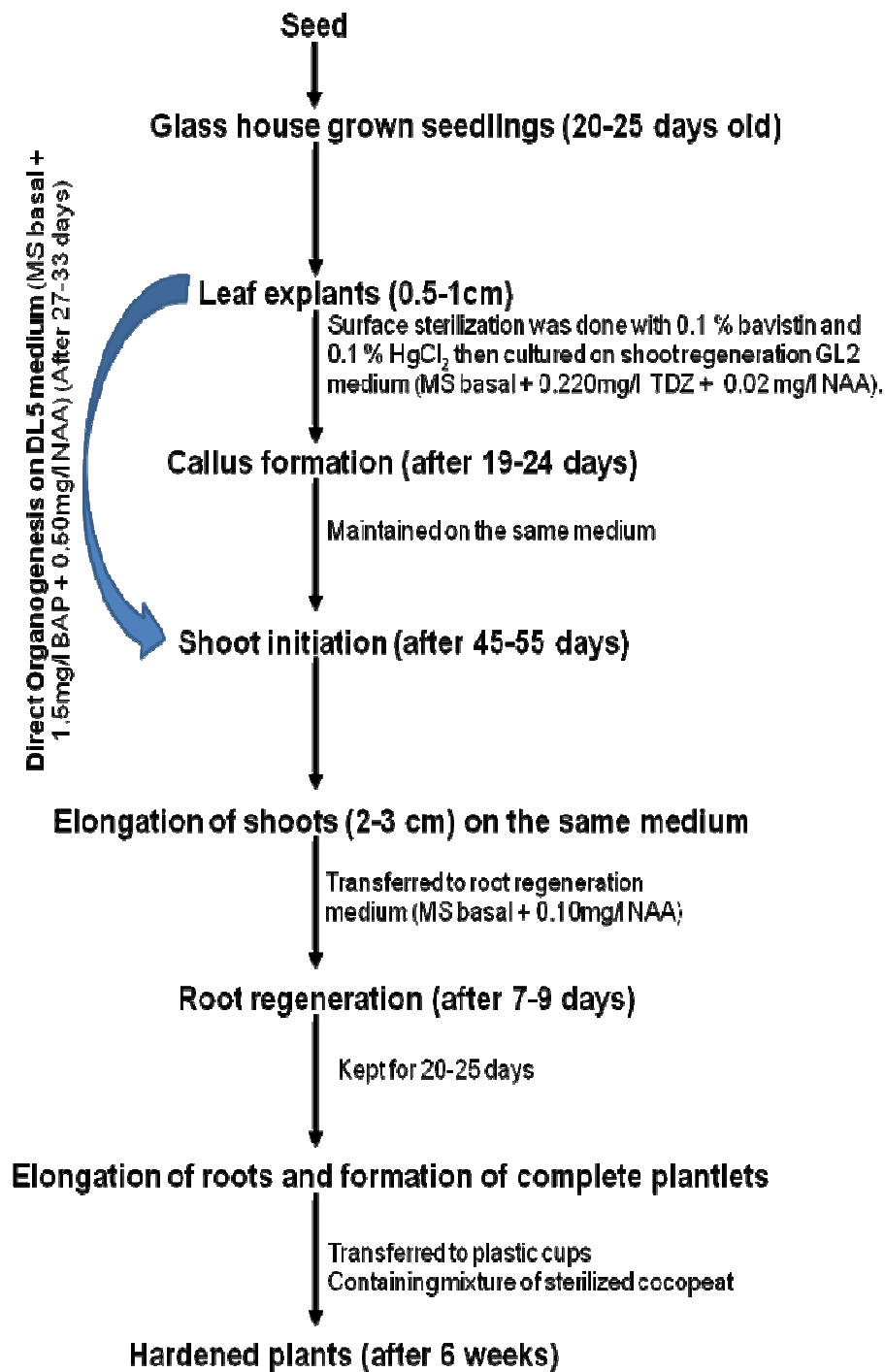


Fig. 40: Flowchart depicting a protocol for plant regeneration from leaf explants in cabbage (*Brassica oleracea* L. var. *capitata* cv. *Pride of India*).

