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Comparative Studies on in Vitro Regeneration, Cell Culture and Artificial Seed Production in Eggplant (Solanum Melongena L.)

Rahman, Md. Motiur

University of Rajshahi

COMPARATIVE STUDIES ON *IN VITRO* REGENERATION, CELL CULTURE AND ARTIFICIAL SEED PRODUCTION IN EGGPLANT (*SOLANUM MELONGENA* L.)

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By

Md. Motiur Rahman

Plant Biotechnology Laboratory
Institute of Biological Sciences
University of Rajshahi
Rajshahi-6205
Bangladesh.

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Dedicated
To My
Beloved
Parents

Declaration

I hereby declare that the whole of the work now submitted as a thesis for the degree of Master of Philosophy in Plant Biotechnology at the Institute of Biological Sciences, University of Rajshahi, Bangladesh is the result of my own investigation.

30.04.07

Md. Motiur Rahman

Candidate

Certificate

I hereby certify that the thesis entitled "Comparative studies on *in vitro* regeneration, cell culture and artificial seed production in eggplant (*Solanum melongena* L.)" has been submitted by Mr. Md. Motiur Rahman in fulfillment for the requirements of the degree of Master of Philosophy in the field of Plant Biotechnology, Institute of Biological Sciences, University of Rajshahi, Bangladesh. It is also certified that the research work embodied in this thesis is original and carried out by Mr. Md. Motiur Rahman and the work or part of it has not been submitted before as candidature for any other degree.

I would like to clarify that the author has completed his work under my supervision.

30/4/07

Dr. M. A. Bari Miah
Professor
Institute of Biological sciences
University of Rajshahi
Rajshahi-6205
Bangladesh.

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ABSTRACTS

Protocol establishment for cell culture and artificial seed production for improvement of agricultural crops is a new venture in Bangladesh. Both the research avenues are equally important to afford opportunities for the application of advance biotechnological tools. Towards the improvement of eggplant in Bangladesh, meristem culture, somatic embryogenesis and artificial seed production were the prime objectives of the present investigation. Widely cultivated two local cultivars namely cv. China and cv. Loda were taken for the present study. Different medium compositions were tested for sorting out efficient combinations for the better establishment of apical meristem culture, vital footstep towards developing virus-free cultivars. Shoot tips of 20-25 days old *in vitro* grown plants of the two cultivars were used for meristem isolation. For primary establishment of isolated meristems, 2.0 mgl^{-1} BAP in MS liquid medium was found to be the most effective. MS semisolid medium having 0.50 mgl^{-1} GA_3 alone was found to be the best and 0.50 mgl^{-1} GA_3 + 2.0 mgl^{-1} BAP appeared moderate formulation for shoot elongation from the primary established meristem. MS semisolid medium fortified with 3.0 mgl^{-1} IBA was found to be the most effective for root induction.

Embryogenic callus induction is the precondition for successful accomplishment of embryogenesis and cotyledon and midrib of the two cultivars were used for this purpose. Auxin-cytokinin combinations were proved more potent for embryogenic callus induction as well as callus growth than that of auxin tried singly in the media. MS medium supplemented with 2.0 mgl^{-1} NAA + 0.05 mgl^{-1} BAP was proved to be the best media formulation for induction of embryogenic calli in eggplant, when cotyledon showed 85.43% and 82.50% and midrib showed 83.30% and 84.50%, respectively for cv. China and cv. Loda. The embryogenic calli were harvested from the experiment and cultured in agarified MS media supplemented with different hormonal treatments to test their regeneration potentialities. Among the different treatments, 2.0 mgl^{-1} Zeatin + 1.0 mgl^{-1} BAP performed the best for regeneration. In this case, cotyledon showed highest regeneration frequency 87.33% and 86.50%, whereas midrib showed 82.15% and 79.75%, respectively for cv. China and cv. Loda.

Best quality of embryogenic calli were isolated and cultured in liquid MS media under continuous shaking in conditioned dark room for cell culture. Cell cultures of the two cultivars were continuously maintained in the lab after transferring them in the fresh media every after two weeks. Growth of eggplant cell culture was studied synchronizing with different days and a growth curve corresponding to the explants was established. The growth curves indicated that eggplant cell continued to grow according to the increased days and their peak period was noted on ten days, afterwards their growth ceased down gradually. Single cells were isolated from cell suspension and cultured in liquid MS media with hormonal supplements to induce single cell derived callus. MS medium supplemented with 2.0 mgl^{-1} NAA + 0.50 mgl^{-1} BAP was proved to be suitable for single cell derived callus induction. The single cell derived calli were cultured in semisolid MS medium having hormonal supplements for somatic embryogenesis. MS medium having 2.0 mgl^{-1} Zeatin + 1.0 mgl^{-1} BAP gave the best result for somatic embryogenesis and shoot regeneration from single cell derived calli. MS medium having 3.0 mgl^{-1} IBA showed highest percentage (93.30%) of root induction in the regenerated shoots.

The present work also describes a regeneration protocol by encapsulating nodal segments and somatic embryos collected from *in vitro* grown sources. The explants (nodes and somatic embryos) were encapsulated with sodium alginate solution (4%) supplemented with growth regulators and subsequently the synthetic seeds were inoculated in hormone free agar gelled MS media. Among different treatments used in alginate bead, 0.10 mgl^{-1} GA_3 + 0.50 mgl^{-1} BAP performed the best for artificial seed germination and highest germination rate was 90% for nodal segments of both cultivars. On the contrary, the somatic embryos showed highest germination rate 65% and 70%, respectively for cv. China and cv. Loda. This result was closely followed by 0.10 mgl^{-1} GA_3 + 0.10 mgl^{-1} BAP appearing as the second performer. Different types of carbon sources (sucrose, mannitol, sorbitol) were also treated in alginate beads and 1% sucrose performed the best for synthetic seed germination (90-95% for node and 60-65% for somatic embryo). Agarified MS medium supplemented with 3.0 mgl^{-1} IBA was found to be the best formulation for root induction in the regenerated shoots. The well-rooted plantlets were gradually acclimatized and successfully established to the field condition.

CONTENTS

ABBREVIATIONS

%	Percentage
µg	Micro gram
µl	Micro liter
°C	Degree Celsius
0.1N	0.1 Normal solution.
2,4-D	2,4-Dichlorphenoxy acetic acid
BAP	6-benzul aminopurine
$CaCl_2$	Calcium chloride
cv.	Cultivar
e.g.	exampli gratia = for example
et al.	et allurio=Other people
etc.	et cetra = and other
Fig.	Figure (s)
gl^{-1}	Gram per litre
HCl	Hydrochloric acid
$HgCl_2$	Mercuric chloride
i.e.	id est = that is
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
KIN	Kinetin (6-furfuryl aminopurine)
KOH	Potassium hydroxide
L.	Linnaeus.
$\overline{X}$	Mean
mg	Milligram
mgl^{-1}	Milligram per litre
min	minute (s)
ml	Mililiter (s)
MS	Murashige and Skoog (1962) medium
NAA	α-Napthalene acetic acid
NaOH	Sodium hydroxide
No.	Number
pH	Negative logrithm of hydrogen ion concentration
S.	*solanum*
SD	Standard Deviation
SE	Standard Error
Sp.	Species
viz.	videlicet = Namely
wt	weight

CHAPTER ONE

Introduction

CHAPTER I

1. GENERAL INTRODUCTION

The present tissue culture revolution stems largely from the fact that plant tissue culture and associated cloning techniques provide the foundation for exploitation of genetic engineering. Tissue culture propagation is also a way to study the mechanisms by which cells differentiate, thereby providing an experimental approach to link genotype and phenotype. "Plant tissue culture" broadly refers to the cultivation in *in vitro* of all plant parts, whether a single cell, a tissue, an organ, artificial seed under aseptic conditions.

1.1. Plant Tissue Culture

The application of advanced biotechniques in agriculture such as tissue culture is now consider very promising for rapid and economic clonal multiplication of fruit and forest trees, production of virus-free stocks of clonal crops, creation of novel genetic variation though somaclonal and gametoclonal variation and transfer of novel and highly valuable genes (usually for herbicide and disease resistance) have opened up exciting possibilities in crop production, protection and improvement. According to Scowcroft *et al.* (1987) tissue culture technique can play an important role for enrichment of genetic variability by creating variation (somaclonal variation) or mutation (by applying radiation or chemical mutagens to *in vitro* cultured plant material).

The term plant tissue culture indicates the growth or maintenance of plant cells, tissues, organs or whole plants *in vitro (vitro* = glass). So, plant tissue culture is a special technique where living small segments of plant such as root tip, shoot tip, leaf, floral parts, seeds or other tissues are cultured on semi-defined or defined nutrient medium inside glass vessels like test tubes, conical flasks or petridishes under aseptic and deliberately modified environmental condition of temperature and light and are ultimately transferred to the field. From this point of view this technique and mode of propagation is called micropropagation. These techniques have the potentials not only to improve the existing cultivars but also for the synthesis of novel plants. The range of

routine technologies has expanded to include somatic embryogenesis as well as the application of bioreactors to mass propagation (Williams, 2002). We can summarize the application of plant tissue culture as pointed below:

Through tissue culture hundreds of plantlets can be grown from a small, even microscopic piece of plant tissue round the year.

- Rapid and large scale multiplication of genetically identical plants (clonal propagation) from a single "superior" stock plant by axilliary bud growth, direct (adventitious) organogenesis, callus to organogenesis and somatic enbryogenesis.
- Production of pathogen frees plants by meristem and callus culture. This also facilitates transfer of plant material through international borders.
- Germplasm storage and long term storage (cryopreservation) of stock plants- *in vitro* gene banks.
- Selection of mutants from spontaneous or induced mutations.
- Recovery of hybrids from incompatible species through either embryo or ovule culture.
- *In vitro* grafting.
- Production of haploid plants through anther or microspore culture. Haploid plants may be used to recover recessive mutations in breeding programmes. Subsequent regeneration of double haploids provides homozygous and thus pure breeding lines.
- Production of rooted microcuttings in recalcitrant woody ornamental species.
- Protoplast culture and somatic fusion.
- DNA transformation system.
- Recovery of regeneration of transformed cells.
- Production of secondary metabolites.

(Taji and Williams, 1996; Williams, 2002).

Now a day, the application of plant tissue culture goes well beyond the bounds of agriculture and horticulture. It has found application in environmental remediation, mining, waste recycling, industrial processing etc. (Williams, 2002).

1.2. History of Tissue Culture

During the last few decades, the technique of plant tissue culture has been developed as a new and powerful tool of crop improvement (Carlson, 1975; Razdan and Cocking, 1981) and received much attention of modern scientists (D' Amato, 1977; Skirvin, 1978; Scowcroft and Larkin, 1982). The history of plant tissue culture is historically linked to the discovery of the cell and subsequent propounding of the cell theory by Schleiden (1838) and Schwann (1839). They mentioned "Omuis Cellular Cellulae", *i e.* a cell may originate from the existing one viz. a cell is totipotent. This means that each cell posses all the genetic information needed for the formation of a complete plant (Gautheret 1985). Tissue culture is based primarily on the principle of totipotency. In 1901, T. Morgan used the term totipotency firstly. Henri-Louis Duhamel du Monceau's (1756) demonstrated spontaneous callus formation on the decorticated region of elm plant (Razdan, 1993). His studies, according to noted biologist Gautheret (1985) could be considered a "forward" for the discovery of plant tissue culture. Trecul (1853) observed callus formation in a number of decorticated trees. He published excellent picture of callus section. In his classic experiments on polarity in cuttings, Vochting (1878) observed that all cells along the stem length are capable of forming roots as well as shoots (Bhojwani and Razdan, 1983).

German botanist Gottlieb Haberlandt (1902) developed the concept of *in vitro* cell culture. He was first to culture isolated, fully differentiated cells in nutrient medium containing glucose, peptone and Knop's salt solution and observed obvious growth in palisade cells. Hanning (1904) initiated a new line of investigation involving the culture of embryonic tissue. He excised nearly mature embryos of some crucifers and grew them to maturity on mineral salt and sugar solution. Few years later Winkler (1908) observed some cell division in segments of string bean and Simon (1908) achieved

success in the regeneration of a bulky callus, buds and roots from poplar stem segments.

A new approach of tissue culture comprising the culture of meristematic cells was conceived simultaneously by Kotte (a student of Haberlandt in Germany) and Robbins (U.S.A). They reported some success with growing isolated root tips independently. Further work by Robbins and Maneval (1924) enabled them to improve root growth, but the first successful report of continuously growing cultures of tomato root tips was made by White (1934). Initially White used a medium containing inorganic salts, yeast extract and sucrose, but later yeast extract was replaced by three B-vitamins, viz. pyridoxine, thiamin and nicotinic acid (White, 1937). This synthetic medium has proved to be one of the basic media for a variety of cell and tissue cultures. In the mid-nineteen-thirteen, identification of auxin as a natural growth regulator was done which gave a big push to the development of plant tissue culture technique. Gautheret (1939) had cultured cambium cells of some tree species (*Salix capraea, Populus nigra)* on Knop's solution containing glucose and cysteine hydrochloride and recorded proliferation of callus for a few months. White (1939) reported similar results in the cultures from tumor tissues of the hybrid *Nictiana glauca* X *N. langsdorffii* and Nobecourt established continuously growing cultures of carrot slices. Finally, the possibility of cultivating plant tissues for an unlimited period, using media enriched with auxins was announced independently by Gautheret, White and Nobecourt (1939).

Skoog (1944), Skoog and Tusi (1951) demonstrated that adenine stimulates cell division and induces bud formation in tobacco tissue. This convinced Skoog and collaborators that nucleic acid, which contains substances such as adenine influence tissue proliferation. Skoog (1955) and collaborators isolated from autoclaved yeast extract a derivative of adenine (6-furfuly amino urine) named kinetin. A substance with kinetin-like properties was isolated from young maize endosperm by Lethum (1963) and named zeatin. Muir (1953) demonstrated that by transferring callus tissues of *Tagetes erecta* and *Nicotiana tabacum* to liquid medium and agitating the cultures on a shaking machine it was possible to break the tissue into single cells and small cell aggregates. Muir *et al. (1954)*

mechanically isolated single cells from the suspension and made them dividing individually on separate filter papers resting on the top of a well-established callus culture.

Skoog and Miller (1957) proposed the concept of hormonal control of organ formation after the discovery of auxin and cytokinin. Vasil and Hildebrandt (1965) raised whole plants starting from single cell of tobacco using coconut milk. Ball (1946) successfully raised transplantable whole plants of *Lupinus* and *Tropaeolum* by cutting their shoot meristem. Guha and Maheswari (1966) cultured immature anthers of *Datura innoxia* and were able to raise embryoids and plantlets. Carlson *et al.* (1972) produced the first somatic hybrid between *Nicotiana glauca* × N. *langsdorffii* by protoplast fusion.
These in brief are some of the milestones in the development of the technique of plant tissue culture. Combined now with molecular biology, plant tissue culture is enhancing our understanding of plant growth, metabolism, cell and tissue differentiation, genetic conservation, cell division, cell structure, hormonal regulation, signal transduction and genetics (Gorst, 1983).

1.3. Tissue Culture of Eggplant

Salehuzzaman and Joarder (1978) reported that the best hybrids were obtained by crossing between varieties containing wide contrasted characters. They suggested possibilities of commercial cultivation in hybrid brinjal program and the varieties containing diverse morphological characters will give hybrid vigour. They studied some agronomic characters in eggplant and found high heritability and high genetic gains.

Inherently low genetic variability of eggplant caused by a predominantly high degree of self-pollination has imposed limitations on using conventional breeding approaches. Eggplants are extremely sensitive to frost, water logging condition and to injury by pesticides or herbicides and have very little defense against a number of fungal disease and also to wilt. *In vitro* selection of plants derived from somatic embryogenesis or organogenesis may possess sufficient useful variability to be used in a brinjal improvement programme. Haploid plants regenerated through anther culture may be used in breeding progamme. These haploids may also be used in changing the ploidy

level by conventional chromosome doubling. Additional unexpected benefits from *in vitro* propagation such as greater vigor, earlier flowering, fuller branching and higher yield can also be maintained (Murashige, 1978).

A few aspects of eggplant tissue culture have been done by several researchers. Yamada *et al.* (1967) used three strains of *Solanum* (BD, BND and KA) to studies on the differentiation in cultured cells and embryogenesis. Kamat and Rao (1978) carried out an extensive study on morphogenesis from hypocotyls sections of 4-week-old seedlings. On a modified MS medium, extensive plant regeneration occurred at several combinations of auxin and cytokine. The best over all success occurred with 5.7 μm IAA and 4.4 μm BAP. Production of haploid eggplant was reported by Isouard *et al.*, (1980). Organogenesis and propagation *in vitro* from vegetative segments of *S. melongena* was reported by Macchia *et al.* (1983) and Fassuliotis *et al.* (1981). Regenerated plants could be easily established on soil in pots. They eventually flowered and set fruits.

Plant regeneration from mesophyll protoplasts of the eggplant and its wild species were carried out by Asao *et al.* (1989). Haploid and diploid plants obtained by culture *in vitro* from eggplant anthers was reported by Isouard *et al.*, (1980). An extensive work has been done by several scientists during these decades. *In vitro* culture of Solanum species for root knot nematode resistance was reported by Fassuliotis et al. (1980). Tissue culture methodology greatly varies with different strains or with different genotypes of eggplant (Yamada *et al.*, 1967). It is necessary to establish specific tissue culture method for individual cultivars of eggplant.

1.3.1. Description of Meristem Culture

Some times the eggplant is infected by systemic diseases caused by fungi, viruses, bacteria, mycoplasma etc. Pathogen attack does not always lead to the death of the plant but very often the infection caused by pathogens considerably reduces the yield and quality of brinjal. While pathogens are nearly always transferred in plants through vegetative propagation, viral diseases occur in virtually all seed propagated as well as

vegetatively propagated crop species (Kartha 1984). Eradication of pathogens is highly desirable to optimize the yields and also to facilitate the movement of materials across the international boundaries.

While eggplant infected with bacteria and fungi may respond to treatments with bactericidal and fungicidal compounds, there is no commercially available treatment to cure virus-infected plants. The therapeutic chemicals capable of eradicating virus from infected plants are not available though the use of virazole and vidarabine (antimetabolites) in the culture medium have resulted in the production of virus-free lily and apple plants (Pierik 1989). To produce disease-free eggplant, a healthy nucleus stock could be developed by selecting out one or more healthy plants and then multiplying them vegetatively. Apical meristems in the infected plants are generally either free or carry a very low concentration of the viruses (Razdan 1993). There are many explanations for this, such as: (a) viruses move readily in plant body through the vascular system which in meristems is absent, (b) a high metabolite activity in the actively dividing meristematic cells does not allow virus replication and (c) a high endogenous auxin level in shoot apices may inhibit virus multiplication.

A Knowledge of the gradient of virus distribution in shoot tips encouraged Holmes (1948) to obtain virus-free plants from infected individuals of Dahlia through shoot-tip cuttings. Morel and Martin (1952) applied tissue culture techniques for elimination of viral infection in Dahlia. Since then meristem culture has now become a popular horticultural practice towards production of pathogen-free plants stock.

1.3.2. Description of Somatic Embryogenesis

For genetically stable species, somatic embryogenesis offers a very fast scaling up system, specially when its possible to produce embryos in bioreactors. Unfortunately this production via fermentors was not simple and to day, only a few model plants are successfully produced by such technology. Several bottleneck limit the use of this interesting technology. One of the main problems is genetic stability. Another difficulty is the loss of embryogenic capacity over time.

According to Sharp *et al.* (1980), somatic embryogenesis may be initiated in two different ways. In some cultures embryogenesis occurred directly in the absence of any callus production from "pre-embryonic determined cells" that are programmed for embryonic differentiation. The second type of development requires some prior callus proliferation, and embryos originate from "induced embryogenic cells" within the callus. The first systemic attempt to grow the embryos of angiosperms *in vitro,* under aseptic conditions, was made by Hanning (1904), who cultured mature embryos of two crucifers, *Cochleria and Raphanus.* Subsequently, many workers raised plants by culturing embryos excised from mature seeds. A stimulus for further progress in the field of embryo culture was provided by Laibach (1925, 1929). The experimental somatic embryogenesis has been reported in tissue culture from more than 30 plant families (Raghavan, 1976; Narayanaswamy, 1977;Ammirato, 1983).

In vitro plant regeneration from various organs of diverse plant groups (Murashige, 1974; 1978), through somatic embryogenesis (Rangaswamy, 1986), organogenesis (Hu and Wang, 1983) and androgenesis (Clapham, 1977; Radojevic and Kovoor, 1986) have been successfully reported in large number of taxa. It has been reported that the calli were generally mixoploidal cell colonies, which were lacking cytological uniformity (Yamada *et al.,* 1967). So, callus derived plant through somatic embryogenesis will have maximum possibilities to create variability, that is "somaclonal variation". This somaclonal variation has attracted considerable interest to the plant breeder. Such lines of produced via tissue culture with valuable agronomic characters can be easily utilized in the breeding of desirable varieties.

1.3.3. Description of Artificial Seed Production

Commercial application of plant tissue culture technology has been demonstrated, however constraints of high labour and other expenses like nutrient medium and hardening and delivery of tissue cultured plants remains to be overcome. In this context, synthetic seed technology assumes greater importance because of low cost and high volume propagation system. Many plant systems are known to produce large number of somatic embryos in tissue culture. Such somatic embryos proceed through similar

developmental stages like that of zygotic embryos and do possess root and shoot meristem connected by a common vascular system. Like zygotic embryos somatic embryo tolerates severe desiccation.

To date synthetic seeds have been prepared by using a suitable encapsulating matrix in many plants. The main thrust is to prepare a simple inexpensive delivery unit of *in vitro* propagated plants. Synthetic seed technology offers many useful advantages on a commercial scale. The resultant plant population from the synthetic seed will be uniform and the direct delivery of somatic embryos will save many subcultures to obtain plantlets from regenerated embryos. The encapsulated embryos could also be packed with pesticides, fertilizers, nitrogen fixing bacteria and even microscopic destroying worms (Redenbaugh *et al.*, 1986; Ohishi *et al.*, 1995)

However, successful development of synthetic seeds involves several important features such as: (1) the ability of *in vitro* cultures to produce large number of somatic embryos over a long time span, (2) synchronized development of somatic embryos and (3) the capacity of the encapsulated embryos to withstand the desiccation shock and to germinate into a plant. The scale-up operation of somatic embryos in a bioreactor followed by mechanization of the process encapsulation is the last step for sowing of synthetic seeds in large numbers. In addition to using somatic embryos, axilliary buds, adventitious buds and shoot tips have also been used in the preparation of synthetic seeds (Redenbaugh, 1993; Bapat and Rao, 1988, 1990; Ganapathi *et al.*, 1992, 1994). The main advantage of using these vegetative propagules is that it can be a useful delivery system for tissue cultured plants by eliminating rooting and hardening phases by directly sowing the encapsulated beads in the soil. Also in a plant species where somatic embryogenesis is not established, these alternative explants could be useful for the production of synthetic seeds.

Redenbaugh *et al.*, (1988) identified various stages in the production of somatic embryo and their utilization as artificial seeds. These stages are as follows:

1. Selection of candidate crops based on both technological and commercial potential.
2. Optimization of somatic embryogenesis system from cultured cells.
3. Optimization of embryo maturation.
4. Automation of embryo production
5. Production of mature, synchronized embryos
6. Encapsulation of embryos with necessary adjuvants
7. Coating of encapsulated embryos
8. Optimization of green house and field conditions for the conversion of embryos to plants
9. Delivery system for artificial seeds in terms of increasing productivity.

The first application of artificial seed technology has been made in alfalfa and celery (Redenbaugh *et al.* 1986). Somatic embryos were encapsulated as single embryo beads (Ca. 4 mm in diameter) to produce individual artificial seeds. The somatic embryos were mixed in sodium alginate and then dropped in a solution of calcium chloride to form calcium-alginate beads. Alfalfa plants from artificial seeds were planted directly in the green house with> 50% conversion (Fuji *et. al.* 1989. 1992) and initial field trials were completed (Redenbaugh, 1991).

Research on artificial seeds in rice is still in infancy and this technology through somatic embryogenesis would offer a great scope for large scale propagation of superior, elite hybrids (Brar *et. al.* 1994). It was reported high frequency somatic embryogenesis from indica rice cultivars (Suprasanna et. al. 1995) and utilized this embryogenic system for the production of artificial seeds. Callus cultures were established to form mature seed embryos on MS (Murashige and Skoog. 1962) medium supplemented with 2 mgl^{-1} 2,4-D. White, compact embryogenic callus developed somatic embryos on medium with low cytokinin. Mature somatic embryos (2-3 mm with scutellum and coleoptile) were used for encapsulation in 3% sodium alginate prepared either in MS or White's medium (White, 1934). The encapsulated embryos were placed on nutrient media and on

different substrates (sterile absorbent cotton, filter paper and macpeat). The encapsulated embryos developed into plantlets on nutrient media and filter paper followed by cotton. Filter paper moistened with MS basal medium gave higher frequency of germination compared to other substrates. The encapsulated embryos developed vigorously and rapidly as compared to non-encapsulated embryos.

Redenbaugh (1991) and Brar *et. al.* (1994) emphasized the need for research on artificial seeds in rice through somatic embryogenesis and outlined its impact on mass propagation of true breeding hybrids which can lead to commercialization of this technology. Also artificial seeds can be useful in handling, transportation and delivery of embryos in a safer and easier way. Encapsulated embryos can be stored for the preservation of valuable germplasm.

Advantage of Artificial Seed over Somatic Embryos for Propagation:

- ❖ Easy of handling in storage
- ❖ Easy to transport
- ❖ Has potential for long-term storage without losing viability.
- ❖ Maintains the clonal nature of the resulting plants.
- ❖ Serve as a channel for new plant lines produced through biotechnological advances to be delivered.
- ❖ Directly to the green house or field.
- ❖ Allows economical mass propagation of elite plant varieties with propagation barrier.

Potential Uses of Artificial Seeds

- ❖ Reduced cost of transplants
- ❖ Direct green house and field delivery of
  - -elite, select genotypes
  - - hand pollinated hybrids
  - -genetically engineered plants
  - -sterile and unstable genotypes.'
- ❖ Large scale monoculture

- ❖ Mixed genotype plantations
- ❖ Carrier of adjuvant such as microorganisms plant growth regulators, pesticides, fungicides, nutrients and antibiotics.
- ❖ Protection of meiotically unstable, elite genotypes.
- ❖ Can be conceivably handled as seed using conventional planting equipment.

1.4. Description of Eggplant

Solanum melongena L. (2n=24), commonly known as eggplant, aubergine, guinea squash or brinjal is one of the most popular and multiuse horticultural vegetable and also cheap source of vitamins and minerals particularly iron. Its total nutritional value is comparable with tomato (Kalloo, 1993). Since, brinjal, a nutritious delicious but low cost vegetable all over the world, so it has a great demand. This crop is cultivated as a vegetable throughout the tropics and as a summer annual in the worm subtropics. Eggplant is one of the most common, popular and principal vegetable crops grown in Bangladesh and other parts of the world. This crop is commonly known as begun in Bangladesh. It can be grown in almost all parts of Bangladesh, all the year round.

1.4.1. Taxonomic Position

Kingdom: Plant Kingdom
Division: Angiospermae
Class: Dicotyledoneae
Order: Solanales
Family: Solanaceae
Genus: *Solanum*
Species: *S. melongena* L.

Eggplant is one of the non-tuberous species of the nightshade family Solanaceae. It is classified in the sub-family Solanoideae, the tribe Solaneae, the genus *Solanum* and the subgenus *Leptosmonum* (Dun.) Bitt. that includes more than 450 species distributed among 22 sections (D'Arcy, 1972; Whalen, 1984).

1.4.2. Origin and Distribution

The eggplant, a normally self-fertilized annual is of uncertain origin (Bose et. al., 1993). Eggplant is a native of the Indian sub-continent, with India as probable centre of origin and Indo-Burma, China, Japan are the secondary centre of eggplant origin (Gleddie *et al.*, 1986). The cultivated eggplant is undoubtedly of Indian origin and has been in cultivation for long time (Thompson and Kelly, 1957). Eggplant has been cultivated in Asia for over 1500 years. Burkill (1935) reported that wild yellow-fruited types after the name *Solanum ferox* are found in Malaysia and it is not certain whether it is a separate species. According to Zeven and Zhukovsky (1975), it originated in India but has a secondary centre of variation in China. In China, it has been known since the last 1500 years. On the basis of a study of this distribution of variability, Vavilov (1928) regarded this crop as being of Indian origin but no date for domestication can be suggested. He added that some secondary variability had also developed in China. It has been known in China since the 5th century B.C. There have been suggestions of an African origin of this crop (Sampson, 1936), but these arose from confusion as to the taxonomy and distribution of wild relatives. Arabs introduced eggplant to the west during the 15th century (Hinata, 1986). This crop was taken to Africa by Arab and Persian travelers before the middle ages in Europe. First records in Europe were in the 15th century (Hedrick, 1919 citing Sturtevant) but this crop did not become generally known there until the 17th century. The early European name " Eggplant" suggests that first introductions were small fruited; an early Italian name "*melazana*" meaning mad apple later become *melongin* and *linnaeus's* Latin '*melogena*' (Heiser, 1969). The French name "aubergine" comes from Arabic by way of the Spanish 'berenjena'. It is also widely cultivated in India, Japan, West Indies, Spain, France, Italy and the USA where the people use it as a vegetable food. Eggplant germplasm resources and collections have been well documented, evaluated and conserved throughout the world (Sarathbabu *et al.*, 1999). It is an economically important crop in Asia, Africa and sub-tropics (India and Central America) and it is also cultivated in some warm temperate regions of the Mediterranean and South America (Sihachakr *et al.* 1993).

The eggplant is spread now to all tropical and subtropical countries (Purseglove, 1968). It grows extensively in India, Bangladesh, Pakistan, China and the Philippines. It is also popular in France, Italy, West Indies, Japan and the USA where the people use it as a vegetable food. In 2001-2002, eggplant production in the world was 26,505,271 mt from an area of 1,539,284 ha (FAO, 2002). Eggplant is an economically important vegetable crop of tropical and temperate parts of the world (**Table 1.1**).

Table 1.1. Worldwide production of eggplant (FAO, 2002 report)

	Area harvested (ha)	Average yield (kg/ha)	Production (mt)
World	1,313,903	169,514	22,272,454
Asia	1,234,002	167,272	20,641,440
Africa	45,260	178,451	807,668
Europe	27,487	242,695	242,695
North & Central America	6,589	227,015	149,580
South America	425	137,647	5,850
Oceania	140	58,571	820

1.4.3. Morphological Characters

The eggplant (2n=24) is an annual herb. It is erect in nature, with multiple branches and strong bushy appearance. Usually it grows up to a height of 4-6 feet.

1. Root: It has a strong, deeply penetrating taproot. Eggplants are usually transplanted and resulting injury to the taproot changes the type of root system.

2. Stem: Stem is pubescent; old plants becoming somewhat woody, somewhat spiny; all parts covered with a gray tomentum.

3. Leaf: The leaf is large, simple alternate and under side of most cultivars is covered with dense wool-like hair. Leaves have 2-5 cm long petiole, estipulate and 5-20 cm long and 5-12 cm wide, entire, ovate with highly lobed or undulated margin; lamina ovate or ovate oblong, apex acute or obtuse, base rounded or cordite, often unequal.

4. Flower: Flower is solitary or in 2-5 flowered cymes. Opposite or sub-opposite leaves, 3-5 cm in diametre, pedicel 1-3 cm long, elongating in fruit. Flowers are hermaphrodite, gamosepalous with persistent 5 lobed calyxes. The calyx is densely covered with

glandular hairs when it is in bud condition, 5-6 lobed gamopetalous corrola, usually of purple-violet colour when open. The species has perfect flower and is self-compatible and inbreeder.

5. Fruit: The fruit is pendant and is a fleshy berry borne singly or in clusters. The colour of the mature fruits varies from purple, purple-black, yellowish, white, green and striped; depending upon the cultivars. The seeds are borne on the fleshy placenta and the placenta with the seeds completely fill the lobular cavity.

1.4.4. *Solanum* and Its Relatives

Genus *Solanum* has eleven species in which maximum species are wild. *Solanum mélongena* and *S. tuberosum* are cultivated every temperate region of the globe. Another solanaceous plant *Lycopersicon esculentum* is also cultivated as vegetable crop in the tropical region.

In Bangladesh, *S. melongena* L, is cultivated everywhere as a stout herb, vernac Baigun, Brinjal or eggplant (Prain, 1963). Other specieses of *Solanum* genus except *S. melongena* are:

i)	*Solanum tuberosum*	vi)	*S. ferox*
ii)	*S. nigrum*	vii)	*S. torvum*
iii)	*S. spirate*	viii)	*S. indicum*
iv)	*S. verbascifolium*	ix)	*S. xanthocarpum*
v)	*S. sisymbrifolium*	x)	*S. trilobatum*

1.4.5. Cultivation Practices

The eggplant is usually grown from seeds; seeds are collected from the ripe fruits. Ripe fruits are squeezed and the seeds are collected after washing and drying. Seedlings are raised in the nursery, which is very well prepared. Seeds are sown in rows or broadcast. About one kg of seed is required for planting one hectare. When the seedlings have grown to a height of 10-12 cm they are planted out. The seedlings are removed carefully from the beds with soil attached to the roots and are set out in rows at a

spacing of 60-90 cm apart and about 45cm from plant to plant in the row. Tall types require larger spacing. The planting is done generally on flat, pre-prepared ground or rarely on ridges or raised on beds. Mixed cropping (intercropping) in early spring is also common with melon, pumpkin and onion. About 6000-6500 seedlings are required for one acre of land. At the time of plantation, 2-3 seedlings are raised per hole, later thinned to a single plant.

Proper irrigation and appropriate manuring are necessary for good yield. Besides a basal dressing of farmyard manure, chemical fertilizers (NPK) are also used with the soil at the time of final field preparation. Nitrogenous fertilizer is given as a top dressing in two split doses with irrigation; first, when the plants are established in the field and secondly in flowering and fruiting stages.

The eggplant requires a long growing season with average day and night temperatures. A temperature of 25^{0}C is reported to be favourable for germination of eggplant seed. For the cultivation of eggplant, a rich, warm, light or sandy loam is preferred. Silk loams and clay loams are also suitable. The soil should be thoroughly prepared before planting. Water logging condition is extremely harmful; therefore, with the proper irrigation and fertilizer use, proper care about drainage system of the field is one of the important prerequisites of eggplant cultivation. Weeding may be done using implements.

Brinjal is a rapidly growing plant. Fruits are ready for harvest after 3-4 months from germination of the seed. Healthy plants bear fruit continuously thereafter for the life time without any disease pressure, but yield is best at the first season. The fruits should be harvested when they are still tender, but after they had attained a good size and when the surface of the fruit is bright and glossy.

Eggplant can yield as much as 20,000-24,000 kg of fresh fruit per acre during the seasonal production; individual fruit can weight 0.15-0.65 kg. Generally eggplants have been preferred for large-scale production and for dooryard plants as a vegetable crop. The average yield per hectre of this crop in this country is only 5.60 tons, which is very

low as compared to that of the other eggplant growing countries of the world (Anonymous, 1993). Like other tropical countries, geographical position and environmental as well as soil condition of Bangladesh indicates its best suitability for large-scale eggplant cultivation.

Important indigenous cultivars of eggplant which have been commercially cultivated in the northern zone of Bangladesh are: Islampuri (Sinnat), Jhumka, Muktakeshi, Altabull, Iree, Ghirtikanchan etc. The fruits of eggplant are large berry and shape varying from pear to oblong tapering. Its length is 6-35 cm with various color i.e., violet, dark purple, green white, pink etc. Inner portion of the fruit is white and spongy, and seeds are embedded in the placentae.

1.4.6. Eggplant Varieties Cultivated in Bangladesh

Generally eggplants have been preferred for large scale production and for dooryard plants as a vegetable crop like other tropical countries. Geographical position and environmental as well as soil condition of Bangladesh indicate its best suitability for large scale eggplant cultivation. Quite a large number of eggplant varieties are now cultivated in Bangladesh and these can be recognized according to shape and colour i.e., violet, dark purple, green, white, pink etc. Different varieties of eggplant are cultivated in Bangladesh. Important cultivars of eggplant which have been commercially cultivated in the northern zone of Bangladesh are Islampuri, grey-violet Ishurdi, round shaped Muktakeshi, Baramasi Shingnath, winter Khatkhatia, Loda, China, black big Laffa are very common. Not only these local varieties but also some recently introduced exotic varieties such as Black Beauty, Pusa Purple, Patari, Japani, Ramjhumki, Jhumki, Uttara, Nayankazal, Irribegun, Lalital, Talbegun, Kegibegun, Katabegun, Sufala, Taherpuri, Suktara and Prolific are now widely grown in our country.

1.4.7. Problems of Eggplant Cultivation in Bangladesh

It is an economically important crop in Bangladesh, but this crop has been suffering from various types of pathogenic diseases, such as tobacco mosaic virus (TMV), cucumber mosaic virus (CMV), tobacco ring spot virus (TRSV), bacterial wilt, damping

off, early blight, phomopsis blight, phytophthora blight, pythium fruit rot, stem rot, verticillium wilt, fusarium wilt etc (Akhtar 2005). In 2001-2002, eggplant production in the world was 26,505,271 Mt from an area of 1,539,284 ha (FAO, 2002). The damage of eggplant, caused by various diseases and pests, is one of the major reasons of this poor yield. A wide range of diseases and parasites, *Fusarium* and *Verticillium* wilt attack eggplant (Sihachakr *et al.* 1994). Although, eggplant has wide variability, it exhibits partial resistance to most of its pests and pathogens and often at low level (Daunay *et al.* 1991). A number of viral, bacterial, fungal and nematode disease attacks eggplant. Not only diseases, but also some insect borers diminish production of eggplant.

1.4.8. Usage and Importance of Eggplant

Eggplant is an economically important horticultural crop due to its high nutritive value, cheap source of vitamins and minerals. In Bangladesh more than 8% of the total vitamin C of vegetable origin comes from brinjal alone. The unripe fruit is primarily used as a cooked vegetable for the preparation of various dishes in different region of the world. Brinjal has been a staple vegetable in our diet since ancient times. Both poor and rich like it. In our country eggplant is used commonly as fry, cooked with fat or oil, as curry, cooked with many spices, as chop, fried with pulse flour deeped into hot oil in a frying pan. Contrary to the common belief, it is quite high in nutritive value and can well be compared with tomato (Choudhury, 1976). The food value of eggplant is considerable. The compositions of food value per 100 gm of edible portion of eggplant are shown in **Table 1.2**. It has got much potential as raw material in pickle making and dehydration industries (Singh *et al.* 1963). Eggplant has been used in traditional medicines (Khan, 1979). For example tissue extracts have been used for treatment of asthma, bronchitis, cholera and dysuria, fruits and leaves are beneficial in lowering blood cholesterol. White brinjal is said to be good for diabetic patients (Choudhury, 1976). Kirtikar and Basu (1933) have mentioned its use in 'ayurveda' as appetizer, aphrodisiac, cardiotonic etc. In 'Unani' system of medicine, roots of eggplant are used to alleviate pain, fruits as cardiotonic, taxative, maturant and reliever of inflammation. The fruit is also considered beneficial to piles when applied externally but contradictory when consumed orally. In

many places, leaves have been found in use as narcotics and seeds as stimulants. In some countries, like Madagascar and South Africa, the leaves, fruits and seeds are used in various purposes and even in curing diseases like syphilis.

Bangladesh is a least development country in the third world. Population explosion is the main problem in this country. The food requirements for the majority of the population of the third world countries like Bangladesh are not adequately met. The gap is widening both in qualitative and quantitative terms. Although eggplant is a staple vegetable in our diet since ancient times, a few necessary steps has been made up for the improvement of this crop. One of the principal methods used for the improvement of this crop is selection for inbred lines and inter varietal crosses. The considerable occurrence of hybrid vigour in eggplants was recorded by Munson (1892) in the USA and in Japan by Nagai and Kida (1926).

Table 1.2: Composition of brinjal (per 100g of edible portion)

Moisture	92.7g	Sodium	3.0 mg
Protein	1.4g	Potassium	2.0 mg
Fat	0.3g	Copper	0.17 mg
Minerals	0.3g	Sulphur	44.0 mg
Fibre	1.3g	Chlorine	52.0 mg
Carbohydrates	4.0g	Vitamin A	124 I.U
Calcium	18mg	Thiamine	0.04 mg
Magnesium	16 mg	Riboflavin	0.11 mg
Oxalic acid	18 mg	Nicotinic acid	0.09 mg
Phosphorus	47 mg	Vitamin C	12.0 mg
Iron	0.9 mg		

(Bose *et. al.* 1993)

1.5. Aim and Objectives

In Bangladesh several studies have been made on biotechnology of a few horticultural plants. But still a large number of horticultural plants had not yet been exposed to plant biotechnology. Eggplant (*Solanum melongena* L.) is one of the important horticultural plants, for which biotechnological development has not been done in Bangladesh.

Keeping this view in mind, the present research work was under taken as a new approach in the advancement of eggplant biotechnology.

- Development of protocol for large scale production of virus free brinjal plantlets through meristem culture.
- Selection of growth regulators along with the proper concentrations and combinations for efficient callus induction and plant regeneration from appropriate explants.
- To standardize suitable media composition for somatic embryogenesis.
- To establish a standard protocol for synthetic seed production using somatic embryos and nodal segments as explants.
- Multiple shoot proliferation from shoot tips and nodes.
- To standardize suitable media composition for proper shoot elongation and root induction.
- Acclimatization and transplantation of *in vitro* regenerated plantlets under *ex vitro* condition.

CHAPTER TWO

Meristem Culture in Eggplant

CHAPTER II

2. APICAL MERISTEM CULTURE OF EGGPLANT

2.1. INTRODUCTION

The eggplant is usually propagated by means of seeds. As a sexual propagule, the seed preserves not only the desirable genotype but virus and other tissue-born pathogens as well. Inborn virus is one of the fatal causes that diminish the yield of this crop. A number of cultivars are grown throughout the country depending upon the yield, consumers' preference about the colour, shape and size of the various cultivars. Its increasing demand among consumer indicates that it has great potential as a vegetable in Bangladesh. To meet the increasing demand of the consumers, it has become imperative to develop varieties with higher yield and good quality suitable for year round cultivation. At the advent of plant biotechnology meristem culture offers a novel tool for production of virus free plants. In 1952, Morel and Martin were successful in regenerating a virus-free dahlia plant by the excision of some meristematic domes from virus-infected shoots. For Stone (1963), only tips between 0.2 and 0.5 mm most frequently produce virus free carnation plants. The explants smaller than 0.2 mm cannot survive and those longer than 0.7 mm produce plants that still contain pathogen (Alam *et al.* 2004).

No definitive explanation can be given to understand this virus eradication (Wang and Charles, 1991). Various explanations have been given: absence of plasmodesm in the meristematic domes, competition between synthesis of nucleoproteins for cellular division and viral replication, inhibitor substances, absence of enzymes preiral replication, inhibitor substances, absence of enzymes present only in the cells of the meristematic zones, and suppression by excision of small meristematic domes. This last proposal could explain why some potato plant showing virus particles in the meristematic domes, could regenerate a virus free plant (Mellor and Stace Smith, 1977).

Meristem culture is a method for obtaining virus free plants has been used in many crops including eggplant (Morel and Martin 1955). Organogenesis via formation of multiple shoot buds was also reported in this crop (Mukherjee *et al.* 1991, Swamy *et al.*,

1998). *In vitro* plant growth depends on the nutrition and the environmental factors (Dougall 1981; Gamborg and Shyurk 1981; Murashige 1977). Hughes (1981) referred to these environmental factors as *in vitro* ecology, where light, temperature and gases are the major elements. Gamborg and Shyurk (1981) divided the nutritional factors into essential components and optional components. The essential components consist of inorganic salts, a carbon source of energy, vitamins and growth regulators. The growth composition and balance is based on Skoog and Miller (1957) concept of auxin and cytokinin ratio organogenesis. Eggplant is an important vegetable crop in Bangladesh, but no research work has been reported on meristem culture of this crop. That is why, this study was conducted to explain an *in vitro* meristem culture, shoot elongation and root induction towards production of virus free plant.

2.2 MATERIALS AND METHODS

2.2.1. MATERIALS

2.2.1.1. Plant Materials

In vitro grown seedlings of two cultivars of *Solanum melongena* L. *viz.* cv. China and cv. Loda, commonly cultivated in the northern region of Bangladesh, were used in the present investigation as experimental materials. The cultivars differ in respect of fruit shape and its colour (**Plate 2.1**). Seeds of meristem donor plants of the two eggplant cultivars were collected from the Bangladesh Agricultural Development Corporation (BADC), Rajshahi, Bangladesh during the main crop season (Aught -September, 2004). *In vitro* grown young seedlings were used as a source of explant for conducting apical meristem culture.

PLATE 2.1 (A-B)

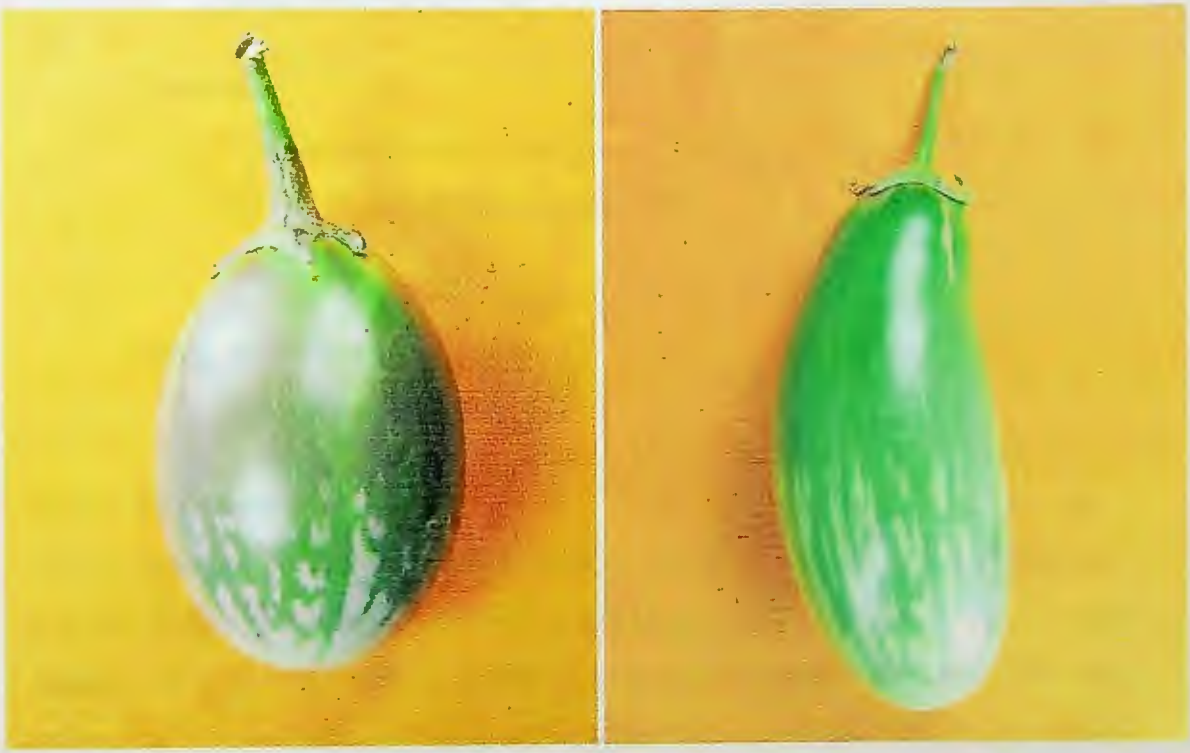

Plate 2.1

Fruits of the experimental cultivars:

A. Cv. China.

B. Cv. Loda.

2.2.1.2. Nutrient Media

In the present investigation MS basal salt formulation (Murashige and Skoog, 1962) was used to conduct all of the experiments.

2.2.1.3. Plant Growth Regulators

The basal media were supplemented with various concentrations of different plant growth regulators. The following plant growth regulators were employed for the present investigation.

Auxins:

2, 4-dichlorophenoxy acetic acid (2,4 -D)

α-Naphthalene acetic acid (NAA)

Indole-3- butyric acid (IBA)

Indole-3-aectic acid (IAA)

Cytokinins:

6-benzyl amino purine (BAP)

Kinetin (6- furfuryl amino purine) (KIN)

Zeatin

Gibberellins:

Gibberellic acid (GA_3)

2.2.1.4. Equipments

Various types of glass vessels were used as container for the plant materials and nutrient medium; the whole constituting a culture systems. Different sizes of culture vessels such as test tubes (125X25m), conical flasks (250, 500 and 1000 ml), separating funnel, pipette as well as other tools such as forceps, spirit lamp, needle, scalpel blade, stereo-microscope, electrical balance, autoclave, laminar air flow machine, cotton, firebox, marker pen, etc. were used to accomplish successful apical meristem culture of eggplant.

2.2.2. METHODS

2.2.2.1. Preparation of Culture Media

The most convenient way to prepare MS medium using commercially available prepacked MS salt formulation supplied by companies such as the Sigma Chemical Company or North Carolina Biological Supply Company. But in this research work MS medium was prepared using all of the individual chemical compounds recommended by Murashige and Skoog (1962). In this way, the first step for the preparation of culture media (MS) was the preparation of the stock solutions. As different media constituents were required in various concentrations, individual stock solutions (I-VIII) were prepared for ready use during the preparation of culture media. Similarly hormonal stock solution was also prepared separately for the ready use. The procedure for the preparation of the MS stock solutions (I-VIII) and individual hormonal stock solution were as follows:

2.2.2.1.1. Preparation of MS Stock Solution

Table-3.1. MS salt formulation for preparing MS stock solutions (I-VIII) according to Murashige and Skoog (1962).

MS Stock solution	Name of the constituents	Amount of constituents (mgl^{-1})	Strength of the stock solution (X)	Amount for the stock solution (gm)	Final volume of the stock solution (ml)	Usable amount (ml)
I	NH_4NO_3 KNO_3 KH_2PO_4	1650 1900 170	10 X	16.50 19.00 1.70	200	20
II	$MgSO_4.7H_2O$	370	10X	3.70	200	20
III	$CaCl_2\ 2H_2O$	440	10X	4.40	200	20
IV	$FeSO_4.7H_2O$ $Na_2EDTA.2H_2O$	27.8 37.3	20 X	0.556 0.746	400	20
V	$MnSO_4.4H_2O$ H_3BO_3 $ZnSO_4.7H_2O$	22.3 6.2 8.6	20 X	0.446 0.124 0.172	400	20
VI	KI $CuSO_4.5H_2O$ $NaMoO_4.2H_2O$ $CoCl_2.6H_2O$	0.83 0.025 0.25 0.025	200 X	0.166 0.005 0.05 0.005	200	1
VII	Myoinositol	100	20 X	2.0	200	10
VIII	Nicotinic acid Pyridoxine HCl Thiamin HCl Glycine	0.50 0.50 0.50 2.00	500 X	0.25 0.25 0.25 1.00	500	1

Stock Solution MS-1: The above tabulated nutrients for stock-I were weighted accurately with the help of an electrical balance and dissolved in 100 ml of distilled water and the final volume was adjusted to 200 ml holding the strength of 10 litre of culture media. So the usable amount of this stock solution was 20 mgl^{-1}. Then the stock solution was stored in refrigerator at 4^0C for the ready use.

Stock Solution MS-II: For stock solution MS-II, 3.70 gm of $MgSO_4.7H_2O$ was weighted accurately with the help of an electrical balance and dissolved in 200 ml of distilled water. This stock solution (MS-II) contained the nutrient of 10 litre culture media. So the usable amount of this stock solution was 20 mgl^{-1}. Then the solution was poured into a reagent bottle and stored in a refrigerator at 4^0C for the ready use.

Stock Solution MS-III: For stock solution MS-III, 4.40 gm of $CaCl_2.2H_2O$ was weighted accurately with the help of an electrical balance and dissolved in 200 ml of distilled water. This stock solution (MS-III) contained the nutrient of 10 litre culture media. So the usable amount of this stock solution was 20 mgl^{-1}. This stock solution was poured into a reagent bottle and stored in the refrigerator at 4^0C for the ready use.

Stock Solution MS-IV: The above tabulated nutrients for stock-IV were weighted accurately with the help of an electrical balance and dissolved in 200 ml of distilled water following its final volume to 400 ml by further addition of distilled water. This stock solution (MS-IV) contained the nutrient of 10 litre culture media. So the usable amount of this stock solution was 20 mgl^{-1}. Then the solution was poured into a reagent bottle and stored at 4^0C wrapping with dark paper for the ready use.

Stock Solution MS-V: The above mentioned amount of nutrients for stock-V was weighted accurately with the help of an electrical balance and dissolved in 200 ml of distilled water and the final volume was made up to 400 ml by adding distilled water. This stock contained the nutrients for 20 litre culture media. So the usable amount of this stock solution was 20 mgl^{-1}. Then the solution was poured into a reagent bottle and stored at 4^0C for the ready use.

Stock Solution MS-VI: The above tabulated nutrients for the stock-VI were weighted and dissolved in 200 ml of distilled water. This stock solution (MS-VI) contained the nutrient of 200 litre culture media and stored at 4^0C. So the usable amount of this stock solution was 1 mgl^{-1}.

Stock Solution MS-VII: For stock solution MS-VII, 20 gm of Myoinositol was weighted accurately with the help of an electrical balance and dissolved in 200 ml of distilled water. This stock solution (MS-VII) contained the nutrients of 20 litre culture media. So the usable amount of this stock solution was 20 mgl^{-1}. Then the solution was poured into a reagent bottle and stored in a refrigerator at 4^0C for the ready use.

Stock Solution MS-VIII: The tabulated amount of nutrients for the stock-VIII were dissolved in 500ml of distilled water and stored at 4^0C temperature. This stock solution (MS-VIII) contained the nutrients of 500 litre culture media. So the usable amount of this stock solution was 1 mgl^{-1}.

2.2.2.1.2. Preparation of Stock Solution for Growth Regulators

In addition to the MS-nutrients, it is generally necessary to add one or more growth regulators such as auxins, cytokinin or gibberellic acid to the media to support desired morphological response of tissue and organs (Bhojwani and Razdan, 1983). The following growth regulators were used in the present investigation:

Table-3.2: Growth regulators were dissolved in appropriate solvent as shown against each of them.

Growth regulators	Amount taken (mgl^{-1})	Dissolving solvents (ml)	Final volume of the stock solution with DW (ml)	Strength of the stock (mg/ml)
2,4-D	10	70% CH_3-CH_2OH	10	1
NAA	10	1.0(N) KOH	10	1
IAA	10	1.0(N) NaOH	10	1
IBA	10	1.0(N) NaOH	10	1
BAP	10	1.0(N) KOH	10	1
KIN	10	1.0(N) HCl	10	1
Zeatin	10	1.0(N) NaOH	10	1
GA_3	10	1.0(N) HCl	10	1

Three types of stock solution was prepared for each of the growth regulators as stated below:

Main Stock (1 mg/ml): To prepare the individual stock solutions for any of the growth regulators, 10 mg of the hormonal solute was placed in a clean test tube and then dissolved in required volume of the appropriate solvent. Final volume of the solution was made up to 10 ml by adding distilled water. The strength of the stock solution was 1mg/ml and this was termed as main stock. The solution was then poured into a reagent bottle, labeled and stored at 0°C. Similar procedure was followed to prepare the stock solution for each of the growth regulators used.

Moderated Stock-1 (0.1 mg/ml): To prepare the stock solution of 0.1mg/ml for any of the hormones, 1 ml of the main stock (1mg/ml) was kept in a reagent bottle and 9 ml of distilled water was added to make its final volume 10 ml. The strength of the stock solution was 0.1mg/ml and this was termed as moderated-1 stock. Then the bottle containing the stock was labeled and stored at 0°C. Similar procedure was followed to prepare the stock solution for each of the growth regulators used.

Moderated Stock-2 (0.01mg/ml): To prepare the stock solution of 0.01mg/ml for any of the hormones, 1 ml of the main stock (1mg/ml) was kept in a reagent bottle and 99 ml of distilled water was added to make its final volume 100 ml. The strength of the stock solution was 0.01mg/ml and this was termed as moderated-2 stock. Then the bottle containing the stock was labeled and stored at 0°C. Similar procedure was followed for preparation of each of the growth regulators.