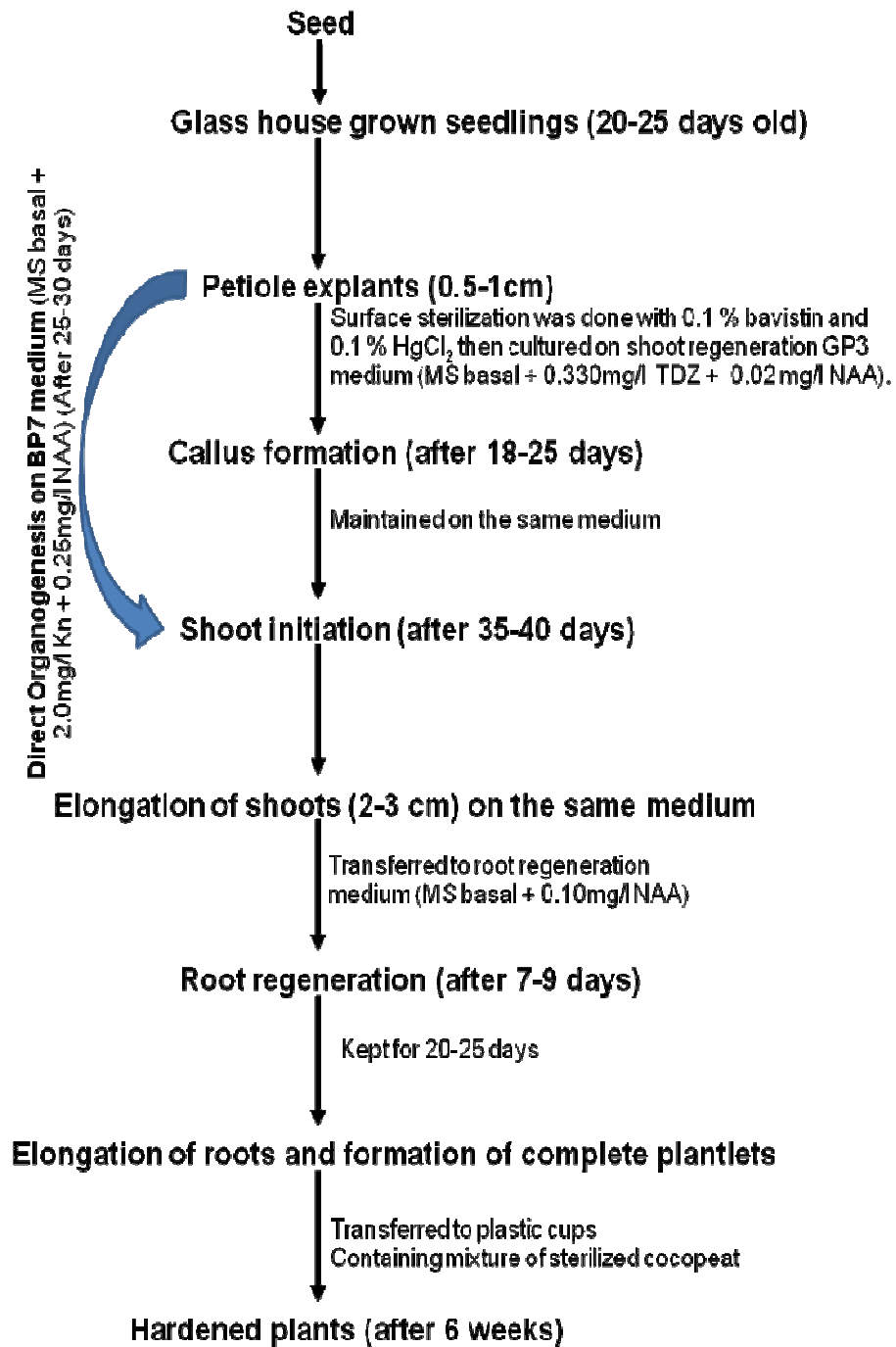


Fig. 41: Flowchart depicting a protocol for plant regeneration from petiole explants in cabbage (*Brassica oleracea* L. var. *capitata* cv. *Pride of India*).

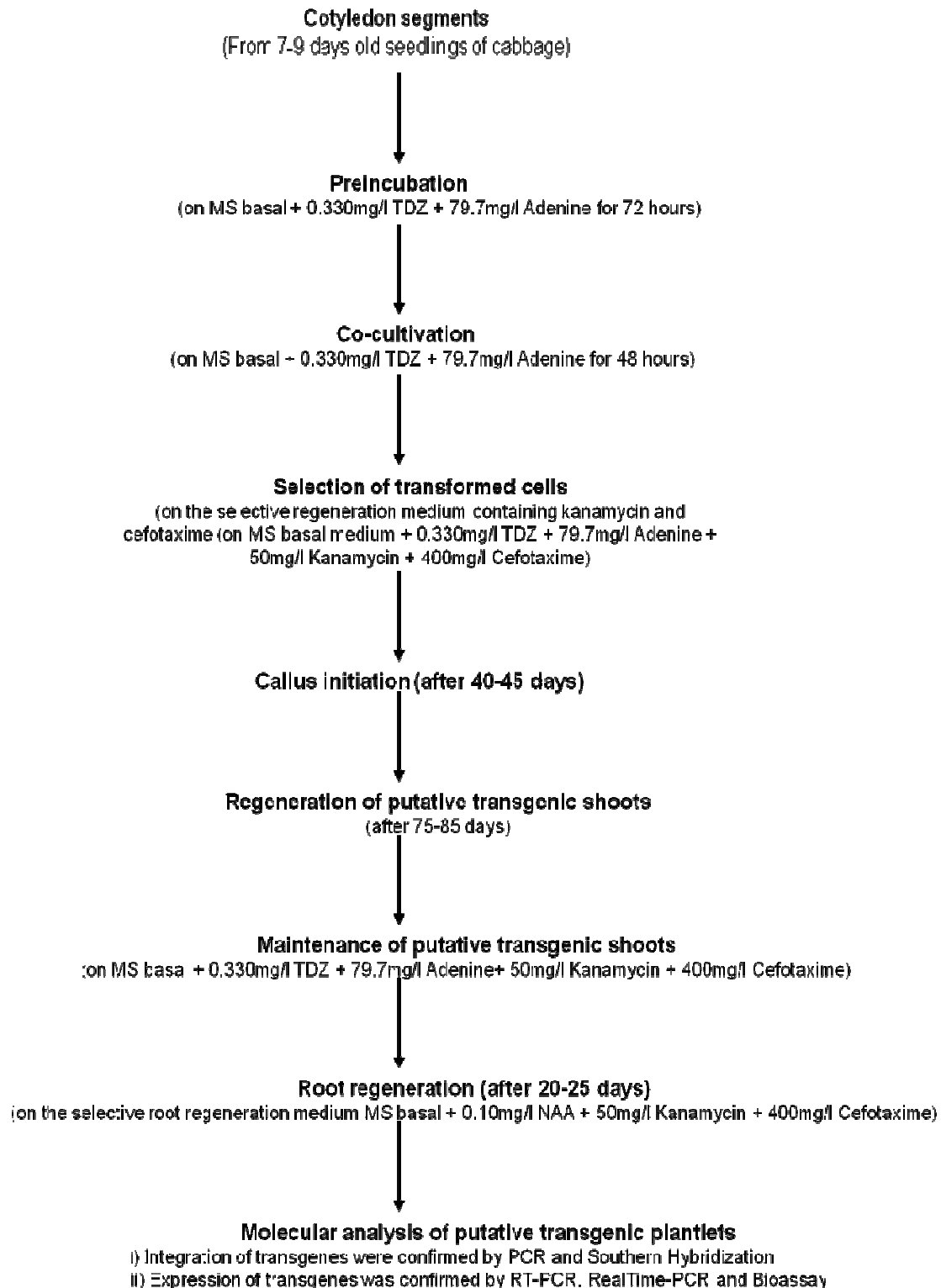


Fig. 42: Flowchart depicting a protocol for *Agrobacterium*-mediated *cryIAa* (insect resistance) gene transfer in cabbage (*Brassica oleracea* L. var. *capitata* cv. *Pride of India*) from cotyledon explants.