2.2.2.1.3. Preparation of one Litre MS Medium

Following steps were followed for the preparation of one litter MS medium.

a) The usable amount for each of the stock solutions (as indicated in Table 3.1) was added to 750 ml of distilled water and mixed well in a conical flask (1000 ml).
b) From the prepared growth regulator stocks, different concentrations of hormonal supplements as required were added either singly or in different combinations to the above solution and mixed thoroughly.
c) 30gm sucrose was added and the final volume of the mixture was made up to 1000 ml (1 litre) with further addition of distilled water.

d) Then the pH of the constituting medium was usually adjusted to 5.7 with the help of a digital pH meter using 0.1(N) KOH or 0.1(N) HCl solution, whichever was necessary.

e) Before autoclaving, 7-8 gm of agar (Carolina Biological Supply Co.) was added for one litre of medium and the total mixture was gently heated in micro oven until the agar melted completely making the turbid solution clear. Care was taken during heating the medium so that the solution was not to be evaporated any way.

f) Required volume of the above hot melted medium was transferred into culture vessels like test tubes, conical flasks and culture bottles through separating funnel. The culture vessels were plugged with non-absorbent cotton plugs, which were inserted tightly to the mouth of culture vessels.

g) The culture vessels containing medium were then autoclaved at 15 lb/sp inch pressure and at the temperature of 121°C for 20 minutes to ensure sterilization. After autoclaving the culture vessels were kept in inoculation chamber to make the media cooled and solidified. The culture vessels containing the media were marked with glass marker pen indicating specific hormonal supplements.

In this procedure, semi-solid MS medium was prepared but in case of MS liquid medium, addition of agar and its subsequent melting were not required.

2.2.2.2. Preparation of Surface Sterilizing Solution

In this investigation, 0.1% mercuric chloride ($HgCl_2$) solution was used for surface sterilization of the experimental seeds. To prepare 0.1% $HgCl_2$ solution, 1mg $HgCl_2$ was taken in a conical flask (250ml) and dissolved in 100 ml sterilized distilled water. Freshly prepared $HgCl_2$ solution was always used for the experimental purpose.

2.2.2.3. Culture Techniques

For the present investigation the following methods were practiced.

2.2.2.3.1. Surface Sterilization of Seeds

The seeds of eggplant were taken in a conical flask and washed thoroughly under running tap water for 30 minutes to reduce the level of dust and surface contaminants.

The floating seeds were discarded. The remaining seeds were taken in a conical flask (250 ml) containing distilled water with few drops of disinfectant *viz.* savlon and few drops of tween-80 (wetting agent) and rinsed with constant shaking for about 10 minutes. This was followed by second washing with autoclaved distilled water to remove all traces of above chemicals. Finally, surface sterilization was carried out in the aseptic condition of a laminar airflow cabinet. Non-judicious application of $HgCl_2$ solution in uncertain duration of time may cause unexpected results like contamination or tissue killing. Hence seeds were then taken into a sterile conical flask and suspended in 0.1% $HgCl_2$ solution for varying periods of 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 and 14 minutes to ensure contamination free viable culture. Then the seeds were washed 4-6 times with double autoclaved distilled water to eliminate all traces of $HgCl_2$.

2.2.2.3.2. Precaution to Ensure Aseptic Condition

Before starting the inoculation programme, the laminar airflow cabinet was switched on allowing airflow and UV light before 30 minutes of culture initiation. Then UV light was switched off and 70% ethyl alcohol was sprayed on the floor of cabinet to reduce the chances of contamination. All the inoculation and aseptic manipulation were carried out in the running laminar airflow cabinet. All the instruments like scalpels, forceps, needles and other requirements like petridishes, filter paper, empty beaker or conical flask, cotton, distilled water were autoclaved before using. In side the laminar flow cabinet, the instruments were again sterilized by dipping into 70% ethyl alcohol and flamed over a spirit lamp for several times. Before starting inoculation, hands were repeatedly washed thoroughly by soaking and spraying with 70% ethyl alcohol to ensure contamination free operation. For surgical operations sufficient care was taken as usual to obtain possible contamination free culture.

2.2.2.3.3. *In Vitro* Seed Germination

Sterilized seeds were taken in a petridish by sterilized forcep. Then seeds were carefully inoculated into culture vessels containing sterilized agar gelled medium. The cotton plugs of the culture vessels were handled inside the laminar airflow cabinet in presence of flaming spirit lamp. Then the inoculation procedure was conducted. 20-25 seeds were inoculated into each conical flask or bottle. During inoculation, special care

was taken so that the seeds must touch the medium equally and not rinsed into the medium. The inoculated flasks or bottles were labeled by a glass marker pen and incubated at 30±2°C for germination of seeds. Shoot tips were collected from 21-25 day old *in vitro* grown seedlings.

2.2.2.3.4. Inoculation of Apical Meristems

The *in vitro* grown seedlings of 21-25 day old were used as the ready source of explants. The shoot tips (1-2 cm) were excised with the help of sharp blade and collected in a petridish. Then, the shoot tips were laid on the sterile tiles using sterile forceps. Shoot tip was hold in one hand under the stereomicroscope with the help of a pair of forceps and the immature leaves and leaf primordial were snapped with slight pressure from the needle. Then the exposed meristem tips that appeared as a shiny dome (0.2-0.3 mm) were gently isolated with sharp blade. The culture tubes were deplugged and a singly isolated meristem tip was carefully placed on the M-shaped filter paper bridge of the culture tubes containing MS liquid medium with hormonal supplements for the establishment of primary meristem culture. During inoculation, special care was taken that the explants must place on paper bridge soaked with liquid medium and not dipped into the medium. After inoculation, the culture vessels were sealed by flame-sterilized cotton plugs and labeled by a glass marker with inoculation date. Then the inoculated culture vessels were ready for incubation and kept in the incubation chamber.

2.2.2.3.5. Culture Incubation

The culture vessels were incubated in a growth chamber containing special culture environment. The test tubes were placed on the shelves of a rack in the growth chamber. Unless mentioned specially, cultures were grown in the growth chamber illuminated by 40 watts white fluorescent tubes fitted at a distance of 30-40 cm from the culture shelves. The cultures were maintained in the culture room at 25±2°C under a 16h photoperiod (cool-white fluorescent tube supplying). Light intensity varied from 2000-3000 lux. The culture tubes were checked daily to record the morphogenic responses of cultured meristems for different experiments conducted in the present investigation.

2.2.2.3.6. Subculture for Primary Established Meristems

After four weeks of inoculation of meristem, the primary established meristems those showed morphogenic response were removed aseptically from the culture tubes and transferred into tubes containing agar gelled MS medium supplemented with different growth regulators as required. During inoculation, special care was taken that the explant must touch the medium equally and did not dip into medium. After 25-30 days, when the regenerated shoots were 2-5 cm in length with 5-10 leaves, they were removed aseptically from the culture tubes. Meristem derived shoots were placed on a sterile tile and the basal end of the shoot was cut into convenient size and transferred into test tubes having rooting medium for root induction.

2.2.2.4. Transplantation

When the plantlets attained a height of 7-9 cm with few leaves and well-developed root system, they were taken out from the culture vessels carefully. Medium attached to the root was gently washed out with running tap water. The plantlets treated with 0.1% Agroson (fungicide) solution and planted in polar pots containing sterile garden soil, sand and compost (2:1:1). After transplantation, the plants along with the polar pots were covered with a large moist polythene bag immediately to prevent desiccation. To reduce sudden shock, the planted polar pots were kept in the controlled environment of growth chamber upto 10 days. To maintain maximum humidity for the plantlets, all polar pots were checked up and the interior of the polythene bags were sprayed with water whenever necessary. After 13-14 days the polythene bags were gradually perforated to expose the plants to natural environment. Finally the covering were completely exposed. After 2 weeks, when the plantlets grew well, they were transferred to normal temperature and kept under sunlight for one hour daily. This duration increased gradually. This is how, the *in vitro* regenerated plantlets were acclimatized carefully. However the plants were successfully adapted with the natural environment and were transferred to the field condition. In this way, the regenerated plants showed 95% survival under natural condition.

2.2.2.5. DATA RECORDING

Data were collected on morphogenic responses using the following parameters and the methods followed for data collection are illustrated bellow:

2.2.2.5.1. Data on Primary Meristem Culture

Meristems were primarily established on paper bridge soaking with liquid MS medium in test tubes and a regular observation was performed. After 2 weeks of culture, the explants, induced to develop adventitious shoots, were counted and its percentage was calculated applying the following formula:

Percentage (%) of meristem explants showing growth rejuvenation

$$= \frac{\text{Number of explants develop adventitious shoots}}{\text{Total number of explants inoculated}} \times 100$$

2.2.2.5.2. Data on Shoot Elongation

The primary established meristems were subcultured on agarified MS medium for further shoot elongation with regular observation. Data were recorded on percentage (%) of explants responded and shoot length (cm) after 14 and 22 days of culture. Shoot length was measured with a centimeter scale and the percentage (%) of explants responded was computed by the following formula.

Percentage (%) of explants responding to shoot elongation

$$= \frac{\text{Number of shoots responded to shoot elongation}}{\text{Total number of shoots inoculated}} \times 100$$

2.2.2.5.3. Data on Root Induction

The elongated shoots were subcultured on agarified MS medium for root induction with regular observation. Data were recorded on percentage (%) of explants responded, average number of roots/shoot and average length (cm) of root after 16 and 28 days of culture. Root length was measured with a centimeter scale and percentage of shoots induced to develop roots were calculated by applying the following formula.

$$\text{Percentage (\%) of shoots induced roots} = \frac{\text{Number of shoots formed root}}{\text{Total number of shoots inoculated}} \times 100$$

2.3. RESULTS

Efficient regeneration protocol for the culture of apical meristem in eggplant was the primary objective of this investigation. A number of experiments were carried out with a view to apical meristem culture, shoot elongation and root induction in eggplant cultivars (cv. China and cv. Loda) of Bangladesh. MS media supplemented with different types of growth regulators namely, auxin (IBA, IAA and NAA), cytokinin (BAP and KIN) and gibberellic acid (GA_3) in different combinations and concentrations were used for above purpose. Details of the results so far obtained from each of the experiments are described under the following heads:

2.3.1. Aseptic Culture of the Donor Plants

In order to raise axenic seedlings, the seeds of the two cultivars were aseptically grown on hormone free medium. Surface sterilization of seeds was accomplished with aqueous solution of 0.1% $HgCl_2$ solution. This experiment was undertaken for standardization of accurate concentration and proper duration of minutes for sterilization of the seeds. Before using $HgCl_2$ solution, the seeds were treated with a few drop of tween-80 (wetting agent) and few drops of savlon for 10 minutes and then the materials were washed 4-5 times with distilled water to eliminate the trace of the chemicals used. The washed seeds were treated by 0.1% $HgCl_2$ solution for various periods of 4, 5, 6, 7, 8, 9,10, 11,12 and 13 minutes. In all cases around 60 seeds were inoculated for germination. Data were collected on contamination free seed germination (%) and the results are shown in **Table 2.3** and **Fig. 2.1.** The highest percentage of contamination free seed germination was 96.67% when treated for 10 minutes by 0.1% $HgCl_2$. The second highest percentage of contamination free seed germination was 86.67% recorded when seeds were treated for 9 minutes with 0.1% $HgCl_2$ solution. Treatment period of 11 minutes also gave good result for viable seed germination (80%). The lowest percentage of contamination free seed germination was 13.33% for 4 minutes and 21.67% for 13 minutes with 0.1% $HgCl_2$ solution. Results of surface sterilization treatment were obtained same for both of the cultivars.

Table 2.3. Standardization of duration for 0.1% $HgCl_2$ treatment on surface sterilization of seeds of eggplant for aseptic culture of the donor plants. Data were collected after 14 days of culture.

Treatment period (Minutes)	No. of seeds sterilized	Contamination free seed germination (%)
4	60	13.33
5	60	36.67
6	60	46.67
7	60	48.33
8	60	73.33
9	60	86.67
10	**60**	**96.67**
11	60	80.00
12	60	36.67
13	60	21.67

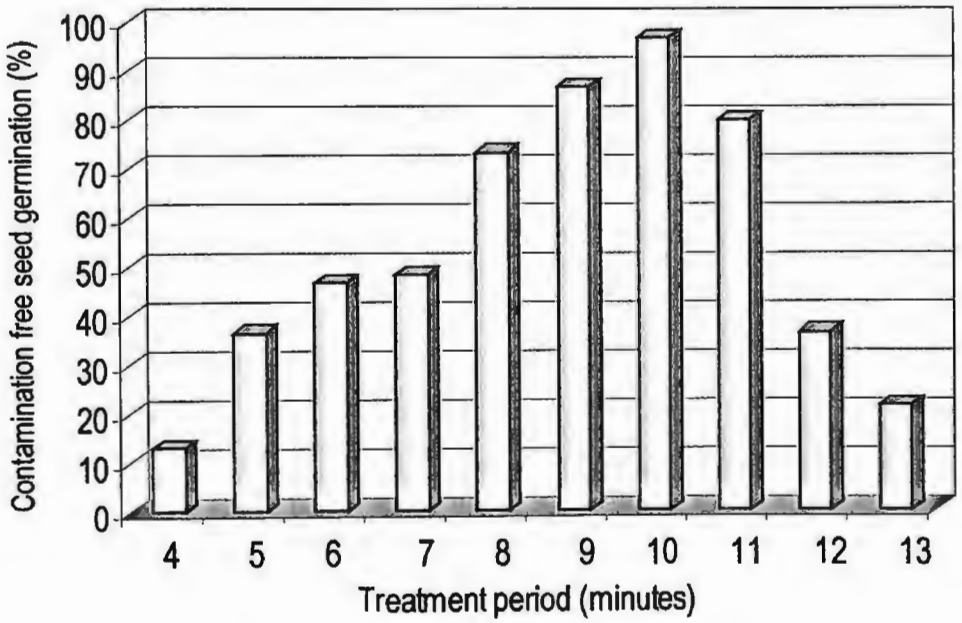

Fig. 2.1: Graph showing the effect of $HgCl_2$ solution (0.1%)on surface sterilization of seeds of eggplant for aseptic culture of the donor plants.

2.3.2. Primary Establishment of Isolated Apical Meristem

To evaluate the best performance for morphogenic responses, the isolated apical meristem were cultured on filter paper bridge in liquid MS medium supplemented with different concentrations and combinations of growth regulators. Data were recorded on days to explant responded, number of explant responded and percentage of explant responded and presented in **Table 2.4.**The isolated meristem about 0.3 mm in size was good for *in vitro* culture. Different treatments for establishment of apical meristem are stated below:

2.3.2.1. Effect of a Control Medium (MS_0)

To ensure of growth for quick responses of apical meristem, a control medium (MS_0) was used in this experiments (**Table 2.4**). In liquid MS_0 medium maximum 30% meristem of cv. China were induced to develop shoots, whereas only 25% was observed in cv. Loda. But their subsequent growth is moderate. The meristems initiate their initial growth by increasing in size and gradually changed to light green colour within 12-16 days.

2.3.2.2. Effect of BAP

MS medium supplemented with ten different concentrations of BAP (0.05, 0.10, 0.50, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0 and 5.0 mgl^{-1}) was treated for primary establishment of isolated meristems. Results of this study have been presented in **Table 2.4**. It has been observed that the cultured meristems started their initial growth by increasing in size and gradually changed to light green in colour within 8-12 days of inoculation. Both of the cultivars exhibited highest 90% response for fresh growth when they were cultured on MS media supplemented with 2.0 mgl^{-1} BAP (**Plate 2.2 A** and **Plate 2.3 A**). In this case the required days for response was minimum i.e. 8-15 in comparison of other treatments. Second highest response (85%) was obtained for both of the cultivars when they were cultured on MS medium containing 1.5 mgl^{-1} BAP. The meristems gave moderate response (20-50%) with maximum duration (10-19 day) for morphogenic changes when they were cultured in media with low (<1.5 mgl^{-1}) or high (>2.0 mgl^{-1}) concentrations of BAP. This experiment indicated that different concentrations of BAP

played a major role in establishing the isolated meristem culture of eggplant cultivars. The isolated meristems cultured on different BAP concentrations showed more or less similar responses for survival percentage and required days for responding. Experimental results revealed that 2.0 mgl^{-1} was appeared as the optimum dose for primary establishment of isolated meristems and the performance declined when the BAP concentration was decreased or increased from the optimum dose.

2.3.2.3. Effect of KIN

Excised meristems were cultured in MS liquid medium supplemented with eight different concentrations of KIN (0.05, 0.10, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0mgl^{-1}) and the experimental data have been presented in **Table 2.4**.The cultured meristem showed their first response by increasing in their sizes. Between the two cultivars, cv. Loda gave better response for this purpose. The meristems resumed new growth within 9-17 days of culture depending on the concentration of KIN. Medium having 0.50 mgl^{-1} KIN gave the best result and in this case, the highest percentage of response was 45% for cv. China and 50% for cv. Loda and its required days were 9-16 and 9-14, respectively. Media supplemented with 0.05 and 2.0 mgl^{-1} gave the lowest percentage of response (15%) with required days 10-16 for cv. China. On the other hand, cv. Loda gave lowest result (5%) with required days of 10-15 when cultured on medium having 2.50 mgl^{-1}KIN. Other treatments showed moderately response but media having low concentration (<0.05) and high concentration (>3.0) did not show any response. This experiment indicated that different concentrations of BAP played a major role in establishing the isolated meristem culture of eggplant cultivars. The isolated meristems cultured on different KIN concentrations showed more or less similar responses for survival percentage and required days for responding. Experimental results revealed that 0.50 mgl^{-1} was appeared as the optimum dose for primary establishment of isolated meristems and the performance declined when the KIN concentration was decreased or increased from the optimum dose.

2.3.2.4. Effect of GA_3

An experiment on the effect of different concentrations of GA_3 (0.05, 0.10, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0mgl^{-1}) in MS medium was also conducted for the primary response of meristem of the two cultivars. Data on responding days, number of explants responded and percentage of explants responded were recorded and the results have been tabulated in **Table 2.4**. Time taken to resume growth varied from 7-16 days. The medium having 0.5 mgl^{-1} GA_3 showed best performance (%) and it was 60% for cv. China and 55% for cv. Loda and this result was performated within 8-14 days of inoculation. On the other hand the lowest percentage was 15% for cv. China and 10% for cv. Loda observed in media having 2.0 mgl^{-1} and 3.0mgl^{-1} GA_3, respectively. Media with high concentrations (>3mgl^{-1}) of GA_3 showed no response. This experiment indicated that GA_3 did not play important role in establishing the isolated meristem culture of the eggplant cultivars. The isolated meristems cultured on different KIN concentrations showed more or less similar responses for survival percentage and required days for responding. Experimental results revealed that 0.50 mgl^{-1} was appeared as the optimum dose for primary establishment of isolated meristems and the performance declined when the KIN concentration was decreased or increased from the optimum dose.

2.3.2.5. Effect of BAP with GA_3

Depending on the supplemented BAP and GA_3 the meristems resumed new growth within 8-16 days of culture. When the excised meristems were cultured in MS liquid medium supplemented with 2.00 mgl^{-1} BAP+0.50 mgl^{-1} GA_3, cv. Loda showed highest 95% of response (**Plate 2.2 B-C**) and the cv. China showed 90% of response (**Plate 2.3 B-C**), both took 8 days to resume the growth. Though both of the cultivars started morphogenic response after 8 days of inoculation, there was a little difference in required time duration i.e. 8-14 days for cv. China and 8-13 days for cv. Loda. Media having 0.50 mgl^{-1} BAP+0.50 mgl^{-1} GA_3 gave the lowest percentage (35-45%) of response within 8-16 days of inoculation. The remaining treatments of BAP with GA_3

also gave satisfactory result in comparison to other treatments for primary establishment of apical meristem.

2.3.2.6. Effect of KIN with GA_3

The isolated apical meristems were cultured in liquid MS media supplemented with 8 different concentrations and combinations of KIN and GA_3. Data on responding days, number of explants responded and percentage of explants responded were recorded and the results are presented in **Table 2.4**.The meristems showed the best response in media having 0.5 mgl^{-1} KIN + 0.5 mgl^{-1} GA_3. In this treatment, the highest percentage of explants responded were 85% for cv. China after 8 days of culture (**Plate 2.2 D**). Whereas the cv. Loda gave highest 90% of growth response after 7 days of culture for the same treatment (**Plate 2.3 D**). The lowest percentage (15-20%) was recorded in media having 0.01 mgl^{-1} KIN + 0.01 mgl^{-1} GA_3 and 1.50 mgl^{-1} KIN + 2.0 mgl^{-1} GA_3 after 12 days of inoculation.

Thus from these experiments it would be concluded that MS media having 2.0 mgl^{-1} BAP+ 0.50 mgl^{-1} GA_3 found most effective for primary establishment of apical meristem and between the two eggplant cultivars, cv. Loda was most responsive (**Fig. 2.2**).

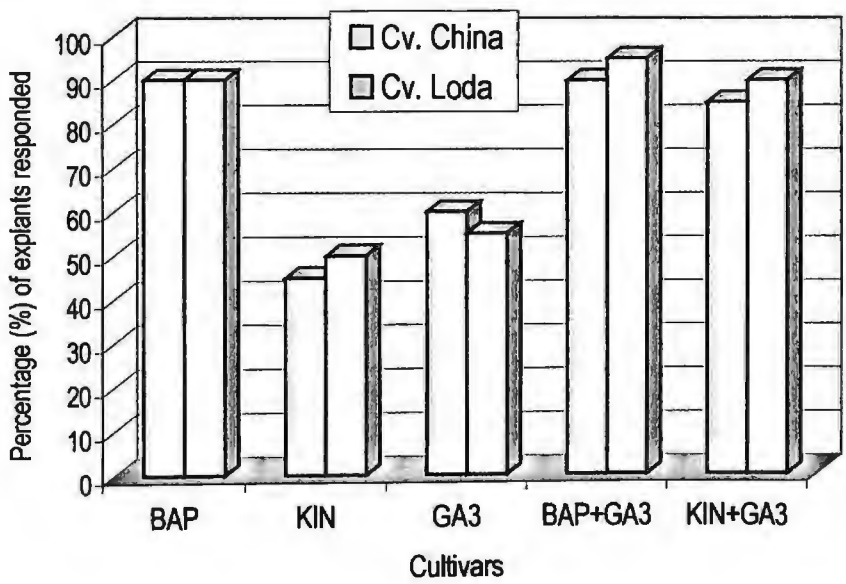

Fig 2.2. Graph showing the average effect of growth regulators on primary response of apical meristems of the two cultivars.

Table 2.4. Effect of different concentrations and combinations of growth regulators in MS liquid media on primary response of meristem isolated from 21-25 day old *in vitro* grown seedlings. Each treatment consisted of 20 explants and data were recorded after 20 days of culture.

Hormonal supplements (mgl^{-1}) used in MS (liq)	Experimental cultivars					
	Cv. China			Cv. Loda		
	Days to responded	No. of explants responded	Percentage (%) of explants responded	Days to responded	No. of explants responded	Percentage (%) of explants responded
00	12-16	6	30	12-16	5	25
BAP						
0.05	00	0	0	0	0	0
0.10	12-16	3	15	11-16	4	20
0.50	12-16	6	30	12-17	8	35
1.00	10-18	10	50	10-16	11	55
1.50	10-18	17	85	10-19	17	85
2.00	**8-15**	**18**	**90**	**8-14**	**18**	**90**
2.50	9-16	11	55	10-15	10	50
3.00	12-18	9	45	11-17	8	40
4.00	12-18	7	35	12-19	8	40
5.00	12-18	3	15	12-18	5	25
KIN						
0.05	0	0	0	0	0	0
0.10	10-16	3	15	10-15	3	15
0.50	**9-16**	**9**	**45**	**9-14**	**10**	**50**
1.00	9-19	5	25	9-16	5	25
1.50	10-16	5	25	9-17	4	20
2.00	10-16	3	15	10-15	3	15
2.50	0	0	0	10-15	1	5
3.00	0	0	0	0	0	0
GA_3						
0.05	7-15	3	15	7-14	4	20
0.10	8-15	7	35	7-15	7	35
0.50	**8-14**	**12**	**60**	**8-14**	**11**	**55**
1.00	7-15	6	30	7-15	7	35
1.50	7-15	3	15	8-15	5	25
2.00	7-15	3	15	8-15	4	20
3.00	00	0	0	9-16	2	10
BAP + GA_3						
0.50+0.50	8-16	7	35	8-16	9	45
1.00+0.50	8-16	10	50	8-15	10	50
2.00+0.50	**8-14**	**18**	**90**	**8-13**	**19**	**95**
3.00+0.50	9-16	13	65	9-17	10	50
KIN + GA_3						
0.05+0.01	12-18	3	15	11-18	4	20
0.05+0.10	12-18	5	25	11-17	7	35
0.10+0.50	9-16	12	60	9-16	13	65
0.50+0.50	**8-16**	**17**	**85**	**7-14**	**18**	**90**
0.50+1.00	10-18	7	35	9-15	8	40
1.00+1.00	10-18	7	35	9-17	6	30
1.00+1.50	12-18	4	20	12-18	4	20
1.50+2.00	12-18	3	15	12-18	3	15

PLATE 2.2

Plate 2.2 (A-D)

Primary establishment of apical meristem of eggplant, cv. China on paper bridge in liquid MS media after 20 days of culture:

A. Leaf initiation in developing meristem on MS + 2.0 mgl^{-1} BAP.

B-C. Leaf initiation in developing meristem on MS + 2.0 mgl^{-1} BAP+0.50$mgl^{-1}GA_3$.

D. Leaf initiation in developing meristem on MS + 0.50mgl^{-1} KIN + 0.50mgl^{-1} GA_3.

PLATE 2.3

Plate 2.3 (A-D)

Primary establishment of apical meristem of eggplant, cv. Loda on paper bridge in liquid MS media after 20 days of culture:

A. Leaf initiation in developing meristem on MS + 2.0 mgl^{-1} BAP.

B-C. Leaf initiation in developing meristem on MS + 2.0 mgl^{-1} BAP+0.50$mgl^{-1}GA_3$.

2.3.3. Shoot Elongation from Primary Established Apical Meristem

The tiny shoots developed from meristem were carefully rescued after 25-30 days of inoculation. Then the shoots were transferred in agar gelled MS medium containing BAP, GA_3 and KIN in different concentrations for observing further shoot development. The summarized results are shown in **Table 2.5.** Three parameters on shoot induction namely, number of shoot responded, shoot formation frequency (%) and mean length (cm) of the longest shoots (after 14 and 22 days of culture) were considered for standardization of suitable media composition for this purpose. A control medium (MS_0) was also tested. Data of those parameters from different treatments were recorded after 20 days of inoculation. Those results are presented under following headings:

2.3.3.1. Effect of BAP on Shoot Elongation

For shoot proliferation, the primary developed meristems were subcultured on MS semisolid medium supplemented with eight different concentrations of BAP (0.05, 0.10, 0.50, 1.0, 1.5, 2.0, 2.5 and 3.0 mgl^{-1}). Among them 1.0 mgl^{-1} BAP supplemented medium showed best response for shoot proliferation. In this medium the longest shoot length was 3.9-9.2 cm in cv. China and 3.9-8.2cm in cv. Loda after 14 - 22 days of inoculation as well as the highest percentage of shoot formation was 85% in cv. China and 90% in cv. Loda. But shoot growth was lowest when they were subcultured on MS medium supplemented with 0.05 mgl^{-1} BAP and average shoot length was obtained in cv. China 1.6 cm after 14 days and 2.1 cm after 22 days of inoculation and the cv. Loda, showed 1.5 cm after 14 days and 3.0 cm after 22 days of inoculation. In this case percentage (%) of explants responded were also lowest and it was 15% for both of the cultivars.

2.3.3.2. Effect of GA_3 on Shoot Elongation

The primary established apical meristems were subcultured on MS semisolid medium supplemented with eight different concentrations of GA_3 (0.05, 0.10, 0.50, 1.0, 1.5, 2.0, 2.5 and 3.0 mgl^{-1}) for shoot proliferation. The results of this experiment are presented in **Table 2.5.**The highest percentage of explants elongated was 95% in cv. China and 90% in cv. Loda when cultured on MS medium having 0.5 0 $mgl^{-1}GA_3$. In this case, the

highest shoot length in cv. China was 4.4 cm after 14 days and 8.1cm after 22 days of inoculation and in cv. Loda, it was 4.5 cm after 14 days and 9 cm after 22 days of inoculation. The lowest percentage of explants elongated was 15 in cv. China and 25 in cv. Loda when cultured on MS medium having 2.5-3.00 $mgl^{-1}GA_3$ as well as the lowest shoot length in cv. China was 2.5 cm after 14 days and 7.2 cm after 22 days of inoculation and in cv. Loda, it was 2.0 cm after 14 days and 5.0 cm after 22 days of inoculation.

So, the medium containing 0.5 GA_3 0 $mgl^{-1}$was proved to be the best concentration for shoot elongation from meristem culture. The media with 2.5-3.0 $mgl^{-1}GA_3$ were not suitable in this respect.

Table 2.5. Effect of different concentrations of BAP and GA_3 singly in MS media on meristem derived shoot elongation. Each treatment consisted of 20 explants.

Hormonal supplements (mgl^{-1}) used in MS (liq.)	Experimental cultivars							
	Cv. China				Cv. Loda			
	No. of shoots responded	Percentage (%) of shoots responded	Average length of shoots (cm) (Days after inoculation)		No. of shoots responded	Percentage (%) of shoots responded	Average length of shoots (cm) (Days after inoculation	
			14 days	22 days			14 days	22 days
Controlled	3	15	1.4	1.4	4	20	1.3	1.5
BAP								
0.05	3	15	1.6	2.1	3	15	1.5	3.0
0.10	6	30	3.1	4.5	7	35	2.0	4.5
0.50	14	70	3.5	6.1	13	65	2.7	7.6
1.00	**17**	**85**	**3.9**	**9.2**	**18**	**90**	**3.9**	**8.2**
1.50	14	70	4.0	8.1	15	75	3.0	7.3
2.00	14	70	3.2	7.6	14	70	2.8	7.0
2.50	11	55	2.9	6.9	11	55	2.1	6.8
3.00	9	45	1.9	5.6	10	50	3.3	5.6
GA_3								
0.05	6	30	2.2	4.8	5	25	2.9	5.8
0.10	11	55	3.0	7.4	10	50	2.8	5.3
0.50	**19**	**95**	**4.4**	**8.1**	**17**	**90**	**4.5**	**9.0**
1.00	12	60	2.6	7.9	14	70	2.5	5.2
1.50	9	45	2.7	7.0	11	55	2.2	4.9
2.00	6	30	2.9	7.5	10	50	2.3	5.1
2.50	3	15	2.6	7.2	5	25	2.4	5.3
3.00	3	15	2.5	7.3	5	25	2.0	5.0

2.3.3.3. Effect of GA_3 with KIN on Shoot Elongation

For shoot induction, the primary established apical meristems were also subcultured on MS media supplement with different concentrations and combinations of GA_3 and KIN and the results of this experiment are presented in **Table 2.6**. MS medium supplemented with 0.5mgl^{-1}GA_3+1.0mgl^{-1}KIN was proved the best formulation because of performing best response. In this treatment the highest mean length (cm) of the shoot were 3.7 cm after 14 days and 7.8 cm after 22 days of inoculation for cv. China and 3.5 cm after 14 days and 7.1 cm after 22 days of inoculation for cv. Loda. Among the different treatments of GA_3+KIN, the medium having 0.1 mgl^{-1}GA_3 + 3.0 mg/l KIN showed the lowest response as shown in the **Table 2.6**.

Thus from these experiments it would be concluded that MS media having 1.0 mgl^{-1} BAP found most effective for shoot elongation from primary established apical meristem and between the two eggplant cultivars, cv. Loda was more responsive than cv. China.

Table 2.6. Effect of different concentrations of GA_3 with KIN in MS media on meristem derived shoot elongation. Each treatment consisted of 20 explants.

Hormonal supplements used (mgl^{-1}) in MS	Experimental cultivars							
	China				Loda			
	No. of shoots responded	Percentage (%) of shoots responded	Average length of shoots (cm) (Days after inoculation)		No. of shoots responded	Percentage (%) of shoots responded	Average length of shoots (cm) (Days after inoculation	
			14 days	22 days			14 days	22 days
GA_3+KIN								
0.1+0.05	4	20	3.2	3.2	4	20	3.0	3.5
0.1+0.10	6	30	3.3	4.6	6	30	2.9	3.8
0.1+0.50	6	30	3.4	4.6	7	35	3.4	3.7
0.1+1.00	10	50	3.9	5.0	9	45	3.8	4.8
0.1+1.50	7	35	3.5	4.9	7	35	3.5	4.8
0.1+2.00	5	25	3.6	5.0	5	25	3.3	4.5
0.1+2.50	5	25	3.8	5.0	5	25	3.0	4.0
0.1+3.00	3	15	2.0	3.0	4	20	2.0	4.0
0.5+0.05	5	25	3.2	3.8	7	35	3.4	4.7
0.5+0.10	7	35	3.3	4.6	7	35	3.5	4.8
0.5+0.50	6	30	3.3	4.6	8	40	3.6	5.0
0.5+1.00	15	75	3.7	7.8	14	70	3.5	7.1
0.5+1.50	8	40	3.5	5.9	12	60	3.5	7.0
0.5+2.00	7	35	3.4	5.0	9	45	3.6	7.0
0.5+2.50	5	25	3.8	5.1	4	20	3.4	5.9
0.5+3.00	4	20	2.2	4.0	5	25	3.0	4.9
1.0+0.05	10	50	3.7	6.4	10	50	3.8	6.9
1.0+0.10	9	45	3.5	5.2	10	50	3.6	5.3
1.0+0.50	9	45	3.4	5.0	9	45	3.5	5.3
1.0+1.00	5	25	3.8	4.8	7	35	3.3	4.8
1.0+1.50	6	30	2.2	3.2	6	30	2.2	4.0
1.0+2.00	5	25	3.4	3.8	6	30	3.3	4.2
1.0+2.50	4	20	3.5	4.6	4	20	2.7	3.8
1.0+3.00	4	20	3.3	4.0	4	20	3.0	4.9
1.5+0.05	9	45	3.5	5.5	10	50	3.6	5.9
1.5+0.10	5	25	3.6	5.9	5	25	3.2	4.9
1.5+0.50	6	30	2.8	4.9	6	30	3.2	5.0
1.5+1.00	4	20	2.4	4.5	4	20	2.9	4.9
1.5+1.50	4	20	2.9	4.6	5	25	2.6	5.0
1.5+2.00	4	20	2.5	4.7	4	20	2.6	4.9
1.5+2.50	4	20	2.5	4.6	4	20	2.5	4.9
1.5+3.00	4	20	2.6	4.7	4	20	2.5	4.8

2.3.4. Root Induction from *In Vitro* Elongated Shoots

For establishment of plantlets, root induction is essential. The primary established meristem derived shoots (height of about 3-4 cm) were transferred onto MS semisolid medium supplemented with various types of growth regulators (Auxin and Cytokinin) either in singly or in combination. A control medium (MS_0) was also tested to ensure the effect of growth regulators on root induction. Three parameters on root induction

namely, number of shoots responded, root formation frequency (%) and mean length (cm) of the induced roots (after 16 and 28 days of culture) were considered to find out standard media formulation for root induction. Data on those parameters from different treatments were recorded after 2 weeks of inoculation. The experimental results are stated under following headings:

2.3.4.1. Effect of IBA, NAA and IAA on Root Induction

For root formation, *in vitro* elongated shoots (about 3.4 cm height) were cultured individually on MS medium supplemented with different concentrations of IBA, NAA or IAA. The summarized results are presented in **Table 2.7** and **Plate 2.4 (A-D)**.

Among the different concentrations of IBA, 3.0 mgl^{-1} was found to be best for root induction and cent percent of root induction was achieved in both of the cultivars. In this treatment, the highest mean number of roots per shoot were 7.2 after 16 days and 12.3 after 28 days of culture in cv. China and 6.9 after 16 days and 12.0 after 28 days of culture in cv. Loda. The average length of roots was 7.3 cm after 16 days and 12.0 cm after 28 days of culture in cv. China and 7.4cm after 16 days and 11.8cm after 28 days of culture in cv. Loda. Further increase or decrease in the concentration of IBA, rooting performance decreased and in above 7.0 mg^{-1} IBA, the shoots developed basal callus instead of root induction.

Among different concentrations of IAA and NAA treated, highest number of roots/shoot, average length (cm) of roots and root induction frequency (%) were recorded in MS medium having 3.0 mgl^{-1} IAA or NAA. In respect of root induction both NAA and IAA were proved to be less effective than IBA and basal callusing was frequent when cultured on MS medium having NAA.

Table- 2.7. Effect of different concentrations and combinations of NAA, IBA and IAA in MS media on root induction from meristem derived elongated shoots. Each treatment consisted of 20 explants (meristem derived shoots).

Hormonal supplements (mgl^{-1}) used in MS.	Experimental cultivars											
	China						Loda					
	No. of explants induced roots	% of explants induced roots	Average No. of roots/shoot (Days after inoculated)		Average length (cm) of root (Days after inoculated)		No. of explants induced roots	% of explants induced roots	Average No. of roots/shoot (Days after inoculated)		Average length (cm)of root (Days after inoculated)	
			16 days	28 days	16 days	28 days			16 days	28 days	16 days	28 days
IBA												
0.5	0	0	0	0	0	0	0	0	0	0	0	0
1.0	2	10	3.1	5.1	3.2	3.9	2	10	3.0	4.9	3.3	4.1
2.0	6	30	4.2	6.4	4.3	7.4	7	35	5.9	6.1	4.4	7.6
3.0	20	100	7.2	12.3	7.3	12.0	20	100	6.9	12.0	7.4	11.8
4.0	15	75	5.3	9.4	6.7	11.3	16	80	4.8	9.1	6.7	10.8
5.0	9	45	4.4	6.9	4.7	7.3	9	45	4.3	6.5	3.9	6.9
6.0	3	15	3.2	4.6	3.8	5.7	4	20	3.4	4.7	3.0	4.7
7.0	0	0	0	0	0	0	0	0	0	0	0	0
8.0	0	0	0	0	0	0	0	0	0	0	0	0
9.0	0	0	0	0	0	0	0	0	0	0	0	0
NAA												
0.5	0	0	0	0	0	0	0	0	0	0	0	0
1.0	0	0	0	0	0	0	0	0	0	0	0	0
2.0	3	15	3.5	4.8	2.6	4.8	4	20	3.7	4.9	2.4	4.6
3.0	6	30	4.1	5.7	3.7	5.9	8	40	4.3	5.8	3.6	5.5
4.0	3	15	3.4	4.9	2.5	4.9	3	15			2.5	4.8
5.0	0	0	0	0	0	0	0	0	0	0	0	0
6.0	0	0	0	0	0	0	0	0	0	0	0	0
7.0	0	0	0	0	0	0	0	0	0	0	0	0
8.0	0	0	0	0	0	0	0	0	0	0	0	0
9.0	0	0	0	0	0	0	0	0	0	0	0	0
IAA												
0.5	0	0	0	0	0	0	0	0	0	0	0	0
1.0	2	10	1.5	2.4	2.0	2.2	2	10	1.4	2.1	2.1	2.3
2.0	6	30	2.3	2.6	2.1	2.9	6	30	2.2	2.4	2.1	2.6
3.0	3	15	1.8	2.3	2.3	2.7	2	10	1.7	2.0	2.2	2.7
4.0	0	0	0	0	0	0	0	0	0	0	0	0
5.0	0	0	0	0	0	0	0	0	0	0	0	0
6.0	0	0	0	0	0	0	0	0	0	0	0	0
7.0	0	0	0	0	0	0	0	0	0	0	0	0
8.0	0	0	0	0	0	0	0	0	0	0	0	0
9.0	0	0	0	0	0	0	0	0	0	0	0	0

2.3.4.2. Effect of IBA with BAP on Root Induction

For root induction, elongated shoots (height of about 3-4 cm) were individually cultured on MS media supplemented with different concentrations and combinations of IBA with BAP. Results of this experiment are presented in **Table 2.8**. The highest percentage of roots/shoot were 85% for both of the cultivars obtained in the treatment of 1.0 mgl^{-1} BAP+ 3.0 mgl^{-1} IBA. In this treatment, the highest mean number of roots per explant were 6.5 after 16 days and 11.9 after 28 days of culture in cv. China as well as 6.4 after 16 days and 11.0 after 28 days of culture in cv. Loda. In the same media, the mean length of the longest roots were 4.4 cm after 16 days and 9.1 cm after 28 days of culture in cv. China as well as 4.5 cm after 16 days and 8.9 cm after 28 days of culture in cv. Loda. This experiment showed that IBA is most effective for root induction. Another treatment of IBA with BAP was carried out to investigate its combine effect on root induction and it was proved that single effect of IBA provided better performance for root induction as shown in Table 2.7.

Table-2.8. Effects of different concentrations and combinations of IBA with BAP in MS medium on root induction. Each treatment contains 20 explants

Hormonal supplements (mgl^{-1}) used in MS: (BAP+IBA)	Experimental cultivars											
	China						Loda					
	No. of explants induced roots	% of explants induced roots	Average No. of roots/shoot (Days after inoculated)		Average length (cm) of root (Days after inoculated)		No. of explants induced roots	% of explants induced roots	Average No. of roots/shoot (Days after inoculated)		Average length (cm) of root (Days after inoculated)	
			16 days	28 days	16 days	28 days			16 days	28 days	16 days	28 days
0.5+0.5	0	0	0	0	0	00	0	0	0	0	0	0
0.5+1.0	2	10	2.1	3.6	2.2	4.3	2	10	2.2	3.6	2.3	4.3
0.5+2.0	5	25	3.1	4.1	2.3	4.3	5	25	3.1	4.0	2.2	4.4
0.5+3.0	6	30	3.2	4.1	2.3	4.4	5	25	3.1	4.1	2.3	4.5
0.5+4.0	3	15	2.9	4.0	2.3	4.2	3	15	2.8	3.9	2.2	4.2
0.5+5.0	0	0	0	0	0	0	0	0	0	0	0	0
1.0+0.5	0	0	0	0	0	0	0	0	0	0	0	0
1.0+1.0	12	55	3.5	6.4	3.3	6.3	11	55	3.4	6.5	0	0
1.0+2.0	11	50	3.3	6.1	2.7	5.9	10	50	3.4	6.4	4.3	8.9
1.0+3.0	17	85	6.5	11.9	4.4	9.1	17	85	6.4	11.0	4.5	8.9
1.0+4.0	6	30	2.7	3.7	2.7	5.2	6	30	2.5	3.9	2.6	5.9
1.0+5.0	1	5	1	1	1.0	0	0	0	0	0	2.6	5.4
1.5+0.5	0	0	0	0	0	0	0	0	0	0	0	0
1.5+1.0	6	30	2.2	2.5	0	0	6	30	2.1	2.5	0	0
1.5+2.0	6	30	2.3	2.5	2.6	5.2	5	25	2.2	2.9	2.6	5.0
1.5+3.0	9	45	2.7	2.9	4.0	8.1	9	45	2.8	3.1	3.9	7.9
1.5+4.0	5	25	2.0	2.3	2.1	3.0	5	25	2.2	2.7	2.1	2.9
1.5+5.0	0	0	0	0	0	0	0	0	0	0	0	0

PLATE 2.4

Plate 2.4 (A-D)

Root induction and shoot elongation from primary established apical meristem of eggplant on MS agarified media:

A. Root induction from elongated shoot of cv. China after 16 day on MS+3.0 mgl^{-1}.
B. Root induction from elongated shoot of cv. China after 28 day on MS+3.0 mgl^{-1}.
C. Root induction from elongated shoot of cv. Loda after 20 day on MS+3.0 mgl^{-1}.
D. Root induction from elongated shoot of cv. Loda after 30 day on MS+3.0 mgl^{-1}.

2.3.5. Acclimatization and Transfer of Plantlets to Soil

In vitro regenerated plantlets of 6-10 cm height with few leaves and suitable root system were initially transplanted in small plastic pots with a view to easy handling during transplantation to natural condition, **Plate 2.5 (A-D)**. It was found that more than 90% of the plantlets were survived during initial establishments and resumed new growth; finally they developed flowers and produced fruits. It was observed that the prevailing atmospheric condition (mostly, temperature and humidity) of transplanting season greatly influenced the initial survivable of meristem derived potted plants. It was noticed that the plantlets with active growth of primary roots showed greater survival and faster initial growth as compared to the plantlets having longer and branched root system at the time of transplantation.

The performances of the both cultivars were more or less similar. Meristem derived plants were healthy and highly vigorous than the source plant stock and no viral diseases and no morphological modifications were observed. Somaclonal variation was not observed among the meristem derived transplanted eggplant of the two cultivars.

PLATE 2.5

Plate 2.5 (A-D)

Acclimatization of the meristem derived plants:

A. Meristem derived plantlets of cv. China in polar pots under natural condition.

B. Meristem derived plant of cv. China growing up in natural condition.

C. Meristem derived plantlets of cv. Loda in polar pots under natural condition.

D. Meristem derived plant of cv. Loda growing up in natural condition.

2.4. DISCUSSION

Eggplant is usually seed propagated and very much susceptible to a number of viral diseases, which cause remarkable decrease in its yield. However, meristem culture is one of the important methods to produce virus free stock plants (Uddin *et al.* 2004). Whereas micropropagation does not permit the micro clones to be free from viruses. The shoots of a apical meristem and first set of primordial leaves are generally not connected to the vascular system of the plant and therefore, are not contaminated by viruses that travel through the vascular system (Prakash and Pierik, 1993, Rahman 1998). If this explant is carefully excised so as not to contaminate it with sap from more mature leaves or stem tissue and it is placed in culture tube a virus-free plant can be established. Many important horticultural crops (e.g. potato, tomato, orchids, lady's finger, citrus, strawberries) are routinely free of viral contamination by using this procedure (Prakash and Pierik, 1993; Ali 1998).

Smith and Murashige (1970) accomplished the first true meristem culture of an isolated angiosperm meristem into a complete plant. Before that time it was believed that the isolated shoot apical meristem of an angiosperm could not direct its own development but rather, relied on subjacent primordial leaves and stems tissue (Ball 1960). Generally, to establish a virus-free plant one can culture the apical dome plus two or four subjacent primordial leaves. This technique is also valuable for the maintenance of carefully defined stock of specific varieties in disease free state. The size of the meristem explant is critical for virus infection because the explant contains shoot apices with vascular tissue instead of true meristem (Adams 1975 and Rahman 1998).

This technique, combined with heat treatment (thermotherapy) or chemical treatment (chemotherapy) has proved to be very effective in virus eradication (De 1992). Heat treatment is done by placing an actively growing plant in a thermotherapy chamber. Using this technique, 80-90% virus free plants have been obtained. Without heat treatment, shoot tips or meristem can be grown on chemotherapeutants added medium for virus eradication (Ali 1998). Commercial production of virus-free eggplant through

meristem culture has been a regular practice in many developed countries. Present investigation was therefore designed to conduct experiment using the explants from field grown plants. The results of which are discussed in following paragraph with an endeavor to justify them.

For the primary establishment of *in vitro* culture, surface sterilization of the explants (shoot tips of eggplant) was essential because of the presence of microbial contaminants remain attached to the aerial surface. For sterilization of explants, many scientists used different types of sterilizing agents with different concentrations. There have been many reports on using calcium hypochlorite (Asaduzzaman 2005) or sodium hypoclorite (Jones 1986) for surface sterilization of explants. There are also many other reports on using $HgCl_2$ for surface sterilization. It is used as one of the surface sterilants and considered as a potent surface-sterilizing agents but its residual inhibitory effect is also greater than the other sterilizing agent commonly used in plant tissue culture (Bhojwani and Razdan 1983, Torres 1988).

$HgCl_2$ solution (0.1%) was used as surface sterilants in this investigation but non-judicious application of $HgCl_2$ solution in uncertain duration may lead to microbial contamination of the culture as well as tissue killing. Problem of tissue killing was occurred due to the toxic nature of $HgCl_2$ and it was overcome by using lower concentration of $HgCl_2$ for a short duration, but in some cases, unsatisfactory results were obtained owing to the problem of tissue killing.

In the present study the maximum number of contamination free seed germination was obtained when they were treated for 9-10 min. On the contrary, when they were treated for long duration (11-13 min) partial or complete tissue killing occurred. Among the different duration of treatment, the seeds when treated for 4-8 min tissue killing was not evident but microbial contamination was present. Therefore 10 min duration was considered as optimum for surface sterilization of seed explants of eggplant. Khatun (2004) also obtained similar result in potato. Balakrisnamurthy *et al.* (1987) that 3.0 min duration of time was optimum for surface sterilization of shoot tip explants of tomato.

In the present investigation, shoot tip explants of 20-25 days old *in vitro* grown plants were excised and cultured onto MS liquid media with different types of growth regulators such as KIN and GA_3 in order to find out the best culture media formulations for the establishment of primary meristem culture and also to find out the comparative performance of *in vitro* meristem culture for the two cultivars of eggplant tested in this investigation. For primary establishment of meristem, liquid MS medium with filter paper bridge was used. Many workers also got satisfactory results with MS liquid medium in different crops (Heller 1963, Stone 1963, Goodwin 1966, Vine 1968, Walkey 1968, Mellor and Smith 1969, Pennazio and Redolfi 1973, Rahman 1998 and Ali 1998). Use of liquid culture methods of eggplant tissue culture has also been reported previously (Goodwin 1966).

Among the various concentrations and combinations of growth regulators, 2.0 mgl^{-1} BAP +0.50 mgl^{-1} GA_3 showed the most quick responses (7-12 days) and highest frequency (90-95%) of growth responses for apical meristem. This result was closely followed by the single treatment of BAP (2.0 mgl^{-1}). Ahmmad (1999) and Khatun (2004) used same growth regulator formulation and obtained satisfactory result for the establishment of meristem culture in potato. Mohammad (2002) and Rahman (1999) also found the same result in potato. However use of NAA, IAA and KIN for establishment of primary meristems was also reported by Merja and Stasa (1997) in potato.

In the present investigation, to develop plantlets from primary establish meristems, different kinds of growth regulators such as BAP, GA_3, KIN were used singly or in combinations for proper growth and development of the culture meristems. Among the different treatments of singly or in combinations, singly use of BAP (1.0, 1.5, 2.0 mgl^{-1}) and GA_3 (0.50mgl^{-1}) were found effective for shoot elongation. Hoque *et al.* (1996) used BAP (2.0 mg/l) for shoot development in potato. Singly use of BA (1.0 mg/l) in modified MS medium was reported effective for plantlet development from meristem tip culture in potato (Marani and Pisi 1977). On the other hand singly use of GA_3 (0.50mgl^{-1}) also showed satisfactory result for shoot elongation. Similar result has also been demonstrated by Banu (1998) in Chrysanthemum and Ahmad (2000) in tomato. GA_3

was proved to be less effective for shoot elongation when KIN was added with it. The physiological basis as to why GA_3+KIN is not effective in activating shoot elongation in eggplant is unknown, however several influences are apparent:

1. Eggplant (like tomatoes) have a very weak apical dominance, thus the necessity for stimulation by exogenous control is less.
2. The shoot used for explants may have high level of endogenous cytokinin (KIN).

In such cases, addition of exogenous cytokinins can be expected to be inhibitory. The toxic effect of an applied cytokinin on axillary shoot growth was demonstrated in *Solanum andigena* (Wang and Wareing, 1979). Woolley and Wareing (1972) treated single nodal cutting of *Solanum andigena* with BA. They found that only pretreated (exhausted from endogenous cytokinin) nodal cuttings responded well to apply BA. Wang and Wareing (1979) treated non-pretreated nodal cutting with different concentrations of BA. They found that applied BA inhibited shoot growth. The higher concentration of cytokinin is responsible for the less shoot growth. BA at 10 mgl^{-1} was very inhibitory to shoot growth, 0.1 mgl^{-1} BA only found to be slightly inhibitory (Khatun 2004).

The shoots regenerated from primary meristem culture were needed to induce roots for their successful establishment to the field. The primary established meristem derived shoots (height of about 3-4 cm) were transferred onto MS semisolid medium supplemented with various types of growth regulators (Auxin and Cytokinin) either in singly or in combination to find out standard media formulation for root induction. Among different concentrations of auxin, 3.0 mg/l IBA was found to be the best for root induction. Percentage of root formation and mean number of roots per shoot were highly influenced by the concentrations of IBA. Efficient effect of IBA on root induction was also observed in Chickpea (Haque, *et al.*, 1984), grape (Chakravorty 1986) and bitter gourd (Rahman, 1998). Mante *et al.* (1989) also reported faster growth of root in *Calotropis gigantea* and *Prunus* sp., respectively. Among different concentrations of BAP with IBA; 1.0 mgl^{-1} BAP + 3.0 mgl^{-1} IBA was best for root induction along with shoot but the frequency of rooting was low as compared with single IBA. Similar results were also demonstrated in other Solanaceae plants by Beck *et al.* (1983) and Rahman (1998).

However, the ratio of cytokinin and auxin seem to play an important role for the morphogenic differentiation (shoot-root and/callus induction) of cultured explants, as suggested by Murashige and Skoog (1962), Steward *et al.* (1969), Thomas and Street (1970), Pareek and Chandra (1981), Beck and Coponetti (1983) and Haider (1992). When sufficient roots were induced, the plantlets were successfully transferred to the field after proper acclimatization.

In the present investigation, morphological characters of tissue culture derived plants were observed at different growing stages. In respect to different growth parameters cv. Loda was found to produce higher plant height and root numbers. Comparatively, better growth performance was also noted for cv. Loda than the cv. China.

After sufficient development of plantlets, gradual acclimatization was essential for subsequent establishment in natural condition. In the present investigation, regenerated plantlets of *S. melongena* L. were established in small plastic pots containing sterile soil and they were subsequently established in natural condition. It was found that more than 95% plantlets of eggplant could tolerate transplantation shock and survive under *in vivo* environment. The acclimatized plants grow normally, produced flowers, born fruits and were similar in performance to the mother plants. Similar results were also observed in eggplant (PTC) and in tomato (Ahmad *et al.* 2000)

The objective of this study was to establish an efficient and reproducible method for production of virus free plantlet of eggplant. Meristem culture techniques, described in this dissertation, have potential use for clonal propagation of eggplant for commercial purpose.

2.5. SUMMARY

The present investigation was conducted with a view to establish a standard method for meristem culture and shoot elongation with root induction in eggplant (*Solanum melongena* L.), as well as their acclimatization in the natural condition.

Apical meristem dissected from 20-25 day old *in vitro* grown plants was cultured onto filter paper bridge in liquid MS media supplemented with different concentrations and combinations of cytokinin (BAP and KIN) and GA_3. Among the different growth regulator formulations 2.0 mgl^{-1} BAP was found to be the best medium formulation for the primary establishment of isolated meristem for both of the cultivars. Addition of 0.50 mgl^{-1} GA_3 to 2.0 mgl^{-1} BAP enhances the efficiency of meristem development. The developing meristems were rescued aseptically from the filter paper bridge and cultured onto agar solidified MS media containing different concentrations and combinations of cytokinin (BAP and KIN) and GA_3 for shoot elongation. Among all of the treatments, semisolid MS medium having 0.50 $mgl^{-1}GA_3$ was found to be the best medium formulation for proper shoot elongation for both of the cultivars. Between the two cultivars of eggplant, cv. China was found to be more responsive caltivar for meristem culture. The elongated shoots were subcultured onto agarified MS medium supplemented with different concentrations and combinations of auxin (IBA, NAA and IAA) and BAP for root induction. Best result (100%) for root induction was obtained in MS having 3.0 mgl^{-1} IBA for both the cultivars. Well rooted plantlets were gradually acclimatized and successfully established in the field condition.

The finding of present investigation would play an important role in eradication of pathogen from infected eggplant variety, where the conventional technique of therapeutic chemicals is not available or not capable for the same.

CHAPTER THREE

Somatic Embryogenesis in Eggplant

CHAPTER III

3. SOMATIC EMBRYOGENESIS IN EGGPLANT

3.1. INTRODUCTION

Eggplant has been a staple vegetable in our diet since ancient times. Contrary to the common belief, it is quite high in nutritive value and can well be compared with tomato (Chowdhury 1976). It is also one of the world most important crops by virtue of volume produced and its contribution to our national economy (Uddin *et al.* 2004). It is grown for home consumption in almost all families near their homestead and more particularly in rural areas, even if may only a few plants in every house (Zahurul 1969). Now a day, tissue culture techniques are widely being used for the improvement of various crops. *In vitro* shoot induction through callus culture can induce genetic and epigenic changes in the regenerated plants. These genetic changes have been coined "somaclonal variation"(Larkin *et al.* 1984). The diverse characteristics of somaclones highlight the fact that somaclonal variation may be an additional tool for crop improvement rather than an interesting scientific phenomenon (Evans *et al.* 1984; Bajaj 1990). Callus induction and its subsequent plant regeneration is a major way to generate somaclonal variation. Moreover, plant regeneration through callus culture is one of the most important steps for any kind of genetic transformation research (Alam *et al.* 2002). In most of the published procedures, eggplant callus formation is promoted by maintenance of explants in the presence of both cytokinins and auxins. For example Hena (1991) and Anisuzzaman (1992) used 2.7 mgl^{-1} BAP and 0.2 mgl^{-1} NAA for callus initiation from leaf tissues of *S. melongena* L. Hansen *et al.* 1999 used an auxin-free shoot regeneration medium containing gibberellic acid and BAP or Zeatin for leaf explants of eggplant.