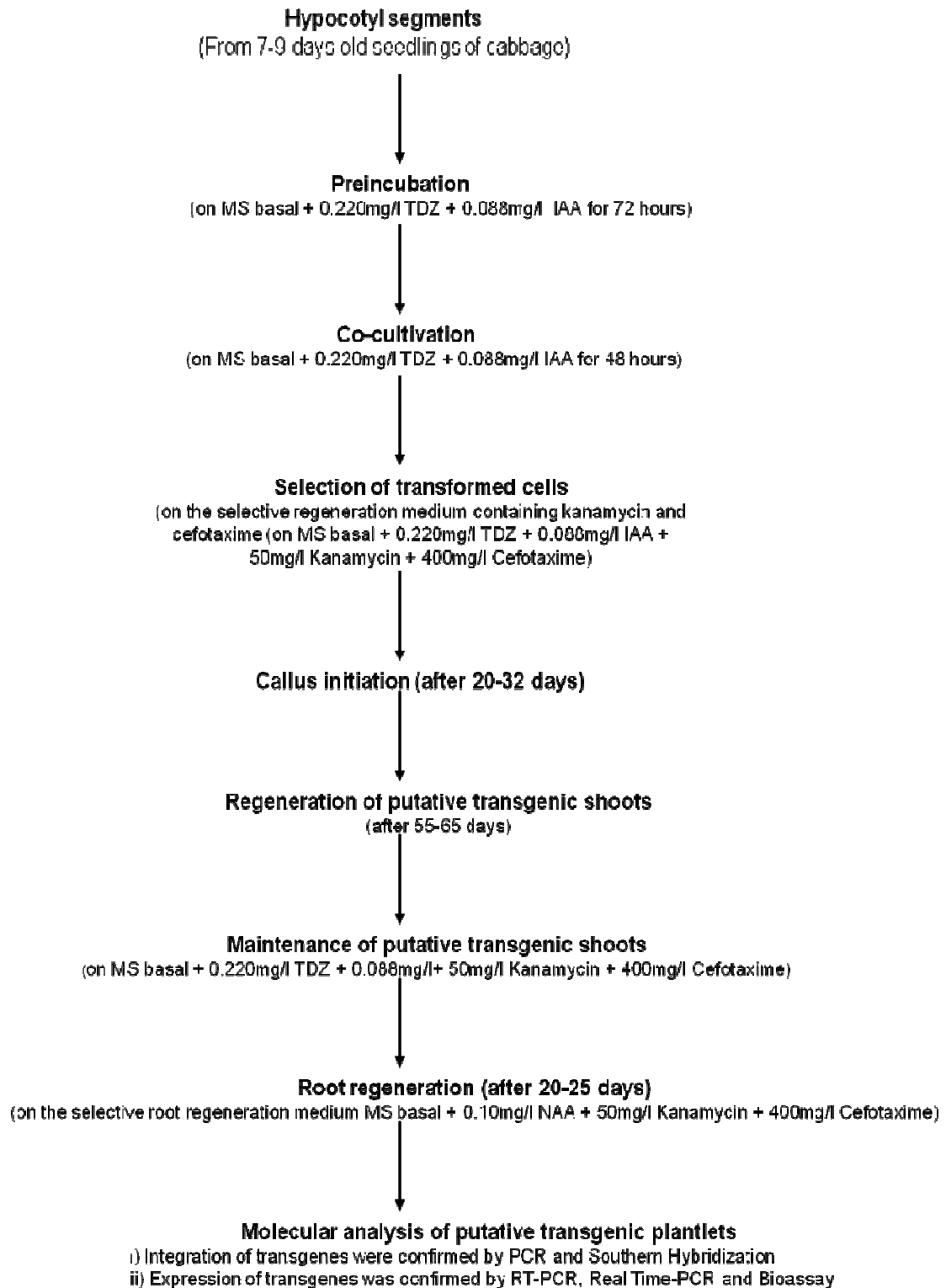


Fig. 43: Flowchart depicting a protocol for *Agrobacterium*-mediated *cryIAa* (insect resistance) gene transfer in cabbage (*Brassica oleracea* L. var. *capitata* cv. *Pride of India*) from hypocotyl explants.

Chapter-7

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DEPARTMENT OF BIOTECHNOLOGY

Title of Thesis : **Studies on *Agrobacterium*-mediated insect resistance gene transfer in Cabbage (*Brassica oleracea* L. var. *capitata*) and molecular analysis of regenerated plantlets.**

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ABSTRACT

Genetic transformation studies were carried out to standardize a protocol for insect resistance gene (*cryIAa*) transfer in cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India). *Agrobacterium tumefaciens* strain containing *npt-II* and *cryIAa* genes in binary vector pBin-1Aa was used for genetic transformation studies. Plant regeneration studies were carried out using four different types of explants viz. cotyledon, hypocotyl, leaf and petiole. Cotyledon and hypocotyl explants were used from seven to nine days old aseptically grown seedlings whereas, leaf and petiole explants were procured from glass house 20-25 days old grown seedlings of cabbage. Hypocotyl explants showed better shoot regeneration as compared to other explants. High efficiency shoot regeneration was obtained in cotyledon (91.11 %), hypocotyl (94.44 %), leaf (91.11 %) and petiole (88.88%) explants on MS medium supplemented with 0.33mg/l TDZ + 79.7 mg/l Adenine, 0.22mg/l TDZ + 0.088mg/l IAA, 0.22mg/l TDZ + 0.02mg/l NAA and 0.33mg/l TDZ + 0.02mg/l NAA, respectively. MS medium supplemented with 0.10mg/l NAA was found best for root regeneration from *in vitro* developed shoots. The regenerated plantlets were acclimatized successfully on cocopeat. Kanamycin sensitivity experiment was carried out to study the effect of antibiotic on relative growth of leaf and petiole tissues and to select transgenic shoots during transformation experiment. Kanamycin sensitivity (10-60mg/l) was checked by fresh weight of the explants which was measured at the interval of 7 days. From the relative growth of the explants it was found that the concentration as low as 10mg/l is toxic to the explants. Effect of different concentrations of cefotaxime was studied on the regeneration potential in cotyledon and hypocotyl explants of cabbage and found no much effect of cefotaxime on regeneration potential. Effect of different concentrations of cefotaxime and kanamycin (50mg/l) were studied on the growth of agrobacterial cells and regeneration potential of cotyledon and hypocotyl tissues after cocultivation. In both the explants the growth of agrobacterial cells were controlled at concentration of 400mg/l cefotaxime and maximum per cent shoot regeneration in cotyledon (35.55 %) and hypocotyl (48.15 %) was obtained on MS medium supplemented with 400mg/l cefotaxime, respectively. Effect of preculturing and co-

cultivation was studied on the transformation frequency. Preculturing of cotyledon and hypocotyl explants for 72 hours and co-cultivation with agrobacterial cells for 48 hours worked out to be the best treatment as it gave the highest transformation frequency (4.66 %) and (14.50 %) in respective explants. Effect of different concentrations of acetosyringone was studied in cotyledon and hypocotyl explants to enhance the transformation frequency. The maximum percent shoot regeneration (18.66 %) and (32.00 %) was obtained from cotyledon and hypocotyl explants cultured on shoot regeneration medium containing 100 μ M acetosyringone at standardized preculturing and cocultivation time interval i.e. 72 hours and 48 hours. The presence/integration of transgene (*cryIAa*) into the genome of cabbage was confirmed by PCR using gene specific primers and Southern blot analysis using radioactive labelled DNA probe. The Southern blot analysis has also been used to confirm copy number of transgene into the genome of cabbage. For PCR analysis, 40 putative transgenic shoots were randomly selected and out of 40 putative transgenic shoots/plantlets, 20 shoots were found to be +ve for the presence/integration of transgene i.e. *cryIAa* into the genome of cabbage. For Southern blot analysis 10 RT-PCR +ve shoots were selected. Out of the 10 PCR +ve shoots, 5 shoots were confirmed +ve for integration of transgene *cryIAa* into the genome of cabbage with 1 to 3 copies of gene insertion. The confirmation of expression of the transgene *cryIAa* into the genome of cabbage at transcriptional level was confirmed by Reverse Transcriptase-PCR and Real Time-PCR and at translational level by Bioassay. A protocol for high frequency plant regeneration and insect resistance gene transfer in cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India) has been standardized.