In plant tissue culture the developmental pathway of numerous well-organized, small embryoids resembling the zygotic embryos from the embryogenic potential somatic plant cell of the callus tissue or cells of suspension culture is known as somatic embryogenesis (De 1995). Under the present study, efforts have been made to establish a protocol for efficient plant regeneration through somatic embryogenesis in

eggplant using cotyledon and midrib. In Bangladesh we have made an intensified efforts to establish the protocol for callus induction, cell culture and their subsequent regeneration in eggplant. Procedures in this chapter describe the establishment of protocols for the initiation and maintenance of callus and plant regeneration through somatic embryogenesis.

The research works undertaken in the present investigation are segmented in the following two sections:

A. Callus induction and regeneration

B. Cell culture and regeneration.

A. CALLUS INDUCTION AND REGENERATION

3.2. MATERIALS AND METHODS

3.2.1. MATERIALS

3.2.1.1. Plant Materials

Two local cultivars of eggplant (*Solanum melongena* L.), namely China and Loda were selected for the present investigation as experimental materials. Cotyledon and midrib isolated from the two cultivars were used in the present investigation as explants for callus induction. Seeds of the two cultivars were collected from the Bangladesh Agricultural Development Corporation (BADC), Rajshahi.

3.2.1.2. Chemicals

All chemical compounds including micro and macronutrients, sugar, agar, $HgCl_2$, alcohol etc. used in the present study were the reagent grade product of Carolina (America) and Fluka (Germany). The vitamins, amino acids and growth regulators were the product of Carollina Biological Supply Company of U.S.A.

3.2.1.3. Other Materials

Culture vessels such as petridishes, conical flask, test-tube, separating funnel, pipette, forceps, cotton, fire box, marker pen, spirit lamp, needle, sharp blade, electronic balance, autoclave, laminar air flow machine etc., were also used in the present experiment.

3.2.2. METHODS

The methods for the preparation of culture media with required supplements have been illustrated in the section of meristem culture (Chapter II). The methods involved in present investigation were described under the following heads.

3.2.2.1. Precaution to Ensure Aseptic Condition

Before starting the inoculation programme, the laminar airflow cabinet was switched on allowing airflow and UV light before 30 minutes of culture initiation. Then UV light was switched off and 70% ethyl alcohol was sprayed on the floor of cabinet to reduce the chances of contamination. All the inoculation and aseptic manipulation were carried out

in the running laminar airflow cabinet. All the instruments like scalpels, forceps, needles and other requirements like petridishes, filter paper, empty beaker or conical flask, cotton, distilled water were sterilized by steam sterilization method, i.e. by autoclaving. Before using the instruments, these were again sterilized by dipping into 70% ethyl alcohol and flamed over a spirit lamp for several times. Before starting inoculation, hands were repeatedly washed thoroughly by soaking and spraying with 70% ethyl alcohol to ensure contamination free operation. For surgical operations sufficient care was taken as usual to obtain possible contamination free condition.

3.2.2.2. Culture Techniques

The following methods were employed in the present investigation.

3.2.2.2.1. Axenic Culture

Seeds of the *S. melongena* cv. Loda and cv. China were thoroughly washed under running tap water for 10 minutes to reduce the level of surface microorganisms. The floating seeds were discarded and the viable seeds were rinsed into a solution of a few drops of tween-80 and Savlon for 10 minutes with constant shaking. Then the seeds were washed carefully 4 to 5 times by the sterilized distilled water. Finally inside the laminar flow cabinet, surface sterilization was done with 0.1% $HgC1_2$ solution for 7 minutes and was washed 4-5 times with double autoclaved distilled water to remove $HgC1_2$ clearly. The sterilized seeds were then inoculated to agarified MS_0 medium in culture bottle and each culture vessel contained 25-30 seeds. Four week old *in vitro* grown seedlings were the ready source of explants and thus provided contamination free explants.

3.2.2.2.2. Explant Culture

The seedlings raised in axenic culture were the source of different kinds of explants. Pair of cotyledons and midrib isolated from *in vitro* grown seedlings were used as explants for initial callus induction. All the inoculation operation was performed in the running laminar air flow cabinet. The explants were treated in different kinds of media with different supplements for callus induction, organogenesis or somatic

embryogenesis and subsequent regeneration as described in result section. The explants were cultured in petridishes (9cm) and each petridish contained 16-20 pieces of explants for both cotyledon and midrib.

Cotyledon Culture: Pair of cotyledons isolated from 5 day old seedlings were used as explants for initial callus induction. The isolated cotyledon was cut into segments of 2-2.5 mm with the help of a sterilized cold scalpel blade and collected in a petridish. At a time, around 20 explants were inoculated in agar gelled MS medium of each petridish. The head of petridish was removed inside laminar flow cabinet and the cotyledonary segments were inoculated in medium with homogeneously distribution. Then the petridish was covered with its head followed by sealing with parafilm and sent to the inoculation chamber. The same technique was followed for the both of the experimental cultivars and 20 petridishes were used for each of the treatments.

Midrib Culture: Midribs isolated from 3-week-old seedlings were used as explants for initial callus induction. The midribs were excised with the aid of scalpel blade and collected in a petridish. Then the isolated midribs were laid on the sterile tile using sterile forceps and were cut into segments of 2-3 mm with the help of a sterile cold scalpel blade. The explants were then placed on the callus inducing MS medium, supplemented with various concentrations and combinations of 2,4-D, NAA, IAA and BAP with 3% sucrose and solidified with 0.7% agar. At a time, around 16 explants were inoculated in agar gelled medium of each petridish. The head of petridish was removed inside laminar flow cabinet and the segmented midribs were inoculated onto media with homogeneously distribution. Then the petridish was covered with its head followed by sealing with parafilm and sent to the inoculation chamber. The same technique was followed for the both of the experimental cultivars and 10 petridishes were used for each of the treatments.

3.2.2.2.3. Culture Incubation

The culture vessels containing the inoculated explants were incubated in dark chamber having a controlled temperature of 25 ± 2°C. A regular observation was carried out to record the response. With the appearance of callus, the petridishes were transferred to

growth chamber under low intensity fluorescent light at the same temperature for further callus development and proliferation.

3.2.2.2.4. Subculture of Callus for Maintenance and Regeneration

After 4 weeks, the induced calli were sub-cultured on fresh callus inducing medium consisting of new hormonal supplements for somatic embryogenesis and regeneration. When the calli attained a convenient size they were rescued aseptically on a sterile petridish in laminar flow cabinet and cut into convenient size by a sterile scalpel, so that no vascular contact with the parental tissue remained and these were inoculated to a freshly prepared medium supplemented with the same or different hormonal combinations, either for the maintenance of callus or for somatic embryogenesis. The sub-cultured calli continued to proliferate and showed various types of response. Within 10-14 days of sub-culture in the same or different growth regulators supplemented media, the calli responded to somatic embryogenesis and further shoot regeneration. Sometime, the bipolar structured embryos were rescued from the culture vessels aseptically and were cultured again on freshly prepared medium without or with different concentrations and combinations of hormonal supplements for subsequent regeneration.

B. CELL CULTURE AND REGENERATION

3.2.3. MATERIALS

Friable greenish (FG) callus containing rapidly dividing cells were used as experimental materials for cell culture. This calli were harvested from *in vitro* grown callus of 18-21 day old and cultured in liquid medium.

3.2.4. METHODS

3.2.4.1. Liquid Media Preparation for Cell Isolation

It is prerequisite to prepare liquid media for the isolation of single cell from friable and rapidly growing calli. The process of preparing MS liquid medium is same as for MS semi-solid medium, except addition of agar and its subsequent melting. MS liquid media were prepared and autoclaved at 15 lb/sq inch pressure and at the temperature of

121°C for 20 minutes to ensure sterilization. After autoclaving the culture medium was taken in running laminar flow cabinet for callus inoculation.

3.2.5. Cell Isolation and Culture Techniques

3.2.5.1. Cell Isolation from Friable Callus

In this investigation, the cells were isolated mechanically from the pre-established friable callus tissue by a needle and subsequent cell suspension culture by a pipette. To make cell suspension, the friable callus is transferred to liquid medium and the medium was continuously agitated by an orbitary shaker.

Due to continuous agitation, the cell clumps were broken and dispensed releasing single cells in the medium resulting cell suspension. After filtration the cell suspension was centrifuged to collect the single cells from the suspension. This procedure is stated below step by step.

1. Some conical flasks (250 ml) were taken and 75 ml of liquid MS medium was kept in each of the conical flasks and autoclaved at 121°C temperature under15 psi for 20 minutes.
2. After autoclaving, the conical flasks containing MS liquid media were taken in running laminar airflow and 4-5 pieces (about 1.5 gm) of pre-established friable greenish calli were transferred aseptically to the conical flasks having 75 ml autoclaved liquid medium (without agar).
3. Then the closures of the flasks were aseptically covered with aluminium foil and the flasks were placed within the clamps of an orbital shaker moving at 200 rpm.
4. After 7 days the contents of each flask were poured through a sterilized sieve having 500μ diameter pore and the filtrate was collected in a big sterilized container. The filtrate contained only free cells that was ensured by making some slides with the filtrate and photographs were taken for each of the slides.

3.2.5.2. Maintenance of Cell Culture

Free cells isolated from callus are grown as single cells under *in vitro* conditions using MS medium having different concentrations and combinations of hormones to observe

the growth/division efficiency of cells. The following steps are taken for this investigation.

1. The pre-filtrate containing single cells was allowed to settle for 20-25 minutes and finally poured off the supernatant.
2. Using the sterilized pipette, 5 ml residue cells was resuspended and dispensed equally in each of the sterilized flasks containing 70 ml freshly prepared liquid medium.
3. The flasks containing cells were placed on shaker with 200 rpm allowing the free cells to grow.
4. To prepare a growth curve of the dividing cells, the weight of single cells was taken 9 times with duration of two days from each flask separately.
5. Finally a growth curve was plotted with the weights of the growing cells.

3.2.5.3. Callus Induction from Isolated Cells

For callus induction, the isolated singe cells from the suspension were subcultured in petridishes containing freshly prepared MS liquid media supplemented with 7 different concentrations and combinations of NAA and BAP. The head of petridish was removed inside the laminar flow cabinet and the single cells were inoculated with a needle in the petridishes containing liquid media. Then the petridish was covered with its head followed by sealing with parafilm and sent to the inoculation chamber having 25 ± 2^0C. The same technique was practiced for both of the experimental cultivars and 7 petridishes were used for each of the treatments. A regular observation was performed to record the callusing response of the single cells.

3.2.6. Somatic Embryogenesis

This experiment was conducted to observe the effect of subculture in agarified MS media on calli derived from the isolated single cells of the two cultivars for somatic embryogenesis and subsequent regeneration. When the single cell derived calli attained a convenient size they were rescued aseptically on a sterile petridish in laminar flow cabinet. Then the calli were cut into convenient size by a sterile scalpel and subcultured on agar gelled MS media with 2.0 mgl^{-1} NAA+0.05 mgl^{-1} BAP and 2.0 mgl^{-1} Zeatin+1.0 mgl^{-1} BAP for somatic embryogenesis. The sub-cultured calli continued to proliferate and showed various types of response. After 21 days of sub-culture, the calli

responded to somatic embryogenesis and further shoot regeneration. Sometime, the bipolar structured embryos were rescued from the culture vessels aseptically and were cultured again on freshly prepared media.

3.2.7. Regeneration

Plants were regenerated by transferring the suitable embryogenic calli in MS semisolid media supplemented with 2,4-D, NAA, GA_3, BAP and Zeatin at various concentrations and combinations. Within 3-4 weeks of inoculation, masses of 15-20 shoots were developed from each callus, which were subdivided and cultured separately for further proliferation and root induction. The cultures were inoculated at 25±2°C under 16/8h light/dark condition. Shoots of 2-3 cm were placed in MS medium supplemented with different concentrations and combinations of IBA and GA_3. MS_0 and ½ strength of MS were also treated for root induction.

3.2.8. Transplantation

After sufficient development of shoot and root system, the plantlets were taken out from the culture vessels carefully. Medium attached to the root was gently washed with running tap water. The plantlets were treated with 0.1% Agroson (fungicide) solution and transplanted to plastic pots containing sterile sand, soil and compost in the ratio of 1:2:1. After transplantation, the plants along with the plastic pots were covered with a large moist polythene bag immediately to prevent desiccation. To reduce sudden shock, the polythene bags with plants, were kept in the controlled environment of growth chamber up to 10 days. To maintain proper humidity for the plantlets, all plastic pots were checked up and the interior of the polythene bags were sprayed with water whenever necessary. After 13-14 days, the polythene bags were gradually perforated to expose the plants to natural environment. Finally the covering were completely exposed. After 2 weeks, when the plantlets grew well, they were transferred in normal temperature and kept under sun light for one hour daily. This duration increased gradually. However the plants were successfully adapted with the natural environment and were transferred to the field condition.

3.2.9. DATA COLLECTION

Data were collected under the following heads:

3.2.9.1. Percentage (%) of Explants Induced to Form Callus: For each combination of growth regulators, around 16-20 pieces of segmented explants were cultured in 20 petridishes separately. Explants those induced to develop callus, were computed after 28 days and in some cases 35 days of culture. Percentage of callus inducing explants was computed by the following formula.

$$\% \text{ of callus forming explants} = \frac{\text{No. of explants formed callus}}{\text{Total number of explants}} \times 100$$

Finally, the obtained percentage values from each treatment were determined as mean±standard error ($\overline{X} \pm SE$) as follows:

$$\text{Mean percentage } (\overline{X}) = \frac{\text{Total percentage values of callus induction}}{\text{Total number of observation (n)}}$$

$$\text{Standard error (SE)} = \frac{SD}{\sqrt{n}}$$

$$\text{Standard deviation (SD)} = \frac{\sqrt{\sum x^2 - (\sum x)^2 / n}}{n-1}$$

3.2.9.2. Percentage of Embryogenic Callus: Embryogenic calli were counted after 25 to 35 days of culture and percentage of embryogenic calli were calculated using the following formula:

$$\text{Percentage (\%) of embryogenic callus} = \frac{\text{No. of embryogenic callus}}{\text{No of explants inoculated}} \times 100$$

3.2.9.3. Fresh Weight of Callus: The mean weight of callus was taken by an electrical balance in the running laminar airflow cabinet and aseptic operation was carried out to avoid unexpected contamination. In this way, the weights were taken in gram for all of the treatments.

3.2.9.4. Fresh Weight of Single Cells (Cell Culture): The fresh weight of cells was measured carefully in the running laminar flow cabinet by electric balance using plastic net of 100μ diameter pore. At first the weight of plastic net was taken and this weight was subtracted from the weight of plastic net with cells. The cells presented in 10 ml liquid medium were weighted in each case. The weights were taken in gram after every two days.

3.2.9.5. Number of Embryos per Callus: Number of embryos per callus was counted under stereomicroscope from the beginning of embryogenesis and average number of embryos was calculated applying following formula:

Average number of embryos per callus, $\bar{X} = \frac{\sum x_i}{N}$

Where, $\bar{x}$ = Average number of embryos (mean)

x_i = Total number of embryos induced from each callus.

N = No. of observation

$\sum$ = Summation.

3.2.9.6. No. of Shoots per Callus: Number of shoots per callus was calculated using the following formula:

$$\text{No. of shoots per callus} = \frac{\text{Number of shoots regenerated}}{\text{Number. of callus inoculated}}$$

3.2.9.7. Percentage of Shoots Induced to Form Root: Percentage of shoots induced to develop roots were calculated by using the following formula:

$$\text{Percentage (\%)of shoots induced root} = \frac{\text{No. of explants formed roots}}{\text{Total No. of shoots culture}} \times 100$$

3.2.9.8. Number of Roots per Shoot: Number of roots per shoot was calculated using the above mentioned formula for number of embryo per callus:

Average number of roots per shoot, $\overline{X} = \frac{\sum x_i}{N}$

Where, $\bar{x}$ = Average number of roots (mean)

x_i = Total number of roots induced from each shoot.

N = No. of observation

$\sum$ = Summation.

3.3. RESULTS

The present investigation was carried out for *in vitro* regeneration through callus induction, cell culture and somatic embryogenesis in eggplant. The objective of this investigation was to establish a protocol for the induction of callus and plant regeneration through somatic embryogenesis of the eggplant cultivars. Details of the results obtained from each of the experiments are described under the following two sections:

Section A: Callus Induction and Regeneration

Section B: Cell Culture and Regeneration

Section A: Callus Induction and Regeneration

3.3.1. Effect of Auxins in MS Media on Callus Induction

The cotyledons and midribs of the two cultivars (cv. China and cv. Loda) were cultured on agarified MS media supplemented with different concentrations and combinations of 2,4-D, NAA, IAA and BAP in order to find out the most suitable medium formulation for maximum callus induction. To evaluate callus induction performance, data were recorded on days to callus initiation, frequency of callus induction (%), weight of callus (gm) and nature of callus. The summarized results are presented in **Table 3.1** and **Table 3.2**. The results of these experiments are described bellow under different heads.

3.3.1.1 Effect of 2,4-D

Seven different concentrations (0.01, 0.05, 0.1, 0.5, 1.0, 2.0 and 3.0 mgl^{-1}) of 2,4-D were used to observe the callusing response of cotyledon and midrib explants of the two cultivars.

In case of cv. China, the highest percentage of callus induction were recorded 97.55% for cotyledon and 96.63% for midrib in 2.0 mgl^{-1} 2.4-D and callus induction was started at 8th and 10th day of inoculation, respectively. In this concentration the recorded callus weight was also highest and it was 1.40 gm and 1.12 gm, respectively. The second

highest results were recorded in the 3.0 mgl^{-1} 2.4-D and the frequencies were 88.89% for cotyledon and 85.33% for midrib having the weight of 1.2gm and 0.99gm, respectively. The lowest 27.18 % of cotyledon and 29.93% of midrib were responded for callus induction in MS media having 0.01 mgl^{-1} 2,4-D after 19-20 days of culture and fresh weight of calli were 0.29 gm and 0.15gm, respectively. In cv. Loda, highest results were obtained in 2.0 mgl^{-1} 2,4-D and it was 97.10% for cotyledon and 96.55% for midrib. In this concentration callus induction was initiated within 8-9 days of culture initiation and after 28 days of culture, the callus weight were 1.35 gm and 1.30 gm, respectively. The second highest results were recorded in the 3.0 mgl^{-1} 2.4-D and in this case, 86.03% cotyledon and 87.89% midrib induced callus after 8-10 days of inoculation and the weight of the induced calli were 1.21 gm and 1.01 gm, respectively after 28 days of inoculation. The lowest 26.73% cotyledon and 27.40% midrib induced callus in 1.0 mgl^{-1} 2,4-D after 18 days of inoculation and the weight of the induced calli were 0.30 gm and 0.28 gm, respectively after 28 days of inoculation.

In all cases of 2,4-D texture of callus was spongy and colour of callus was white for both of the explants as well as the fresh weights of callus were varied in different hormonal concentrations. The rate of callusing (%) increased gradually with the increment of hormonal concentration and decreased when the concentration was increased above the optimum dose (2.0 mgl^{-1}).

3.3.1.2. Effect of NAA

MS media supplemented with seven concentrations (0.01, 0.05, 0.1, 0.5, 1.0, 2.0 and 3.0 mgl^{-1}) of NAA were treated for callus induction from cotyledon and midrib explants and the data were recorded after 4 weeks of inoculation.

In case of cv. China the highest rate (%) of callus inducing explants were 99.00 % for cotyledon and 99.73% for midrib initiated at 8 days of inoculation when cultured in 2.0 mgl^{-1} NAA supplemented medium. In this case the fresh weight of callus were 1.38 mg and 1.40 gm, respectively. The second highest rate (%) of callus induction were 95.63 % for cotyledon and 95.75% for midrib initiated at 8 days of inoculation when cultured in 3.0

mgl^{-1} NAA supplemented medium. In this case the fresh weight of callus were 1.19 mg and 1.20 gm, respectively. Lowest 32.11 % of cotyledon and 29.96 % of midrib responded to callus initiation after 17-18 days of inoculation when cultured in 0.01 mgl^{-1} NAA supplemented medium and the fresh weight was 0.29 mg for both of the explants. In case of cv. Loda, the highest rate (%) of callus induction were 98.60 % for cotyledon and 98.50% for midrib initiated at 8 days of inoculation when cultured in 2.0 mgl^{-1} NAA supplemented medium and the fresh weight was 1.40 mg and 1.39 gm, respectively. This result was closely followed by the treatment of 3.0 mgl^{-1} NAA showing 95.25% of both explants responded to callus initiation after 8 days of inoculation and the callus weights were 1.22 gm and 1.20 gm, respectively. The lowest 29.34% of cotyledon and 32.86% of midrib responded to callus initiation after 17-18 days of inoculation when cultured in 0.01 mgl^{-1} NAA supplemented medium and the fresh weight was 0.30 mg and 0.31 gm, respectively. In case of NAA alone, the texture of callus was friable and colour was whitish for both of the explants, which is not favorable for somatic embryogenesis. The rate of callusing (%) increased gradually with the increment of hormonal concentration and decreased when the concentration were increased above the optimum dose (2.0 mgl^{-1}).

3.3.1.3. Effect of IAA

To observe the effect of IAA on callus induction from cotyledon and midrib of the two cultivars, seven different concentrations (0.01, 0.05, 0.1, 0.5, 1.0, 2.0 and 3.0 mgl^{-1}) of IAA were used and the obtained results are presented in **Table 3.1** and **Table 3.2**.

In case of cv. China, the highest percentage of callus induction were recorded 85.43% for cotyledon and 87.43% for midrib in 3.0 mgl^{-1} IAA started within 13-14 days of inoculation as well as the recorded callus weight were also highest in this case and it was 0.78 gm and 0.79 gm, respectively. This result was closely followed by the treatment of MS+2.00 mgl^{-1} IAA. In MS medium having 0.01 mgl^{-1} IAA, lowest results were 31.24 % for cotyledon and 30.05% for midrib responding callus induction after 20 days of culture and fresh weight of calli were 0.22 gm for cotyledon and 0.24 gm for midrib. In cv. Loda, the highest percentage of callus induction were recorded 86.88% for

cotyledon and 85.20% for midrib in 3.0 mgl^{-1} IAA started at 13 days of inoculation. In this case the recorded callus weight was also highest and it was 0.75 gm and 0.70 gm, respectively. This result was closely followed by the treatment of MS+2.00 mgl^{-1} IAA. The lowest 29.13 % cotyledon and 31.19% midrib were responded for callus induction in MS media having 0.01 mgl^{-1} IAA after 20 days of culture and fresh weight of calli were 0.23 gm and 0.24 gm, respectively.

It is remarkable that, the callus induction frequency (%) was increased with the increment of the concentration of IAA and decreased when the concentration were increased above the optimum dose (3.0 mgl^{-1}) (Data not shown). But in all cases of IAA the nature of calli was hard and its colour was white, which is not favorable for somatic embryogenesis. More over required days to callus initiation was longer than that for 2,4-D and NAA.

3.3.2. Effect of Auxin with BAP in MS Media on Callus Induction

Among seven different concentrations of auxins treated in the previous treatments, 1.0, 2.0 and 3.0 mgl^{-1} were found to be better than the others. Hence these three concentrations were selected for further experiment in combination with three different concentrations (0.05, 0.10 and 0.50 mgl^{-1}) of BAP to evaluate the effect of auxin influenced by BAP. The results obtained are shown in **Table 3.1** and **Table 3.2**.

3.3.2.1. Effect of 2,4-D with BAP

MS media supplemented with three different concentrations of 2,4-D with BAP were treated to evaluate the effect of 2,4-D influenced by BAP on callus induction from cotyledon and midrib of the two cultivars. In case of cv. China, the best results in respect of callus induction were 75.43% for cotyledon and 75.50% for midrib found in medium having 2.0 mgl^{-1} 2,4-D + 0.05 mgl^{-1}BAP and its callus weight were 1.19 gm and 0.99 gm, respectively. In this case callus induction were initiated at 10 days of inoculation, that is higher than the time required for optimum dose of single 2,4-D (2.0 mgl^{-1}). When the explants were cultured in medium having 0.10 mgl^{-1} BAP with above mentioned three different concentrations of 2,4-D, the second highest results on callus induction were 71.70% for cotyledon and 73.18% for midrib

observed in media supplemented with 2.0 mgl^{-1} 2,4-D+0.10 mgl^{-1} BAP and weight of callus were 1.00 gm and 0.96gm, respectively. In this case callus induction was initiated at 13 day and 14 day of culture, respectively. The treatment of 3.0 mgl^{-1} 2,4-D+0.10 mgl^{-1} BAP gave the lowest percentage of callus induction 55.18% for cotyledon and 53.35% for midrib and the lowest callus weight were 0.86 gm and 0.80 gm, respectively. This lowest result was higher than that obtained in any treatment of single 2,4-D. Media having 2.0 mgl^{-1} 2,4-D+ 0.50 mgl^{-1} BAP showed callus induction frequency 71.70% for cotyledon and 70.45% for midrib after 15-16 days of culture.

The same concentrations of auxin (1.0, 2.0 and 3.0 mgl^{-1}) and BAP (0.05, 0.10 and 0.50 mgl^{-1}) were also treated for cv. Loda. In cv. Loda, the best results in respect of callus induction were 74.75% for cotyledon and 73.50% for midrib found in medium having 2.0 mgl^{-1} 2,4-D + 0.05 mgl^{-1} BAP and its callus weight were 1.20 gm and 1.02 gm, respectively. In this case, callus induction were initiated at 13 day and 12 day of inoculation, respectively, that is higher than the time required for optimum dose of single 2,4-D (2.0 mgl^{-1}). This results were closely followed by the results obtained in 2.0 mgl^{-1} 2,4-D + 0.10 mgl^{-1} BAP as presented in Table 3.2. MS+ 3.0 mgl^{-1} 2,4-D+0.10 mgl^{-1} BAP gave the lowest percentage of callus induction 55.75% for cotyledon and 54.50% for midrib and the lowest callus weight were 0.85 gm and 0.76 gm, respectively. For the both cultivars, the nature of calli was either spongy or hard and its colour was either white or greenish and the hard calli of spongy or white are not suitable for somatic embryogenesis. Media having 2.0 mgl^{-1} 2,4-D+ 0.50 mgl^{-1} BAP showed callus induction frequency 69.25% for cotyledon and 71.25% for midrib after 15 days of culture.

In all the concentrations, the nature of the calli for both of the cultivars were found as spongy-whitish (SW) or friable-greenish (FG) or hard-greenish (HG) and the friable-greenish (FG) callus is the most favorable for somatic embryogenesis. Here high concentration of BAP with 2,4-D was found ineffective on callus induction for the two cultivars.

3.3.2.2. Effect of NAA with BAP

MS media supplemented with three different concentrations (1.0, 2.0 and 3.0 mgl^{-1}) of NAA with BAP (0.05, 0.10 and 0.50 mgl^{-1}) were treated to evaluate the effect of NAA influenced by BAP on callus induction from cotyledon and midrib of the two cultivars (**Plate 3.1 A-F**).

In case of cv. China, the best results in respect of callus induction were 85.43% for cotyledon and 83.30% for midrib found in medium having 2.0 mgl^{-1} NAA + 0.05 mgl^{-1} BAP and its callus weight were 1.27 gm and 1.18 gm, respectively. In this case callus induction were initiated at 8-9 days of inoculation and the callus was friable greenish (FG) which is suitable for somatic embryogenesis. The second highest result on callus induction were 75.25% for cotyledon and 74.25% for midrib observed in medium having 3.0 mgl^{-1} NAA+0.05mgl^{-1} BAP and weight of callus were 0.99 gm and 1.01gm, respectively. In this case callus induction was initiated at 9 days of culture. The treatment of 1.0 mgl^{-1}NAA +0.10 mgl^{-1} BAP gave the lowest percentage of callus induction, 52.33% for cotyledon and 51.33% for midrib providing the lowest callus weight 0.75 gm and 0.71 gm, respectively and the nature of callus of both explants was hard white (HW), which is not favorable for somatic embryogenesis. Media having 2.0 mgl^{-1} NAA+ 0.50 mgl^{-1} BAP showed callus induction frequency 65.33% for cotyledon and 67.33% for midrib after 11-12 days of culture.

In case of cv. Loda, the best results for callus induction were 82.50% for cotyledon and 84.50% for midrib found in medium having 2.0 mgl^{-1} NAA + 0.05 mgl^{-1} BAP and its callus weight were 1.30 gm and 1.16 gm, respectively. In this case callus induction were initiated at 8-9 days of inoculation and the callus was friable greenish (FG) which is suitable for somatic embryogenesis. The second highest result on callus induction was found to be 73.75% for cotyledon and 74.75% for midrib observed in 3.0 mgl^{-1} NAA+0.05 mgl^{-1} BAP and weight of callus were 0.90 gm and 1.06 gm, respectively. In this case callus induction was initiated at 9 day of culture. The treatment of 1.0 mgl^{-1}NAA +0.10 mgl^{-1} BAP gave the lowest percentage of callus induction, 50.50% for cotyledon and 51.25% for midrib providing the lowest callus weight 0.78 gm and 0.74 gm, respectively and the nature of callus of both

explants was hard white (HW), which is not favorable for somatic embryogenesis. Media having 2.0 mgl^{-1} NAA+ 0.50 mgl^{-1} BAP showed callus induction frequency 66.00% for cotyledon and 63.75% for midrib after 11 days of inoculation.

3.3.2.3. Effect of IAA with BAP

MS media supplemented with three different concentrations of IAA (1.0, 2.0 and 3.0 mgl^{-1}) with 0.05 mgl^{-1} BAP were treated to evaluate the effect of IAA influenced by BAP on callus induction from cotyledon and midrib of the two cultivars. When explants of cv. China were cultured on the media supplemented with 1.0 mgl^{-1} IAA + 0.05 mgl^{-1} BAP, 61.15% of cotyledon and 57.35% of midrib induced callus having the callus weight of 0.35 gm for cotyledon and 0.30 gm for midrib initiated at 14 days and 16 days of culture, respectively. This result was closely followed by the treatment of 3.0 mgl^{-1} IAA+0.05 BAP and in this case, 59.15% of cotyledon and 56.63% of midrib produced respectively 0.31 gm and 0.32 gm callus and the days to callus initiation were 13 day and 16 day, respectively. The lowest results on callus induction were obtained in the treatment of 2.0 mgl^{-1} IAA + 0.05 mgl^{-1}BAP. When the explants of cv. Loda were cultured on the media supplemented with 1.0 mgl^{-1} IAA + 0.05 mgl^{-1} BAP, 56.75% of cotyledon and 60.25% of midrib induced callus of 0.30 gm and 0.31 gm, respectively. In this case, the days to callus initiation were 15 day and 17 day of culture, respectively. This result was closely followed by the treatment of 3.0mgl^{-1} IAA + 0.05 mgl^{-1} BAP and in this case, 55.75% of cotyledon and 58.25% of midrib produced callus of 0.31 gm and 0.32 gm, respectively and the days to callus initiation were 14 day and 16 day, respectively. The lowest results on callus induction were obtained in the treatment of 2.0mgl^{-1} IAA + 0.05 mgl^{-1}BAP. In all cases of IAA + BAP, the nature of callus of both explants was hard white (HW), which is not favorable for somatic embryogenesis.

This experiment revealed that combine effect of auxin (2,4-D, NAA and IAA) with BAP is more effective for embryogenic callus (friable greenish) induction. The average effect of growth regulators with MS media on callus induction from cotyledon and midrib of the two cultivars has been presented in **Fig 3.1**.

Table 3.1. Effect of auxin (2,4-D, NAA and IAA) singly or in combination with BAP on callus induction in cv. China. Each treatment consisted of 20 explants and data were recorded after 28 days of culture.

Growth regulators used in the treatment		Explants inoculated							
		Cotyledon				Midrib			
Combination	Concentration (mg^{-1})	Days to Callus initiation	% of explants responded (M±SE)	Weight / Callus (gm)	Nature of Callus	Days to Callus initiation	% of explants responded (M±SE)	Weight / Callus (gm)	Nature of Callus
2,4-D	0.01	19	27.18±1.18	0.29	SW	20	26.93±0.86	0.15	SW
	0.05	14	28.80±1.22	0.52	SW	15	29.0±1.10	0.53	SW
	0.10	12	29.99±0.94	0.75	SW	12	30.75±0.95	0.70	SW
	0.50	12	46.03±1.94	0.75	SW	12	46.75±1.81	0.80	SW
	1.00	11	58.25±2.07	1.01	SW	12	59.25±1.45	0.99	SW
	2.00	**8**	**97.55±1.51**	**1.40**	**SW**	**10**	**96.63±1.12**	**1.12**	**SW**
	3.00	8	88.89±2.61	1.20	SW	10	85.33±1.27	0.99	SW
NAA	0.01	17	32.11±2.74	0.29	FW	18	29.96±2.64	0.29	FW
	0.05	15	33.15±2.97	0.49	FW	15	32.33±2.82	0.52	FW
	0.10	12	44.85±2.50	0.71	FW	12	43.10±1.80	0.69	FW
	0.50	11	46.86±1.49	0.69	FW	12	44.11±1.41	0.75	FW
	1.00	9	61.91±3.87	1.07	FW	10	61.41±4.11	1.01	FW
	2.00	**8**	**99.00±0.41**	**1.38**	**FW**	**8**	**99.73±0.18**	**1.40**	**FW**
	3.00	8	95.63±1.73	1.19	FW	8	95.75±2.29	1.20	FW
IAA	0.01	20	31.25±0.85	0.22	HW	20	30.05±1.37	0.24	HW
	0.05	18	34.85±0.92	0.29	HW	18	35.40±0.87	0.29	HW
	0.10	17	45.61±0.95	0.29	HW	18	45.18±1.04	0.31	HW
	0.50	16	56.40±1.81	0.52	HW	18	57.20±2.50	0.48	HW
	1.00	14	66.10±1.66	0.61	HW	15	65.43±1.21	0.64	HW
	2.00	14	80.48±3.43	0.69	HW	14	78.40±0.73	0.71	HW
	3.00	**13**	**85.43±3.33**	**0.78**	**HW**	**14**	**87.43±0.75**	**0.79**	**HW**
2,4-D+BAP	1.0+0.05	12	60.25±1.53	0.81	SW	13	58.48±0.94	0.78	SW
	2.0+0.05	**10**	**75.43±1.17**	**1.19**	**SG**	**10**	**75.50±0.67**	**0.99**	**SG**
	3.0+0.05	10	61.95±1.07	0.99	SW	10	67.08±1.40	0.81	SW
	1.0+0.10	14	60.75±1.20	0.80	SW	15	55.25±0.90	0.75	SW
	2.0+0.10	13	71.70±1.53	1.00	HG	14	73.18±1.35	0.96	HG
	3.0+0.10	12	55.18±0.10	0.86	HW	12	53.35±1.67	0.80	HW
	2.0+0.50	16	71.70±1.25	1.09	HW	15	70.45±1.65	0.90	HW
NAA+BAP	1.0+0.05	11	53.45±1.31	0.85	SW	12	52.45±1.27	0.84	SW
	2.0+0.05	**9**	**85.43±2.27**	**1.27**	**FG**	**8**	**83.30±0.87**	**1.18**	**FG**
	3.0+0.05	9	75.25±0.95	0.99	FG	9	74.25±0.80	1.01	FG
	1.0+0.10	12	52.33±0.89	0.75	HW	13	51.33±0.98	0.71	HW
	2.0+0.10	12	67.33±1.15	1.15	HG	12	68.18±0.61	1.02	HG
	3.0+0.10	12	65.18±0.10	0.90	HG	13	63.10±1.60	0.90	HG
	2.0+0.50	11	65.33±0.95	1.02	HG	12	67.33±0.33	0.89	HG
IAA+BAP	**1.00+0.05**	**14**	**61.15±1.11**	**0.35**	**HW**	**16**	**57.35±1.09**	**0.30**	**HW**
	2.00+0.05	15	51.30±0.85	0.38	HW	17	50.23±1.04	0.39	HW
	3.00+0.05	13	59.15±1.86	0.36	HW	16	56.63±0.84	0.42	HW

Note: SW=Spongy-whitish; FW= Friable-whitish; HW=Hard-whitish; SG=Spongy-greenish; FG= Friable-greenish (best callus) and HG=Hard-greenish.

Table 3.2. Effect of auxin (2,4-D, NAA and IAA) singly or in combination with BAP on callus induction in cv. Loda. Each treatment consisted of 20 explants and data were recorded after 28 days of culture.

Growth regulators used in the treatment		Explants inoculated							
		Cotyledon				Midrib			
Combination	Concentration (mg^{-1})	Days to Callus initiation	% of explants responded (M±SE)	Weight / Callus (gm)	Nature of Callus	Days to Callus initiation	% of xplants responded (M±SE)	Weight/ Callus (gm)	Nature of Callus
2,4-D	0.01	18	26.73±0.71	0.30	SW	18	27.40±0.67	0.28	SW
	0.05	14	28.75±0.96	0.50	SW	15	29.25±1.00	0.52	SW
	0.10	13	30.38±1.25	0.70	SW	13	29.75±0.74	0.68	SW
	0.50	13	48.00±1.12	0.73	SW	12	47.25±1.47	0.78	SW
	1.00	12	59.25±0.82	1.10	SW	12	58.25±2.247	1.00	SW
	2.00	**8**	**97.10±0.47**	**1.35**	**SW**	**9**	**96.55±1.14**	**1.30**	**SW**
	3.000	8	86.03±0.83	1.21	SW	10	87.89±3.07	1.01	SW
NAA	0.01	18	29.34±2.39	0.30	FW	17	32.86±1.31	0.31	FW
	0.05	15	31.83±3.08	0.51	FW	16	34.75±1.76	0.52	FW
	0.10	13	42.75±1.71	0.75	FW	12	45.05±2.27	0.74	FW
	0.50	11	43.85±1.48	0.60	FW	13	46.36±2.33	0.75	FW
	1.00	9	61.90±3.17	1.09	FW	9	62.41±1.63	1.11	FW
	2.00	**8**	**98.60±0.62**	**1.40**	**FW**	**8**	**98.50±0.29**	**1.39**	**FW**
	3.000	8	95.25±1.32	1.22	FW	8	95.25±2.43	1.20	FW
IAA	0.01	20	29.13±1.74	0.23	HW	20	31.19±1.20	0.24	HW
	0.05	18	35.73±1.13	0.30	HW	18	34.48±1.44	0.30	HW
	0.10	17	43.95±0.89	0.28	HW	17	45.68±1.67	0.30	HW
	0.50	17	56.88±1.64	0.50	HW	18	56.30±2.28	0.49	HW
	1.00	15	65.20±1.67	0.55	HW	15	65.75±1.63	0.65	HW
	2.00	14	78.00±0.65	0.60	HW	14	80.38±2.84	0.68	HW
	3.00	**13**	**86.88±0.49**	**0.75**	**HW**	**13**	**85.20±2.06**	**0.70**	HW
2,4-D+BAP	1.0+0.05	12	60.00±2.68	0.85	SW	13	60.80±1.49	0.80	SW
	2.0+0.05	**13**	**74.75±0.95**	**1.20**	**SG**	**10**	**73.50±1.85**	**1.02**	**SG**
	3.0+0.05	10	65.25±1.65	1.05	SW	11	62.25±1.11	0.83	SW
	1.0+0.10	13	54.00±2.58	0.84	SW	15	61.13±1.05	0.79	SW
	2.0+0.10	12	72.50±1.71	1.10	HG	12	71.75±2.06	0.99	HG
	3.0+0.10	13	55.75±0.63	0.85	HW	12	54.50±1.71	0.76	HW
	2.0+0.50	15	69.25±1.65	1.10	HW	15	71.25±1.65	0.89	HW
NAA+BAP	1.0+0.05	13	51.50±2.36	0.78	SW	13	52.25±1.65	0.85	SW
	2.0+0.05	**8**	**82.50±0.96**	**1.30**	**FG**	**9**	**84.50±2.26**	**1.16**	**FG**
	3.0+0.05	9	73.75±0.85	0.90	FG	9	74.75±1.49	1.06	FG
	1.0+0.10	13	50.50±1.56	0.78	HW	13	51.25±2.50	0.74	HW
	2.0+0.10	12	67.25±2.50	1.10	HG	12	65.50±2.60	1.08	HG
	3.0+0.10	12	62.75±1.84	0.92	HG	13	64.25±1.65	0.92	HG
	2.0+0.50	11	66.00±2.12	1.09	HG	11	63.75±2.72	0.91	HG
IAA+BAP	**1.00+0.05**	**15**	**56.75±1.25**	**0.30**	**HW**	**17**	**60.25±3.52**	**0.31**	**HW**
	2.00+0.05	16	48.75±1.11	0.34	HW	17	50.50±1.26	0.33	HW
	3.00+0.05	14	55.75±1.18	0.31	HW	16	58.25±2.14	0.32	HW

Note: SW=Spongy-whitish; FW= Friable-whitish; HW=Hard-whitish; SG=Spongy-greenish; FG= Friable-greenish (best callus) and HG=Hard-greenish.

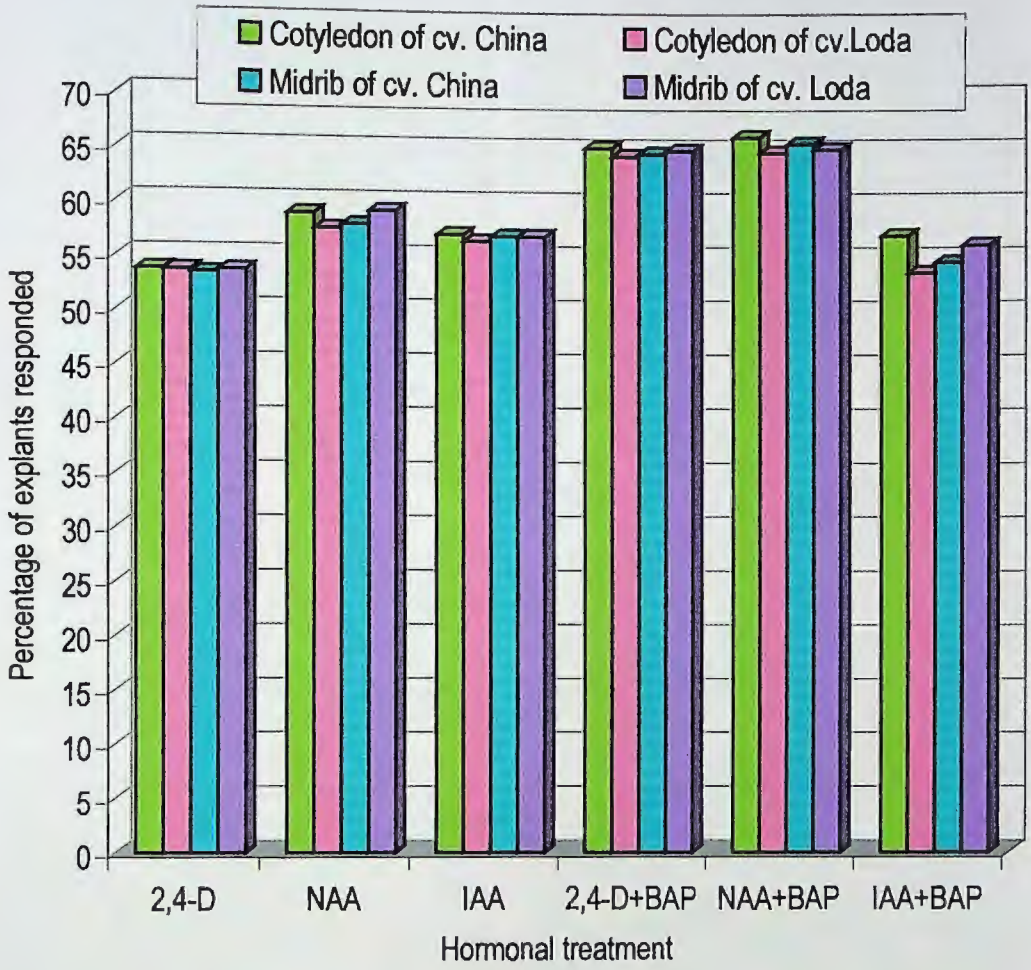

Fig 3.1: Graph showing the average effect of growth regulators with MS media on callus induction for the two cultivars.

PLATE 3.1

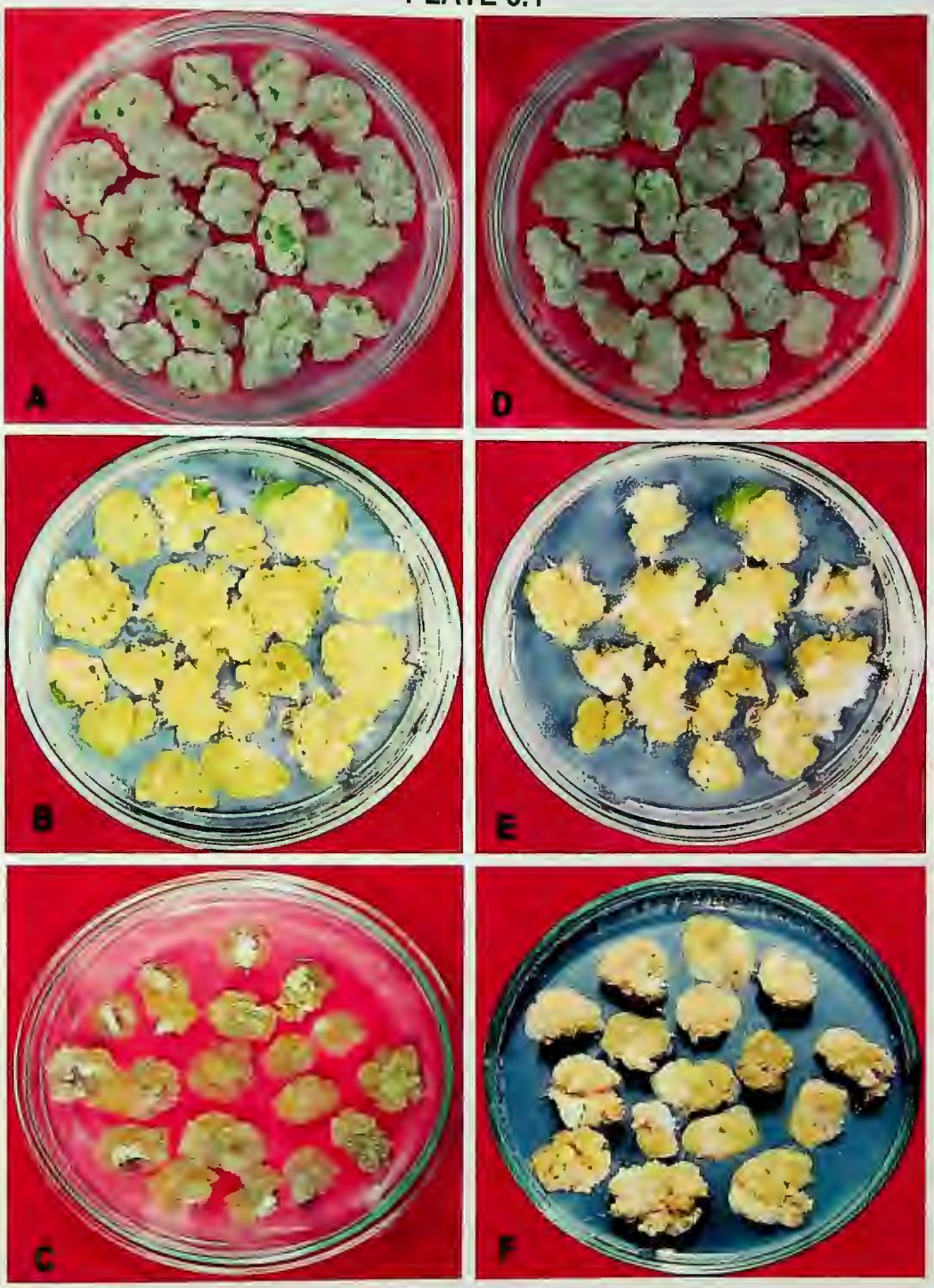

Plate 3.1 (A-F)

Induction of callus on MS agarified media having 2.0 mgl^{-1} NAA+0.05mgl^{-1}BAP:

A. Embryogenic callus induced from midrib of cv. China.
B -C. Embryogenic callus induced from cotyledon of cv. China.
D. Embryogenic callus induced from midrib of cv. Loda.
E -F. Embryogenic callus induced from cotyledon of cv. Loda

for somatic embryogenesis and shoot regeneration when subcultured on MS consisting of 0.05 mgl^{-1} GA_3 +1.0 mgl^{-1}BAP **(Plate 3.2 F)**. In case of cv. China, the number of embryos/callus were 13 for cotyledon and 14 for midrib within 9-12 days of subculture and shoot regeneration were 64.18% and 62.35%, respectively. In case of cv. Loda, the number of embryos/callus were found to be 15 for both of the explants within 9-12 days of subculture and shoot regeneration were 64.50% and 61.95%, respectively. Morphological responses were also found in case of the calli harvested from 1.0 mgl^{-1}NAA +0.05 mgl^{-1}BAP of the pre-culture when subcultured in 0.05 $mgl^{-1}GA_3$ +1.0 mgl^{-1}BAP. Other subcultural treatments of 0.05 $mgl^{-1}GA_3$ +1.0 mgl^{-1}BAP did not show any morphogenic response.

Calli derived from the preculture of NAA + BAP were also subcultured on semisolid MS media supplemented with 2.0 mgl^{-1}Zeatin +1.0 mgl^{-1}BAP. The calli harvested from 2.0 mgl^{-1}NAA +0.05 mgl^{-1}BAP of the pre-culture gave best morphogenic responses when subcultured on MS containing 2.0 mgl^{-1}Zeatin +1.0 mgl^{-1}BAP. In case of cv. China, the number of embryos/callus were found to be 18 for cotyledon and 17 for midrib within 10-12 days of subculture and shoot regeneration were 87.33% and 82.15%, respectively **(Plate 3.3 B & C)**. In case of cv. Loda, the number of embryos/callus were found to be 19 for cotyledon and 17 for midrib within 10-13 days of subculture and shoot regeneration were 86.50% and 79.75%, respectively **(Plate 3.3 E & F)**. The second highest result was observed in case of the calli harvested from the previous treatment of pre-culture (2.0 mgl^{-1}NAA +0.50 mgl^{-1}BAP) when subcultured in 2.0 mgl^{-1}Zeatin +1.0 mgl^{-1}BAP. In case of cv. China, the number of embryos/callus were found to be 16 for cotyledon and 14 for midrib within 10-12 days of subculture and shoot regeneration were 81.2% and 79.0%, respectively. In case of cv. Loda, the number of embryos/callus were found to be 17 for cotyledon and 16 for midrib within 11-13 days of subculture and shoot regeneration were 79.25% and 81.50%, respectively. Morphological responses were also found to be satisfactory in case of the calli harvested from 1.0 mgl^{-1}NAA +0.05 mgl^{-1}BAP of the pre-culture when subcultured in 2.0 mgl^{-1}Zeatin +1.0 mgl^{-1}BAP. The lowest result was observed in case of the calli harvested from the pre-cultural treatment of 3.0 mgl^{-1}NAA

+0.50 mgl^{-1}BAP when subcultured in 2.0 mgl^{-1}Zeatin +1.0 mgl^{-1}BAP. In case of cv. China, the number of embryos/callus were 10 for cotyledon and 9 for midrib within 13-15 days of subculture and shoot regeneration were 55.08% and 56.10%, respectively. In case of cv. Loda, the number of embryos/callus were found to be 11 for both of the explants within 11-15 days of subculture and shoot regeneration was found to be 55.75% and 56.50%, respectively.

Table 3.3. Effect of sub-culture on somatic embryogenesis and regeneration efficiency from the unorganized calli derived from midrib and inter node in eggplant. Each treatment consisted of 20 explants (best calli obtained from the midrib and inter node) and data were recorded after 52 days (28+24) of culture.

Combination of growth regulators used in mgl^{-1}		China						Loda					
		Cotyledon			Midrib			Cotyledon			Midrib		
Pre-culture (28 days)	Sub-culture (24 days)	Days to embryos initiation	No. of embryos / callus	% of shoots regenerated from the calli	Days to embryos initiation	No. of embryos / callus	% of shoots regenerated from the calli	Days to embryos initiation	No. of embryos / callus	% of shoots regenerated from the calli	Days to embryos initiation	No. of embryos / callus	% of shoots regenerated from the calli
2,4-D+BAP													
1.00+0.05	2.0 2,4-D +0.05 BAP	–	–	–	–	–	–	–	–	–	–	–	–
2.00+0.05		–	–	–	–	–	–	–	–	–	–	–	–
3.00+0.05		–	–	–	–	–	–	–	–	–	–	–	–
1.00+0.10		–	–	–	–	–	–	–	–	–	–	–	–
2.00+0.10		–	–	–	–	–	-	–	–	–	–	–	-
3.00+0.10		–	–	–	–	–	–	–	–	–	–	–	–
2.00+0.50		–	–	–	–	–	–	–	–	–	–	–	–
1.00+0.05	0.05 GA_3+1.0 BAP	–	–	–	–	–	–	–	–	–	–	–	–
2.00+0.05		–	–	–	–	–	–	–	–	–	–	–	–
3.00+0.05		–	–	–	–	–	–	–	–	–	–	–	–
1.00+0.10		–	–	–	–	–	–	–	–	–	–	–	–
2.00+0.10		–	–	–	–	–	-	–	–	–	–	–	-
3.00+0.10		–	–	–	–	–	–	–	–	–	–	–	–
2.00+0.50		–	–	–	–	–	–	–	–	–	–	–	–

(Table Continued)

Table 3.3. Effect of sub-culture on somatic embryogenesis and regeneration efficiency from the unorganized calli derived from midrib and inter node in eggplant. Each treatment consisted of 20 explants (best calli obtained from the midrib and inter node) and data were recorded after 52 days (28+24) of culture (continue).