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APPENDIX-I

COMPOSITION OF MODIFIED MURASHIGE AND SKOOG'S (1962) BASAL MEDIUM (MS MEDIUM)

CONSTITUENTS	AMOUNT (mg/l)
INORGANIC CONSTITUENTS	
Major constituents	
NH ₄ NO ₃	1650.000
KNO ₃	1900.000
CaCl ₂ . 2H ₂ O	440.000
MgSO ₄ . 7H ₂ O	370.000
KH ₂ PO ₄	170.000
Na ₂ EDTA	37.000
FeSO ₄ . 7H ₂ O	27.000
Minor Constituents	
MnSO ₄ . 4H ₂ O	22.300
ZnSO ₄ . 7H ₂ O	8.600
H ₃ BO ₃	6.200
KI	0.830
Na ₂ MoO ₄ . 2H ₂ O	0.250
CuSO ₄ . 5H ₂ O	0.025
CoCl ₂ . 6H ₂ O	0.025
ORGANIC CONSTITUENTS	
Thiamine HCl	0.100
Nicotinamide	0.500
Pyridoxine HCl	0.500
Glycine	2.000
Inositol	100.000
Sucrose	3.000%

APPENDIX-II

SOLUTIONS, CHEMICALS AND REAGENTS FOR MOLECULAR ANALYSIS OF TRANSFORMANTS

1. 1M Tris-HCl Buffer (pH 8.0) (SRL)

121.1g of Tris salt was dissolved in sterilize distilled water and pH was adjusted to 8.0 using HCl. The volume was made up to 1000ml using sterilize distilled water and solution was autoclaved prior to usage.

2. 0.5M EDTA (pH 8.0) (SRL)

186.2g of EDTA dissolved in sterilize distilled water and the pH was raised to 8.0 by the addition of NaOH. The solution was stirred vigorously with a magnetic stirrer and after complete dilution of salt the volume was made up to 1000 ml with sterilized distilled water. Solution was autoclaved prior to usage.

3. 5M Sodium Chloride (NaCl) solution (SRL)

29.20 gm of sodium chloride was dissolved in 70 ml of double distilled water and the final volume was made to 100ml and sterilized by autoclaving.

4. CTAB 10% Solution (N-cetyl NNN-trimethyl ammonium (GENEI) bromide)

10 gm of CTAB was dissolved in 70ml of double distilled water at 65⁰C and the final volume was made to 100ml

5. β -mercaptoethanol solution (SRL)

100% of solution was provided by the manufactures and stored in an amber coloured bottle.

6. Alkaline transfer buffer:

0.4 N NaOH (SRL)

1 M NaCl (SRL)

7. Denaturation solution

1.5 M NaCl

0.5 M NaOH

8. 2.5 N HCl

Add 25 ml of concentrated HCl to 91 ml of sterile H₂O. Store the diluted solution at room temperature.

9. Nuteralization solution

0.5 M Tris-Cl (pH 7.4) (SRL)
1.5 M NaCl

10. Prehyridization Buffer/Hybridization Buffer

0.5 M Sodium Phosphate (pH 7.2)
7% SDS
1mM EDTA (pH 7.0)
0.5 M phosphate buffer is 134g of Na₂HPO₄·7H₂O, 4 ml of 85 % H₃PO₄, H₂O to 1 Lt.

11. Chloroform:Isomyl alcohol(24:1) (SRL)

720µl of chloroform and 30µl of isoamyl alcohol were mixed by vortexing. The resulted solution was kept in an amber coloured container.

12. Ethanol 70% (MERCCK)

70ml of absolute alcohol was mixed well with 30ml of sterilize distilled water to make it 100ml.

13. TAE buffer: 50X Tris-acetate TAE

242gm of tris base and 57.1ml of glacial acetic acid were taken in a container. 100ml of 0.5M EDTA and 200ml of water was added and stirred on a magnetic stirrer until a clear solution was formed. The volume was adjusted to 1 litre and sterilized by autoclaving.

14. Ethidium bromide (GENEI)

10mg of ethidium bromide (10mg/ml) was added to 100ml of sterile distilled water, and stirred on a magnetic stirrer until the dye was completely dissolved.

The container was wrapped in aluminium foil or the solution was transferred to dark bottle and stored at room temperature.

15. 6X loading dye (GENEI)

Weigh 667mg of sucrose and 4.2gm of bromophenol blue and dissolve it in 1ml of double distilled water, mixed it and wrapped the container with aluminium foil.

16. Alkaline Solution-I:

50mM Glucose (SRL)
25mM Tris-Cl(pH-8.0)
10mM EDTA(pH-8.0)

17. Alkaline Solution-II:

0.2N NaOH
1% SDS (w/v) (GENEI)

18. Alkaline Solution-III:

5M Potassium Acetate: 60ml (SRL)
Glacial Acetic Acid: 11.5ml (SRL)
Water: 28.5ml

19. 20X SSC Buffer

Dissolve 175.3 g of NaCl and 88.2 g of Sodium citrate in 800 ml of Distilled water. Adjust the pH to 7.0 with a few drops of 14N solution of HCl. Adjust the volume to 1lt. with distilled water and sterilized by autoclaving.

APPENDIX-III

LIST OF EQUIPMENTS USED

1. Autoclave
2. B.O.D. Incubator
3. Cooling centrifuge
4. Culture room (with temperature controller and timer for maintaining temperature and photoperiodic conditions)
5. Deep freezer (-20⁰C, -40⁰C)
6. Distillation apparatus
7. Electrophoresis unit
8. Gel documentation
9. Hot air oven
10. Laminar air flow
11. Orbital shaking incubator
12. pH-meter
13. Refrigerator
14. Thermocycler (PCR – Polymerase Chain Reaction)
15. Transilluminator
16. UV-Spectrophotometer
17. Vortex mixer
18. Weighing balance
19. Real Time Thermocycler
20. Hybridization Oven

APPENDIX-IV

1. Effect of various concentrations and combinations of BAP+IAA, BAP+NAA, Kn+IAA and Kn+NAA (in MS medium) on shoot regeneration from cotyledon explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India)

a) Per cent shoot regeneration

Source of variation	d.f.	SS	MS	F
Treatment	9	2458.0	2731.1	456.80
Error	20	119.56	5.997	
Total	29	2470.0		

b) Average number of shoots per explant

Source of variation	d.f.	SS	MS	F
Treatment	9	29.305	3.256	387.17
Error	20	0.682	0.841	
Total	29	29.473		

2. Effect of various concentrations of TDZ (in MS medium) on shoot regeneration from cotyledon explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India)

a) Per cent shoot regeneration

Source of variation	d.f.	SS	MS	F
Treatment	9	849.45	94.384	9.27
Error	20	203.69	10.184	
Total	29	1053.1		

b) Average number of shoots per explant

Source of variation	d.f.	SS	MS	F
Treatment	9	1.790	0.198	14.94
Error	20	0.266	0.013	
Total	29	36.7587		

3. Effect of various concentrations and combinations of TDZ and Adenine (in MS medium) on shoot regeneration from cotyledon explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India)

a) Per cent shoot regeneration

Source of variation	d.f.	SS	MS	F
Treatment	9	6328.2	703.13	53.10
Error	20	264.83	13.241	
Total	29	6593.0		

b) Average number of shoots per explant

Source of variation	d.f.	SS	MS	F
Treatment	9	19.705	2.189	152.50
Error	20	0.2871	0.014	
Total	29	19.992		

4. Effect of various concentrations and combinations of TDZ and NAA (in MS medium) on shoot regeneration from cotyledon explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India)

a) Per cent shoot regeneration

Source of variation	d.f.	SS	MS	F
Treatment	9	1917.7	213.07	4.14
Error	20	1028.7	51.434	
Total	29	2946.4		

b) Average number of shoots per explant

Source of variation	d.f.	SS	MS	F
Treatment	9	8.880	0.986	84.79
Error	20	0.232	0.016	
Total	29	9.113		

5. Effect of various concentrations and combinations of TDZ and IAA (in MS medium) on shoot regeneration from cotyledon explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India)

a) Per cent shoot regeneration

Source of variation	d.f.	SS	MS	F
Treatment	9	1447.9	1608.8	102.39
Error	20	31.424	1.571	
Total	29	1451.1		

b) Average number of shoots per explant

Source of variation	d.f.	SS	MS	F
Treatment	9	12.752	1.416	262.88
Error	20	0.107	0.0053	
Total	29	12.860		

6. Effect of various concentrations and combinations of of BAP+IAA, BAP+NAA, Kn+IAA and Kn+NAA (in MS medium) on shoot regeneration from hypocotyl explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India)

a) Per cent shoot regeneration

Source of variation	d.f.	SS	MS	F
Treatment	9	1843.8	2048.7	170.23
Error	20	240.70	12.035	
Total	29	1867.9		

b) Average number of shoots per explant

Source of variation	d.f.	SS	MS	F
Treatment	9	22.387	2.487	168.07
Error	20	0.296	0.0148	
Total	29	22.683		

7. Effect of various concentrations of TDZ (in MS medium) on shoot regeneration from hypocotyl explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India)

a) Per cent shoot regeneration

Source of variation	d.f.	SS	MS	F
Treatment	9	2842.4	315.82	30.61
Error	20	206.36	10.318	
Total	29	3048.8		

b) Average number of shoots per explant

Source of variation	d.f.	SS	MS	F
Treatment	9	9.053	1.005	75.67
Error	20	0.265	0.0132	
Total	29	36.7587		

8. Effect of various concentrations and combinations of TDZ and Adenine (in MS medium) on shoot regeneration from hypocotyl explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India)

a) Per cent shoot regeneration

Source of variation	d.f.	SS	MS	F
Treatment	9	3082.7	342.52	28.80
Error	20	237.85	11.892	
Total	29	3320.5		

b) Average number of shoots per explant

Source of variation	d.f.	SS	MS	F
Treatment	9	4.789	0.532	49.71
Error	20	0.214	0.070	
Total	29	36.7587		

9. Effect of various concentrations and combinations of TDZ and NAA (in MS medium) on shoot regeneration from hypocotyl explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India)

a) Per cent shoot regeneration

Source of variation	d.f.	SS	MS	F
Treatment	9	2278.1	253.12	47.54
Error	20	106.48	5.323	
Total	29	2384.5		

b) Average number of shoots per explant

Source of variation	d.f.	SS	MS	F
Treatment	9	3.699	0.411	57.03
Error	20	0.144	0.0072	
Total	29	36.7587		