Combination of growth regulators used in mgl^{-1}		China						Loda					
		Cotyledon			Midrib			Cotyledon			Midrib		
Pre-culture (28 days)	Sub-culture (24 days)	Days to embryos initiation	No. of embryos / callus	% of shoots regenerated from the calli	Days to embryos initiation	No. of embryos / callus	% of shoots regenerated from the calli	Days to embryos initiation	No. of embryos / callus	% of shoots regenerated from the calli	Days to embryos initiation	No. of embryos / callus	% of shoots regenerated from the calli
NAA+BAP													
1.00+0.05	2.0 NAA +0.05 BAP	–	–			–	–	–	–	–	–	–	–
2.00+0.05		**10-12**	**16**	**75.10±0.63**	10-12	**13**	**70.33±1.19**	**9-12**	**17**	**75.75±0.75**	10-12	17	**71.00±1.08**
3.00+0.05		–	–	–	–	–	–	–	–		–	–	–
1.00+0.10		–	–	–	–	–	–	–	–		–	–	–
2.00+0.10		–	–	–	–	–	–	–	–		–	–	–
3.00+0.10		–	–	–	–	–	–	–	–		–	–	–
2.00+0.50		–	–	–	–	–	–	–	–		–	–	-
1.00+0.05	0.05 GA$_3$+1.0 BAP	11-13	12	31.18±2.96	11-13	13	31.20±2.62	10-13	14	31.50±3.10	10-13	13	31.75±2.39
2.00+0.05		9-12	13	64.18±1.23	11-12	14	62.35±1.18	9-12	15	64.50±1.32	10-12	15	61.95±1.07
3.00+0.05		–	–	–	–	–	–	–	–		–	–	–
1.00+0.10		–	–	–	–	–	–	–	–		–	–	–
2.00+0.10		–	–	–	–	–	–	–	–		–	–	–
3.00+0.10		–	–	–	–	–	–	–	–		–	–	–
2.00+0.50		–	–	–	–	–	–	–	–		–	–	-
NAA+BAP													
1.00+0.05	2.0 Zeatin+1.0 BAP	11-14	15	71.10±1.74	10-14	14	78.25±0.80	10-13	14	71.50±1.55	9-13	15	77.88±1.01
2.00+0.05		**10-12**	**18**	**87.33±0.93**	**10-12**	**17**	**82.15±0.97**	**10-13**	**19**	**86.50±0.87**	**10-13**	**17**	**79.75±3.28**
3.00+0.05		13-15	10	55.08±1.22	13-15	9	56.10±0.85	12-15	11	55.75±1.11	11-14	11	56.50±0.65
2.00+0.50		10-12	16	81.20±0.72	10-12	14	79.00±1.48	11-13	17	79.25±1.32	10-12	16	81.50±0.65

PLATE 3.2

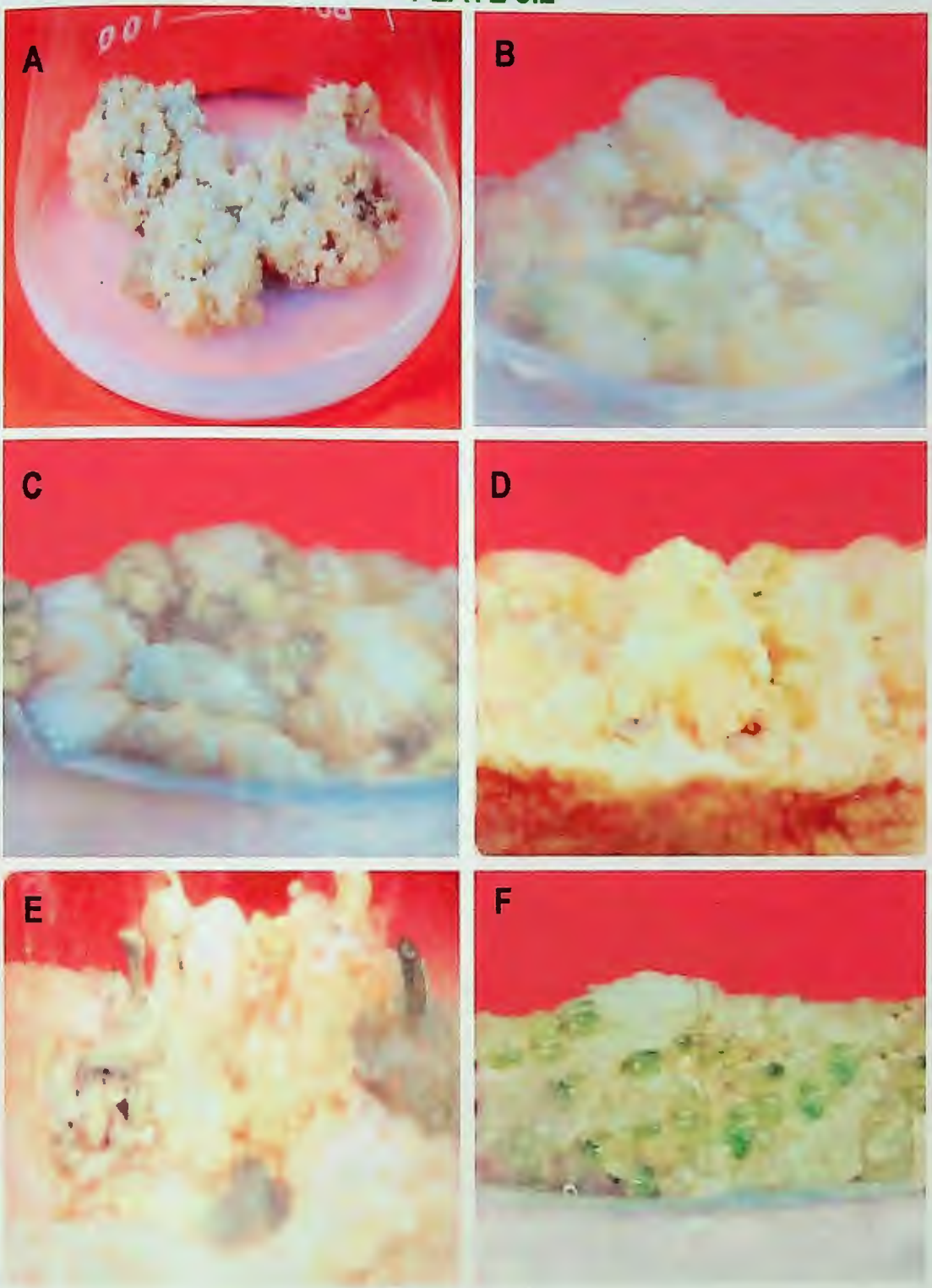

Plate 3.2 (A-F)

Subculture of callus on MS agarified media:

A. Spongy whitish callus of cv. China on MS + 2.0mgl^{-1} 2, 4-D + 0.05 mgl^{-1} BAP.
B-C. Hard whitish callus of cv. Loda on MS + 2.0mgl^{-1} 2, 4-D + 0.05 mgl^{-1} BAP.
D-E. Brownish callus of cv. China on MS + 0.05 mgl^{-1} GA_3 +1.0 mgl^{-1} BAP.
F. Embryo initiation on callus of cv. Loda on MS + 0.05 mgl^{-1}GA_3 +1.0 mgl^{-1} BAP.

PLATE 3.3

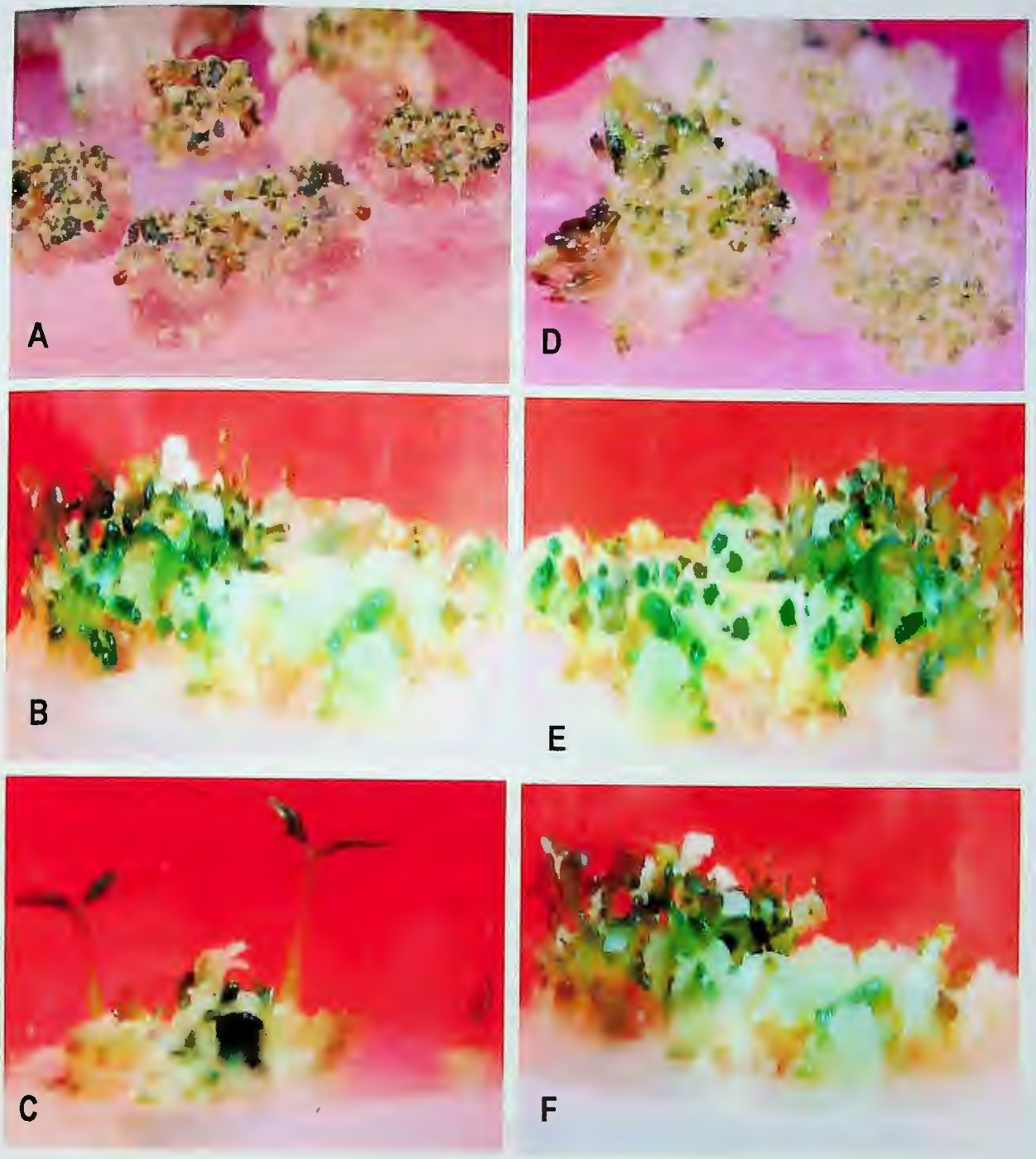

Plate 3.3 (A-F)

Plant regeneration through somatic embryogenesis on MS agarified media:

A. Initial stage of shoot regeneration from midrib derived callus of cv. China on MS+2.0mgl^{-1} NAA+0.05mgl^{-1} BAP.
B. Shoot regeneration in midrib derived callus of cv. China on MS+2.0mgl^{-1} Zeatin+1.0mgl^{-1} BAP.
C. Shoot regeneration in cotyledon derived callus of cv. China on MS+2.0mgl^{-1} Zeatin+1.0mgl^{-1} BAP.
D. Initial stage of shoot regeneration from midrib derived callus of cv. Loda on MS+2.0mgl^{-1} NAA+0.05mgl^{-1} BAP.
E. Shoot regeneration in midrib derived callus of cv. Loda on MS+2.0mgl^{-1} Zeatin+1.0mgl^{-1} BAP.
F. Shoot regeneration in cotyledon derived callus of cv. Loda on MS+2.0mgl^{-1} Zeatin+1.0mgl^{-1} BAP.

Section B: Cell Culture and Regeneration

3.3.4. Cell Culture in Liquid Media

The experiment on cell culture was conducted to observe the growth of cells of the two cultivars of eggplant. Friable greenish (FG) and embryogenic calli containing rapidly dividing cells were harvested from the pre-established *in vitro* culture in semisolid MS medium having 2.0 mgl^{-1} NAA+0.05 mgl^{-1} BAP. It is prerequisite to prepare liquid media for the isolation of single cells from friable and rapidly growing calli. Conical flasks measuring 250ml were used for cell suspension culture. Around 1gm of the harvested calli was taken in each of the conical flasks containing 75ml of MS liquid medium supplemented with 2.0 mgl^{-1} NAA+0.05 mgl^{-1} BAP to initiate cell suspension. Data were recorded on cell weight per 10ml cell suspension 9 times with the interval of every 2 days of culture. The experimental observation revealed that the cells grew gradually in the suspension culture of all treatments. The obtained results are tabulated in **Table 3.4** and presented in **Plate 3.4 (A-F)**.

In case of cv. China, the cells continued to grow until 15th day and then the cell division was ceased for cotyledon explant and the peak period of cell division was observed within 5^{th} day (0.138gm) to 7^{th} day (0.202gm) of culture. The cells of midrib continued to grow until 17th day and then the cell division was ceased and the peak period of cell division was found within 7^{th} day (0.159gm) to 9^{th} day (0.198gm) of culture. The grand mean weights of cells/10ml suspension were found to be 0.205±0.018 gm for cotyledon and 0.198±0.0147 gm for midrib.

In case cv. Loda, the cells continued to grow until 15th day and then the cell division was ceased for cotyledon explant and its peak period of cell division was observed within 5^{th} day (0.154gm) to 7^{th} day (0.195gm) of culture. The cells of midrib continued to grow until 17th day and then the cell division was ceased and the peak period of cell division was found within 5^{th} day (0.153gm) to 7^{th} day (0.209gm) of culture. The grand mean weights of cells/10ml suspension were found to be 0.202±0.011gm for cotyledon and 0.214±0.017gm for midrib of cv. Loda.

Table 3.4. Effect of 2.0 mgl^{-1} NAA+0.05 mgl^{-1} BAP on cell culture of eggplant in liquid MS media. Data were collected 9 times after every 2 days of culture.

Period	Weight (gm) of cells per 10ml cell suspension ($\bar{x}$±SE) for cv. China		Weight (gm) of cells per 10ml cell suspension ($\bar{x}$±SE) for cv. Loda	
	Cotyledon	Midrib	Cotyledon	Midrib
3rd day	0.091±0.036	0.128±0.052	0.135±0.007	0.109±0.024
5th day	0.138±0.050	0.142±0.004	0.154±0.004	0.153±0.016
7th day	0.202±0.027	0.159±0.003	0.195±0.009	0.209±0.019
9th day	0.219±0.030	0.198±0.003	0.208±0.006	0.223±0.021
11th day	0.237±0.025	0.208±0.002	0.219±0.008	0.244±0.020
13th day	0.239±0.026	0.234±0.003	0.222±0.005	0.247±0.020
15th day	0.240±0.026	0.235±0.083	0.227±0.007	0.248±0.019
17th day	0.241±0.026	0.239±0.036	0.227±0.005	0.249±0.020
19th day	0.241±0.005	0.238±0.019	0.227±0.005	0.249±0.020
Grand Mean±SE	0.205±0.018	0.198±0.0147	0.202±0.011	0.214±0.017

The mean values of cells of the two explants for both eggplant cultivars were calculated and plotted in graph **(Fig. 3.2)**. The results presented in the graph indicated that the two cultivars, China and Loda showed the similar trend of cell growth in the treated medium composition and their growths were found highest within 11th to 13th day of suspension culture. After 13th day, their growths were going to be stationary in the artificial medium.

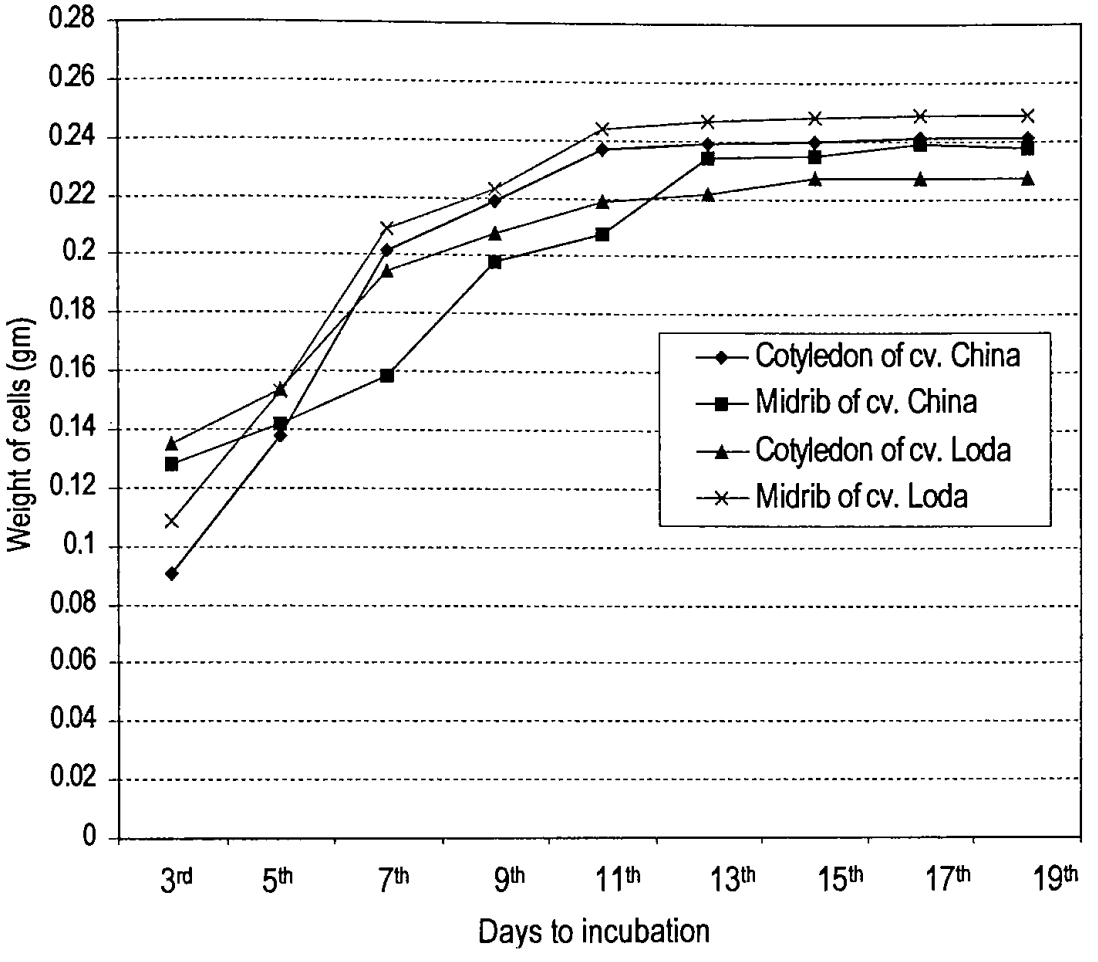

Fig. 3.2. Graph showing the growth pattern of cells in suspension culture of eggplant in liquid MS media.

PLATE 3.4

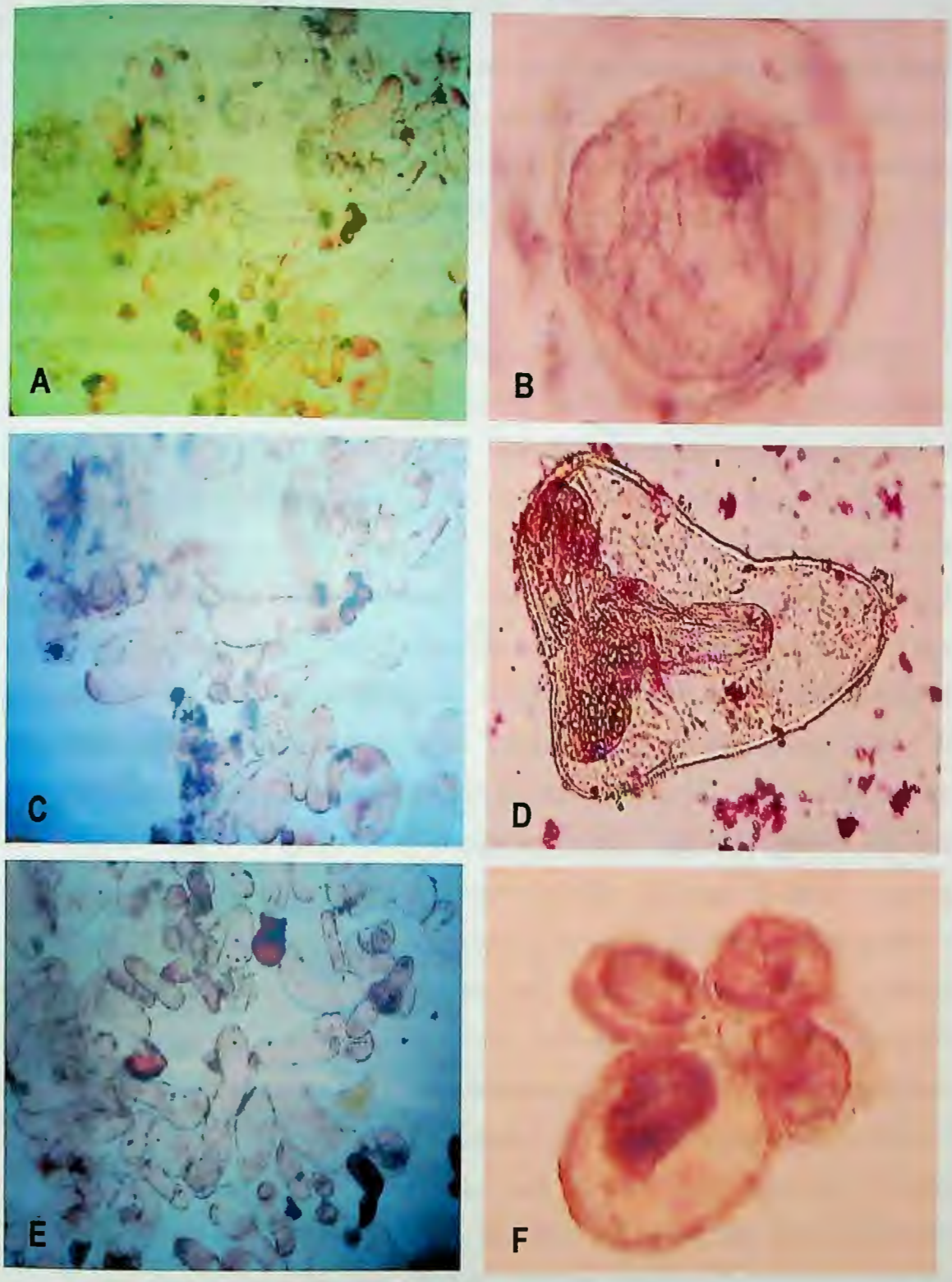

Plate 3.4 (A-F)

Microscopic observation showing the single cells isolated from the suspension culture:

A-C. Isolated cells of cv. China.

D-F. Isolated cells of cv. Loda.

3.3.5. Callus Induction from Isolated Cells

For callus induction, the isolated singe cells of the two cultivars were cultured on liquid MS media supplemented with 7 different concentrations and combinations of NAA and BAP. Data were recorded on the average number of callus/petridish, average weight/callus and days to callus initiation and the obtained results on callus induction are presented in **Table 3.5** and **Fig. 3.3**.

The best result in respect of callus induction was found in medium having 2.0 mgl^{-1} NAA + 0.05 mgl^{-1} BAP for both of the cultivars. MS media supplemented with three different concentrations of NAA (1.0, 2.0 & 3.0 mgl^{-1}) with BAP were treated to evaluate the effect of NAA influenced by BAP on callus induction from cotyledon and midrib derived single cells of the two cultivars. In case of cv. China, the best results on the average number of callus/petridish were 13.0 for cotyledon and 12.3 for midrib found in medium having 2.0 mgl^{-1} NAA + 0.05 mgl^{-1} BAP and the average weight/callus were 0.77 gm and 0.79 gm, respectively. In this case callus induction were initiated at 25 day and 26 day of inoculation **(Plate 3.5 A-C)**. In case of cv. Loda, the best results on the average number of callus/petridish were 12.7 for cotyledon and 12.0 for midrib found in medium having 2.0 mgl^{-1} NAA + 0.05 mgl^{-1} BAP and the average weight/callus were 0.81 gm and 0.80 gm, respectively as well as the callus induction were initiated at 24 day and 27 day of inoculation **(Plate 3.5 D-F)**. In case of cv. China, the second highest results on the average number of callus/petridish were 11.4 for cotyledon and 10.9 for midrib observed in media supplemented with 1.0 mgl^{-1} NAA+0.05 mgl^{-1} BAP and weight/callus were 0.70gm and 0.71gm, respectively. In this case, callus induction was initiated at 30 day of culture for both of the explants. In case of cv. Loda, the second highest results on the average number of callus/petridish were 10.9 for cotyledon and 11.1 for midrib observed in media supplemented with 1.0 mgl^{-1} NAA+0.05 mgl^{-1} BAP and weight/callus were 0.76gm and 0.72gm, respectively. In this case, callus induction was initiated at 31 day for cotyledon and 33 day for midrib. In case of cv. China, the treatment of 3.0 mgl^{-1}NAA+0.10 mgl^{-1} BAP gave the lowest number of callus/petridish 9.4 for cotyledon and 9.7 for midrib and the required days to callus initiation were also higher i.e., 37 day for both of the explants. In this case the

weight/callus were 0.45 gm and 0.48 gm, respectively. The same treatment also gave lowest result for cv. Loda and the lowest number of callus per petridish were 10.0 for cotyledon and 9.8 for midrib and the required days to callus initiation were also higher i.e., 36 day and 37 day, respectively. In this case the lowest weight/callus were 0.44 gm and 0.46 gm, respectively. In all these cases, the nature of the induced calli for both of the cultivars was found as friable creamy.

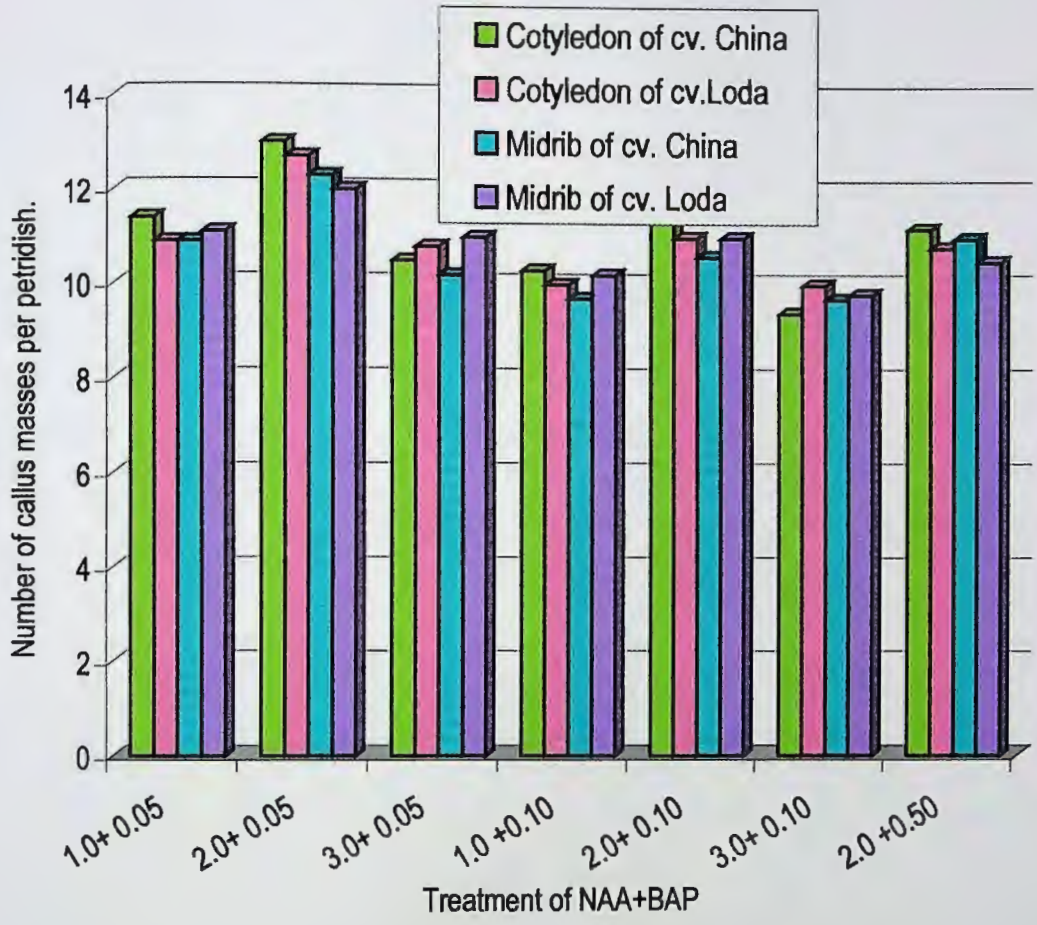

Fig 3.3. Graph showing the average effect of NAA + BAP with MS media on callus induction from the isolated single cells of the two cultivars.

Table 3.5. Callus induction in MS liquid media having 7 different combinations of NAA+BAP using isolated single cells from cell culture in liquid MS containing 2.0 mgl^{-1} NAA+ 0.05 mgl^{-1} BAP.