10. Effect of various concentrations and combinations of TDZ and IAA (in MS medium) on shoot regeneration from hypocotyl explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India)

a) Per cent shoot regeneration

Source of variation	d.f.	SS	MS	F
Treatment	9	4607.0	511.89	144.30
Error	20	70.948	3.547	
Total	29	17.7187		

b) Average number of shoots per explant

Source of variation	d.f.	SS	MS	F
Treatment	9	10.991	1.221	103.0
Error	20	0.237	0.0118	
Total	29	11.228		

11. Effect of various concentrations and combinations of of BAP+IAA, BAP+NAA, Kn+IAA and Kn+NAA (in MS medium) on shoot regeneration from leaf explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India)

a) Per cent shoot regeneration

Source of variation	d.f.	SS	MS	F
Treatment	9	1914.2	2126.9	167.50
Error	20	253.96	12.698	
Total	29	1939.6		

b) Average number of shoots per explant

Source of variation	d.f.	SS	MS	F
Treatment	9	20.558	2.284	310.35
Error	20	0.147	0.0073	
Total	29	20.705		

12. Effect of various concentrations of TDZ (in MS medium) on shoot regeneration from leaf explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India)

a) Per cent shoot regeneration

Source of variation	d.f.	SS	MS	F
Treatment	9	2585.6	287.29	40.83
Error	20	140.71	7.035	
Total	29	2726.3		

b) Average number of shoots per explant

Source of variation	d.f.	SS	MS	F
Treatment	9	2.692	0.299	16.67
Error	20	0.358	0.017	
Total	29	3.050		

13. Effect of various concentrations and combinations of TDZ and Adenine (in MS medium) on shoot regeneration from leaf explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India)

a) Per cent shoot regeneration

Source of variation	d.f.	SS	MS	F
Treatment	9	1726.2	191.80	20.39
Error	20	188.16	9.4082	
Total	29	1914.4		

b) Average number of shoots per explant

Source of variation	d.f.	SS	MS	F
Treatment	9	5.675	0.630	40.69
Error	20	0.309	0.0015	
Total	29	5.984		

14. Effect of various concentrations and combinations of TDZ and NAA (in MS medium) on shoot regeneration from leaf explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India)

a) Per cent shoot regeneration

Source of variation	d.f.	SS	MS	F
Treatment	9	4246.4	471.82	24.0
Error	20	393.12	19.656	
Total	29	4639.5		

b) Average number of shoots per explant

Source of variation	d.f.	SS	MS	F
Treatment	9	11.342	1.260	104.64
Error	20	0.240	0.0012	
Total	29	11.582		

15. Effect of various concentrations and combination of TDZ and IAA (in MS medium) on shoot regeneration from leaf explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India)

a) Per cent shoot regeneration

Source of variation	d.f.	SS	MS	F
Treatment	9	6321.8	702.42	278.12
Error	20	50.512	2.525	
Total	29	6372.3		

b) Average number of shoots per explant

Source of variation	d.f.	SS	MS	F
Treatment	9	3.092	0.343	44.78
Error	20	0.153	0.0076	
Total	29	3.246		

16. Effect of various concentrations and combinations of BAP+IAA, BAP+NAA, Kn+IAA and Kn+NAA (in MS medium) on shoot regeneration from petiole explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India)

a) Per cent shoot regeneration

Source of variation	d.f.	SS	MS	F
Treatment	9	1495.8	1662.0	214.35
Error	20	155.08	7.753	
Total	29	1511.3		

b) Average number of shoots per explant

Source of variation	d.f.	SS	MS	F
Treatment	9	17.597	1.955	267.0
Error	20	0.146	0.0073	
Total	29	17.774		

17. Effect of various concentrations of TDZ (in MS medium) on shoots regeneration from petiole explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India)

a) Per cent shoot regeneration

Source of variation	d.f.	SS	MS	F
Treatment	9	9997.4	1110.8	167.50
Error	20	132.63	6.631	
Total	29	10130.0		

b) Average number of shoots per explant

Source of variation	d.f.	SS	MS	F
Treatment	9	13.332	1.481	84.70
Error	20	0.349	0.00174	
Total	29	13.682		

18. Effect of various concentrations and combinations of TDZ and Adenine (in MS medium) on shoot regeneration from petiole explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India)

a) Per cent shoot regeneration

Source of variation	d.f.	SS	MS	F
Treatment	9	4140.8	460.09	59.82
Error	20	153.83	7.691	
Total	29	4294.7		

b) Average number of shoots per explant

Source of variation	d.f.	SS	MS	F
Treatment	9	14.484	1.609	141.21
Error	20	0.227	0.0013	
Total	29	14.711		

19. Effect of various concentrations and combinations of TDZ and NAA (in MS medium) on shoot regeneration from petiole explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India)

a) Per cent shoot regeneration

Source of variation	d.f.	SS	MS	F
Treatment	9	4983.7	553.75	69.58
Error	20	159.17	7.958	
Total	29	5142.9		

b) Average number of shoots per explant

Source of variation	d.f.	SS	MS	F
Treatment	9	18.913	2.101	233.23
Error	20	0.802	0.0090	
Total	29	19.093		

20. Effect of various concentrations and combinations of TDZ and IAA (in MS medium) on shoot regeneration from petiole explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India)

a) Per cent shoot regeneration

Source of variation	d.f.	SS	MS	F
Treatment	9	3422.0	380.20	85.45
Error	20	88.996	4.449	
Total	29	3511.0		

b) Average number of shoots per explant

Source of variation	d.f.	SS	MS	F
Treatment	9	9.587	1.063	91.89
Error	20	0.231	0.0011	
Total	29	9.819		

21. Effect of different concentrations of various auxins on per cent root regeneration from *in vitro* developed shoots of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India)

Source of variation	d.f.	SS	MS	F
Treatment	8	3478.8	434.84	11.33
Error	18	690.95	38.386	
Total	26	4169.7		

22. Effect of kanamycin on the relative growth (fresh weight) of leaf explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India)

Source of variation	d.f.	SS	MS	F
Treatment (T)	6	38823.0	6470.5	2097.5
Days (D)	5	64431.0	012886.0	4177.4
D x T	30	28332.0	9444.1	306.1
Error	84	259.12	3.0848	
Total	125	131610.0		

23. Effect of kanamycin on the relative growth (fresh weight) of petiole explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India)

Source of variation	d.f.	SS	MS	F
Treatment (T)	6	21007.0	3501.2	8016.6
Days (D)	5	31626.0	6325.2	1448.25
D x T	30	23560.0	7853.5	179.81
Error	84	36.687	0.4367	
Total	125	76197.0		

24. Effect of different concentration of cefotaxime on the regeneration potential of cotyledon explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India) (without co-cultivation)

a) Per cent shoot regeneration

Source of variation	d.f.	SS	MS	F
Treatment	3	959.69	319.86	390.3
Error	8	0.6555	0.00819	
Total	11	17.7187		

b) Average number of shoots per explant

Source of variation	d.f.	SS	MS	F
Treatment	3	8.242	2.747	24.42
Error	8	0.900	0.112	
Total	11	9.142		

25. Effect of different concentration of cefotaxime on the regeneration potential of hypocotyl explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India) (without co-cultivation)

a) Per cent shoot regeneration

Source of variation	d.f.	SS	MS	F
Treatment	3	92.283	30.761	37.57
Error	8	6.549	0.8186	
Total	11	98.833		

b) Average number of shoots per explant

Source of variation	d.f.	SS	MS	F
Treatment	3	1.440	0.480	32.0
Error	8	0.120	0.015	
Total	11	1.560		

26. Effect of various concentrations of cefotaxime and kanamycin (50mg/l) on the regeneration potential of cotyledon explants in cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India) (after co-cultivation)

a) Per cent shoot regeneration

Source of variation	d.f.	SS	MS	F
Treatment	5	5155.5	1031.1	228.37
Error	12	54.12	4.51	
Total	17	5209.62		

b) Average number of shoots per explant

Source of variation	d.f.	SS	MS	F
Treatment	5	16.2	3.24	66.95
Error	12	0.576	0.048	
Total	17	16.776		

27. Effect of cefotaxime and kanamycin (50mg/l) on the regeneration potential in hypocotyl tissue of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India) (after co-cultivation)

a) Per cent shoot regeneration

Source of variation	d.f.	SS	MS	F
Treatment	5	3717.95	743.59	239.3
Error	12	37.32	3.11	
Total	17	3755.27		

b) Average number of shoots per explant

Source of variation	d.f.	SS	MS	F
Treatment	5	11.50	2.30	65.83
Error	12	0.420	0.035	
Total	17	11.920		

28. Effect of preculture and co-cultivation time on transformation frequency in cotyledon explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India)

Source of variation	d.f.	SS	MS	F
Treatment	2	14.209	7.104	800.43
Cocultivation (C)	2	4.5091	02.254	254.01
Preculturing (P)	2	0.0352	0.0017	1.990
C x P	4	9.0181	3.354	254.01
Error	16	0.1420	0.0088	
Total	26	27.914		

29. Effect of preculture and co-cultivation time on transformation frequency in cotyledon explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India)

Source of variation	d.f.	SS	MS	F
Treatment	2	34.945	17.473	1687.04
Cocultivation (C)	2	10.927	5.463	527.53
Preculturing (P)	2	0.0038	0.00192	1.850
C x P	4	21.855	5.463	527.53
Error	16	0.1657	0.00103	
Total	26	67.931		

30. Effect of preculture and co-cultivation time on transformation frequency in hypocotyl explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India)

Source of variation	d.f.	SS	MS	F
Treatment	2	201.88	100.94	2158.18
Cocultivation (C)	2	89.549	44.775	957.30
Preculturing (P)	2	0.0019	0.00099	0.000
C x P	4	102.61	25.653	548.48
Error	16	0.748	0.00467	
Total	26	394.81		

31. Effect of preculture and co-cultivation time on transformation frequency in hypocotyl explants of cabbage (*Brassica oleracea* L. var. *capitata* cv. Pride of India)

Source of variation	d.f.	SS	MS	F
Treatment	2	366.42	183.21	5.74
Cocultivation (C)	2	333.31	166.65	5.33
Preculturing (P)	2	66.612	33.306	1.04
C x P	4	269.86	67.464	2.11
Error	16	510.69	31.918	
Total	26	1546.9		

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(Geetika Gambhir)