Growth regulators (NAA+BAP) mgl^{-1}	China						Loda					
	Cotyledon			Midrib			Cotyledon			Midrib		
	Days to callus initiation	No. of callus / petridish($\bar{x}$)	Weight/ Callus (gm)	Days to callus initiation	No. of callus / petridish($\bar{x}$)	Weight/ Callus (gm)	Days to callus initiation	No. of callus / petridish($\bar{x}$)	Weight/ Callus (gm)	Days to callus initiation	No. of callus / petridish($\bar{x}$)	Weight/ Callus (gm)
1.0+ 0.05	30	11.4	0.70	30	10.9	0.71	31	10.9	0.76	33	11.1	0.72
2.0+ 0.05	**25**	**13.0**	**0.77**	**26**	**12.3**	**0.79**	**24**	**12.7**	**0.81**	**27**	**12.0**	**0.80**
3.0+ 0.05	39	10.5	0.70	38	10.2	0.71	36	10.8	0.63	33	11.0	0.58
1.0 +0.10	34	10.3	0.58	34	9.7	0.60	35	10.0	0.55	34	10.2	0.56
2.0+ 0.10	36	11.4	0.63	36	10.6	0.68	37	11.0	0.67	35	11.0	0.63
3.0+ 0.10	37	9.4	0.45	37	9.7	0.48	36	10.0	0.44	37	9.8	0.46
2.0 +0.50	45	11.2	0.40	40	11.0	0.44	47	10.8	0.45	45	10.5	0.47

PLATE 3.5

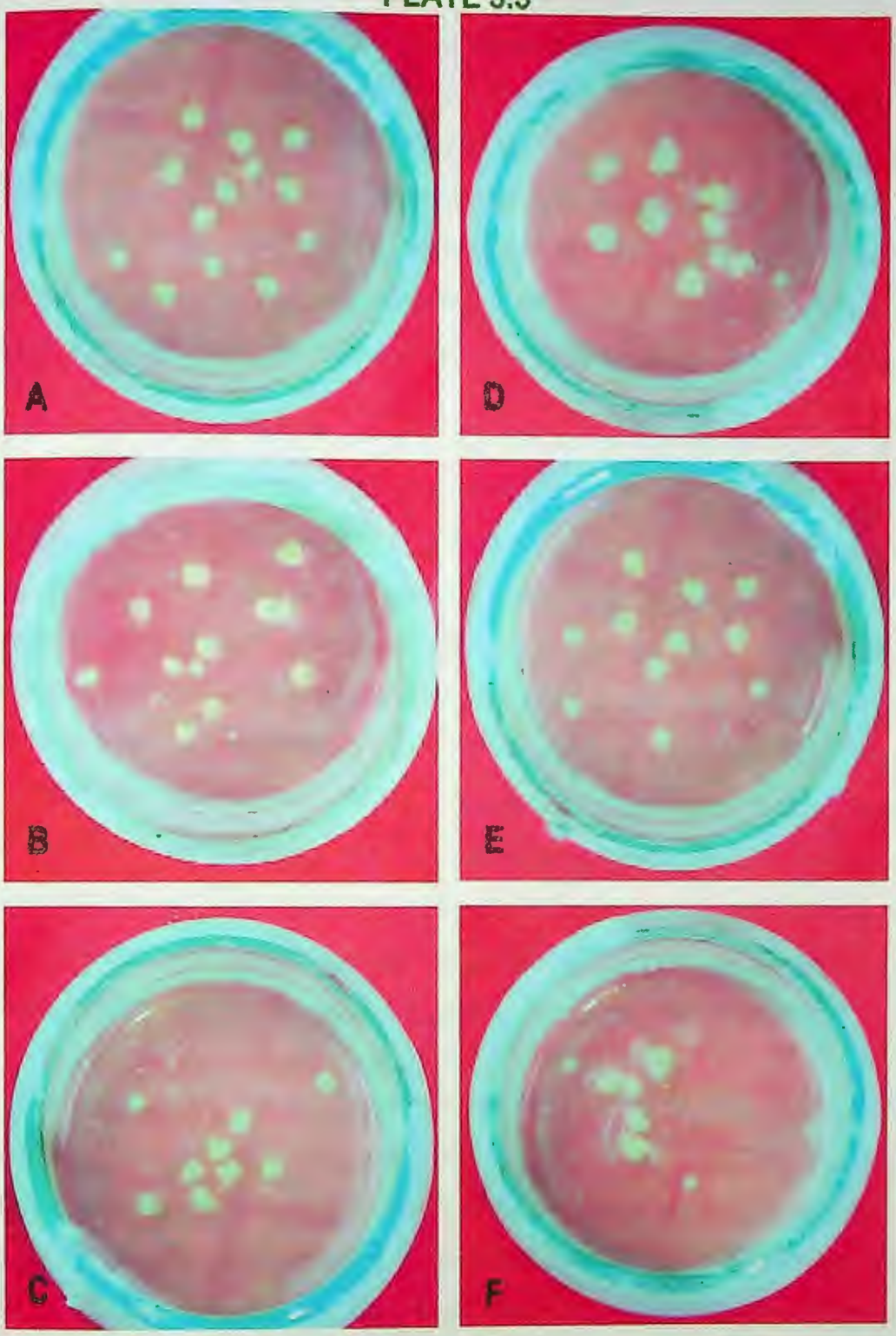

Plate 3.5 (A-F)

Callus induction from isolated single cells from cell culture in liquid MS media having 2.0 mgl^{-1} NAA+0.05 mgl^{-1} BAP:

- **A.** Single cells derived callus masses using midrib explants of cv. China.
- **B-C.** Single cells derived callus masses using cotyledon explants of cv. China.
- **D.** Single cells derived callus masses using midrib explants of cv. Loda.
- **E-F.** Single cells derived callus masses using cotyledon explants of cv. Loda.

3.3.6. Somatic Embryogenesis and Shoot Regeneration

This experiment was conducted to observe the effect of subculture in agarified MS media on calli derived from isolated single cells of the two cultivars for morphological differentiation. Since it is pre-established in previous section (**3.3.3.2.**) that 2.0 mgl^{-1} NAA+0.05 mgl^{-1} BAP and 2.0 mgl^{-1} Zeatin+1.0 mgl^{-1} BAP are the suitable formulation for somatic embryogenesis and shoot regeneration, this two combinations were used in this experiment. The results obtained are presented in **Plate 3.6 (A-F)**.

When calli were subcultured on semisolid MS media supplemented with 2.0 mgl^{-1} NAA +0.05 mgl^{-1} BAP, friable greenish callus proliferation was occurred after 15 days of inoculation for both of the cultivars. In case of cv. China, embryo initiation took place after 15 days of subculture in MS having 2.0 mgl^{-1} NAA+0.05 mgl^{-1} BAP. In the same treatment embryo initiation also took place for the cv. Loda after 15 days of subculture. When the calli were subcultured in MS containing 2.0 mgl^{-1} Zeatin+1.0 mgl^{-1} BAP, friable greenish calli were induced within 15 days of subculture and subsequently embryo initiation took place after 15 days of subculture for both of the cultivars. Some of the induced somatic embryos were isolated and a microscopic observation was performed (**Plate 3.7 A-F**). In another treatment, 0.05 mgl^{-1} GA_3+1.0 mgl^{-1}BAP was also used for the same purpose. In this case, only callus proliferation was occurred but no embryo initiation was took place within 30 days of subculture (**Plate 3.8 A & D**).

Calli derived from the isolated single cells gave best morphogenic responses on MS containing 2.0 mgl^{-1} Zeatin +1.0 mgl^{-1}BAP. In case of cv. China, the number of embryos/callus were found to be 20 for cotyledon and 19 for midrib within 15 days of subculture and after 30 days shoot regeneration were 90% and 85%, respectively (**Plate 3.8 B & C**). In case of cv. Loda, the number of embryos/callus were found to be 18 for cotyledon and 20 for midrib within 15 days of subculture and after 30 days shoot regeneration were 90% and 80%, respectively (**Plate 3.8 E & F**).

PLATE 3.6

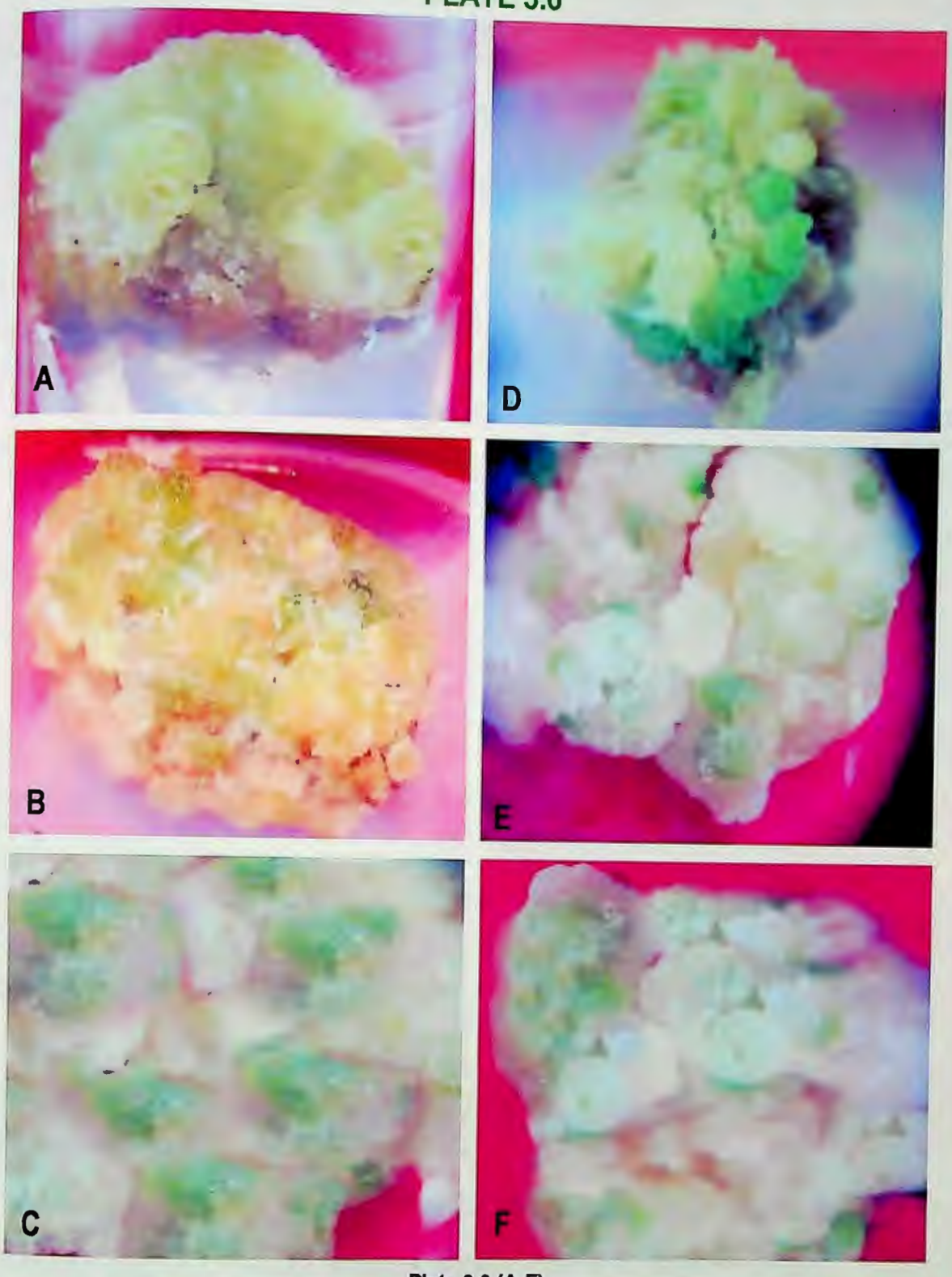

Plate 3.6 (A-F)

Subculture of single cell derived callus on MS agarified media:

A. Greenish callus proliferation in cotyledon derived callus of cv. China on 2.0 mgl^{-1} NAA+0.05 mgl^{-1} BAP.
B. Embryo initiation in cotyledon derived callus of cv. China on 2.0 mgl^{-1} NAA+0.05 mgl^{-1} BAP.
C. Embryo initiation in midrib derived callus of cv. China on 2.0 mgl^{-1} Zeatin + 1.0 mgl^{-1} BAP.
D. Embryo initiation in cotyledon derived callus of cv. Loda on 2.0 mgl^{-1} NAA+0.05 mgl^{-1} BAP.
E-F. Embryo initiation in midrib derived callus of cv. Loda on 2.0 mgl^{-1} Zeatin+1.0 mgl^{-1} BAP.

PLATE 3.7

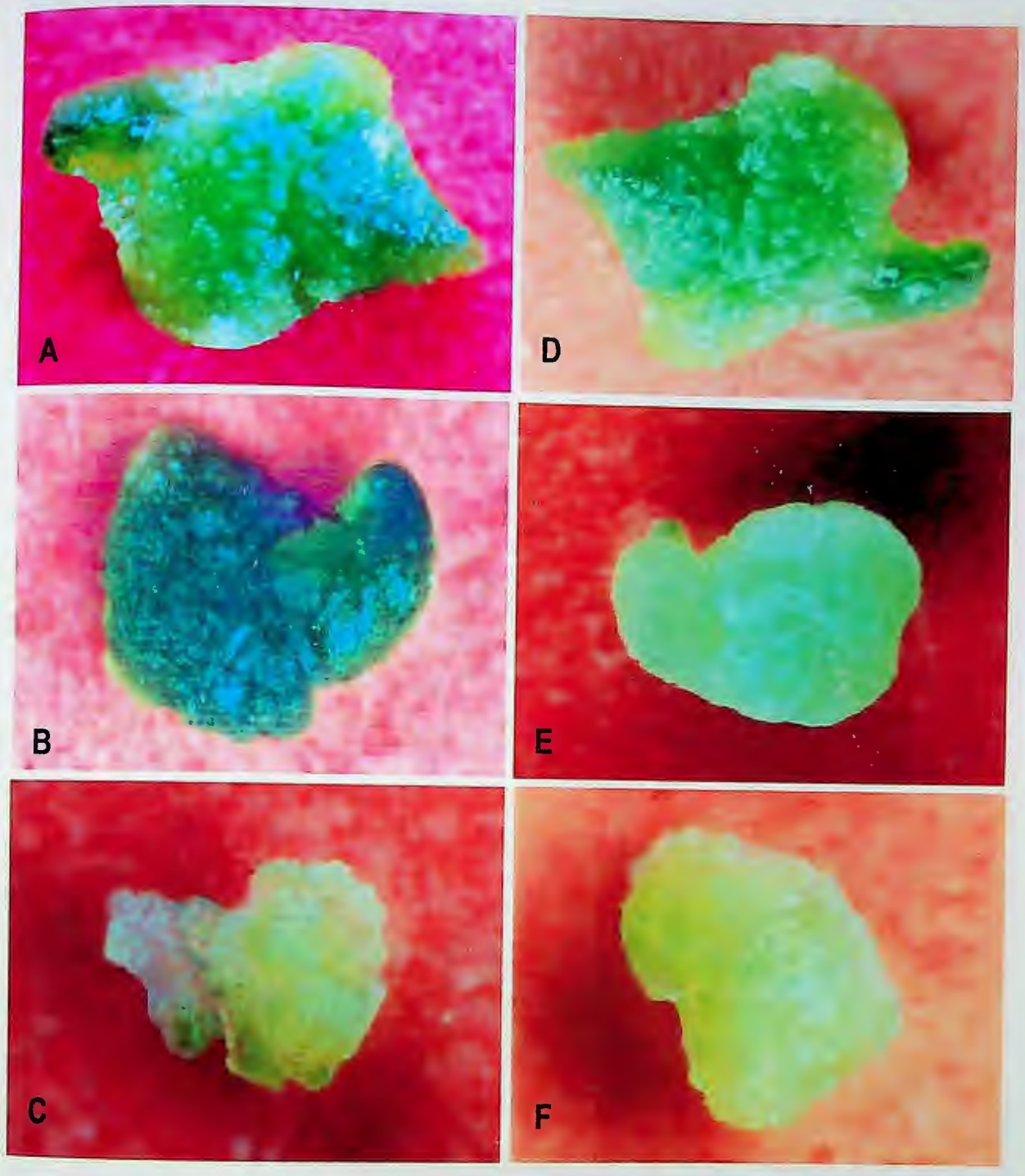

Plate 3.7 (A-F)

Different shapes of somatic embryos isolated from embryogenic callus:

A-B. Somatic embryos isolated from midrib derived callus of cv. China.
C. Somatic embryo isolated from cotyledon derived callus of cv. China.
D. Somatic embryo isolated from midrib derived callus of cv. Loda.
E-F. Somatic embryos isolated from midrib derived callus of cv. Loda.

PLATE 3.8

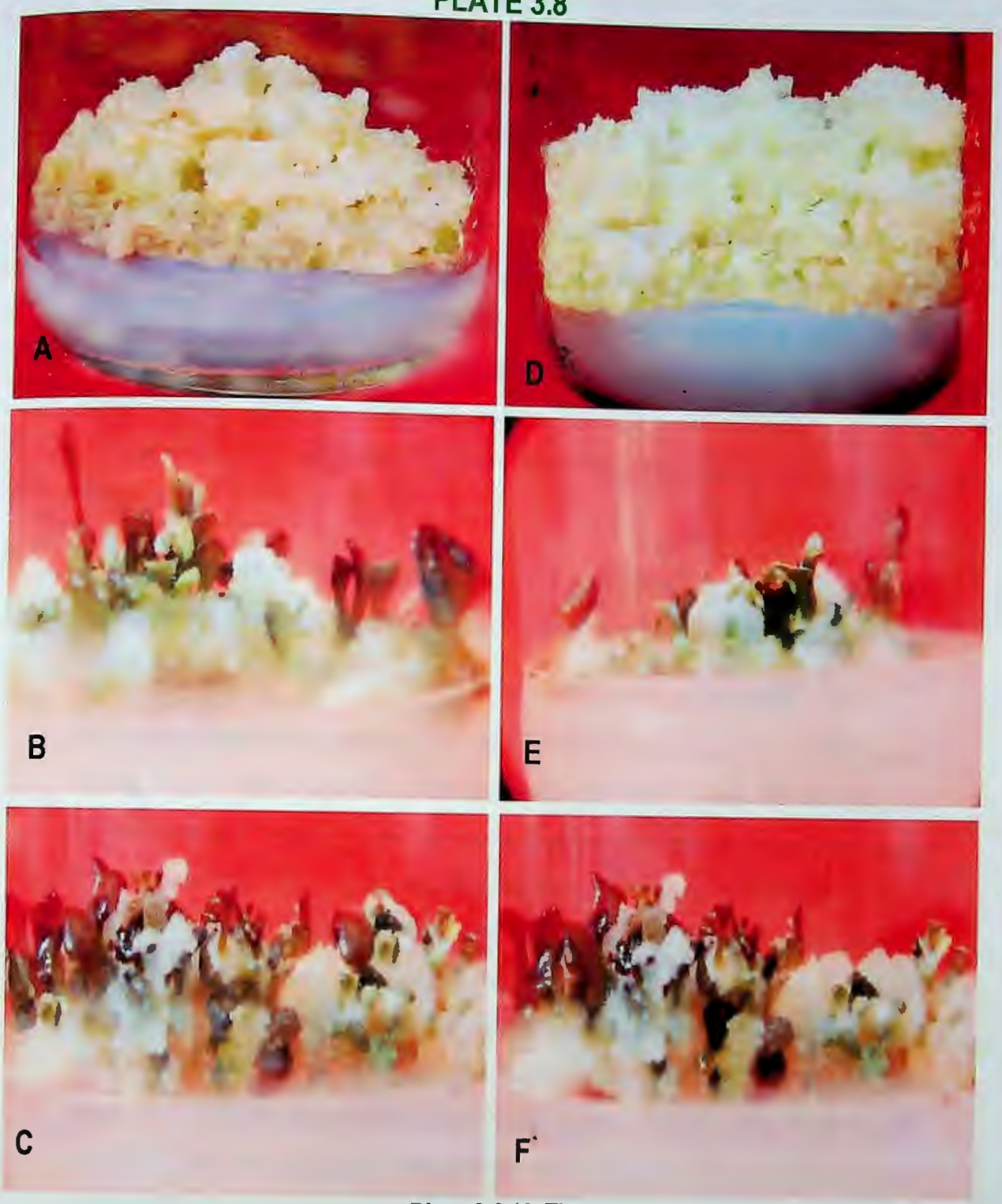

Plate 3.8 (A-F)

Shoot regeneration through somatic embryogenesis after 30 days of subculture:

A. No shoot regeneration occurred in midrib derived callus of cv. China on MS+0.05 mgl^{-1} GA_3+1.0 mgl^{-1}BAP.
B. Shoot regeneration in cotyledon derived callus of cv. Loda on MS+2.0mgl^{-1} Zeatin +0.05mgl^{-1}BAP.
C. Shoot regeneration in midrib derived callus of cv. China on MS+2.0mgl^{-1} Zeatin+0.05mgl^{-1}BAP.
D. No shoot regeneration occurred in midrib derived callus of cv. Loda on MS+0.05 mgl^{-1} GA_3+1.0 mgl^{-1}BAP.
E. Shoot regeneration in cotyledon derived callus of cv. Loda on MS+2.0mgl^{-1} Zeatin +0.05mgl^{-1}BAP.
F. Shoot regeneration in midrib derived callus of cv. Loda on MS+2.0mgl^{-1} Zeatin +0.05mgl^{-1}BAP.

3.3.7. *In vitro* Root Induction in the Regenerated Shoot

In field condition to establish the shoots regenerated from somatic embryogenesis, root induction is a prerequisite. Hence the next phase of this investigation was to unearth a suitable media formulation for optimization of root proliferation. In this investigation somatic embryo regenerated shoots were subcultured onto agarified MS media having different concentrations and combinations of growth regulators. Control medium (MS_0 and $1/2MS_0$) was also tested to ensure the effect of growth regulators on root induction. Four parameters on root induction namely, days to root initiation, root induction frequency (%), number of shoots responded (%) and average length (cm) of the induced roots were considered to find out standard media formulation for root induction (**Table 3.6**).

In case of both of the cultivars, full MS_0 media was found to be more effective than $1/2MS_0$ media for *in vitro* root induction in eggplant and the shoots induced root lately i.e. the required days was 25-35.

Among the different concentrations of IBA, best result was observed in MS medium having 3 mgl^{-1} IBA for both of the cultivars (**Plate 3.9: A-E**). In this concentration, the highest percentage of shoots induced root (%) was same (93.3%) for both of the cultivars and the average length of roots were 4.30 cm for cv. China and 4.93 cm for cv. Loda. Root induction was initiated within 7-16 days of subculture and the number of roots/shoot were 13.9 and 12.6, respectively. Second highest result was observed when 2 mgl^{-1} IBA was used. In this concentration, the percentage of shoots induced roots were 80.0% for cv. China and 83.7% for cv. Loda and average length of the induced roots was respectively 2.90 cm and 3.38 cm. Root induction was initiated within 8-20 days of subculture and the number of roots/shoot were 8.9 and 8.8, respectively. Other treatments of IBA also showed satisfactory result as compared to NAA and IAA. Among various concentrations of IBA, 0.10 mgl^{-1}IBA gave the lowest result for root induction as shown in Table 3.6.

In this investigation three concentrations of NAA (0.1, 0.5 and 1.0 mgl^{-1}) were also treated for root induction. But in all cases of NAA, it failed to induce root for both of the cultivars. For induction of roots on regenerated shoots, 5 different concentrations of IAA were also used in the MS medium. When 2.0 mgl^{-1} IAA was used, the highest percentage of root induction, number of roots/shoot, required time for root induction and average length of root were 73.5%, 8.8, 8-17 days and 2.6 cm, respectively for cv. China and 73.3%, 8.4, 8-19 and 3.23 cm, respectively for cv. Loda. This result was closely followed by the treatment of 3.0 mgl^{-1} IAA as shown in Table 3.6. Medium having 0.1 mgl^{-1}IAA showed the lowest result in respect of all parameters considered for root induction. This experiment showed that IBA is most effective for root induction in eggplant.

Table 3.6: Effect of different concentrations auxin in MS media on root induction in the regenerated shoots through somatic embryogenesis.

Growth regulators (mgl^{-1})	China				Loda			
	Days to Root initiation	Shoots induced roots (%)	No. of root/ shoot	Average length of root in cm	Days to Root initiation	Shoots induced roots (%)	No. of root/ shoot	Average length of root in cm
$1/2MS_0$	-	-	-	-	21-36	40.0	1.9	1.00±0.07
MS_0	25-35	53.3	5.9	1.03±0.20	25-34	62.5	2.4	1.93±0.13
IBA								
0.1	10-28	66.7	4.8	1.85±0.17	9-23	73.3	3.9	2.33±0.17
0.5	10-25	73.3	4.1	2.10±0.18	8-20	80.0	4.1	2.23±0.19
1.0	8-20	76.7	7.1	2.23±0.17	8-21	80.0	7.0	3.03±0.18
2.0	9-20	80.0	8.9	2.90±0.19	8-19	83.7	8.8	3.38±0.22
3.0	**7-16**	**93.3**	**13.9**	**4.30±0.21**	**8-16**	**93.3**	**12.6**	**4.93±0.19**
4.0	8-22	73.3	7.9	3.60±0.12	7-24	80.0	7.5	2.90±0.20
5.0	10-25	70.0	6.9	3.05±0.19	7-20	71.20	7.2	0.95±0.16
NAA								
0.1	-	-	-	-	-	-	-	-
0.5	-	-	-	-	-	-	-	-
1.0	-	-	-	-	-	-	-	-
IAA								
0.1	12-15	53.3	3.5	2.08±0.13	13-18	46.7	3.4	1.98±0.20
0.5	13-20	60.0	6.9	2.13±0.12	12-25	60.0	5.2	2.03±0.17
1.0	9-18	72.8	7.5	2.03±0.15	8-15	71.5	6.5	2.45±0.37
2.0	8-17	73.5	8.8	2.60±0.18	8-19	73.5	8.4	3.23±0.19
3.0	7-20	72.3	7.9	2.13±0.17	7-20	66.7	7.9	3.05±0.19

PLATE 3.9

Plate 3.9 (A-E)

Root induction from somatic embryo derived shoots in MS agar gelled media:

A-C. Root induction in somatic embryo derived shoots of cv. China in MS+3.0mgl^{-1} IBA.

D-E. Root induction in somatic embryo derived shoots of cv. Loda in MS+3.0mgl^{-1} IBA.

PLATE 3.10

Plate 3.10 (A-D)

Root induction from somatic embryo derived shoots in agar gelled MS media:

A-B. Well rooted plantlets of cv. China in MS+3.0mgl^{-1} IBA.

C. Well rooted plantlets of cv. Loda in MS+3.0mgl^{-1} IBA.

D. Fully developed multiple shoot with root induction of cv. Loda in MS+2.0 mgl^{-1} IBA+1.0mgl^{-1}KIN+0.05 mgl^{-1}BAP.

3.3.8. Acclimatization and Transfer of Plantlets to Natural Condition

In vitro regenerated plantlets of 6-10 cm height with few leaves and suitable root system were acclimatized carefully and transferred to the *in vivo* environment. *In vitro* regenerated plantlets showed more than 90% survival under the natural condition. The acclimatized plants grew normally, produced flowers, born fruits and were similar in performance to the mother plants.

3.4. Discussion

Eggplant (*Solanum melongena* L.) is one of the cheapest and important seed propagated crops in Bangladesh. This is the vegetable that conquered the world and about 85% brinjal produce all over the world (Khatun 2004). Eggplant has great adaptability to a very wide range of climatic conditions, high yield and versatility as a food; hence its production is ever increasing.

Callus induction is a prerequisite on the way to generate somaclonal variation. The term callus refers to tissue arising from the unorganized proliferation of cells from segmented plant organs (explants). Callus formed during *in vitro* culture has some similarities to tissue arising *in vitro* morphology, cellular structure, growth and metabolism between callus derived through tissue culture and natural wound callus. It has now been well established that any tissue can be changed into callus, it is cultured on a suitably defined medium under controlled conditions. The history of callus growth from eggplant tissue goes back as early as 1939 when Nobecourt obtained callus proliferation from segmented plant parts. Skoog and Miller (1957) demonstrated hormonal control of differentiation and laid the foundation of clonal propagation of plants through tissue culture techniques. Since then, efforts have been made throughout the world to differentiate eggplant callus. At fast Lam (1975) and Behnke (1995) were able to induce callus in eggplant. An exogenous supply of growth regulators is often recommended to initiate callus from different explants. Exogenous supplies of auxin and often in combination with cytokinin to media are essential for callus induction. Rao and Lee (1968) reported that intermediate levels of auxin and cytokinin usually promote callusing in eggplant.

However, many factors such as genotypes, compositions of the nutrient medium, physical growth factors such as light, temperature, humidity and endogenous supply of growth regulators are important for callus induction (Pierik 1987). For callus culture of dicot plants there are many reports with many plant species. On the contrary, for monocot plants, it has been believed that callus induction is very difficult, because they have no secondary growth, which occurs through the activity of vascular cambium

(Maeda 1980). Auxins are usually required for the induction of callus from a variety of tissue explants, except cambial tissues that can proliferate without an exogenous supply of auxin (Minocha 1987).

In the present investigation MS medium was found to be effective for callus induction and plant regeneration. This is in agreement with that of the findings of Reddy and Reddy (1993), Kartha *et al.* (1981), Narasimhulu and Reddy (1983). In this experiment, different concentrations of auxins and cytokinins were used either singly or in combinations for callus induction using cotyledon and midrib explants from *in vitro* grown plants. It was revealed that *in vitro* callus induction strictly depends on exogenous hormone supplementation. In absence of exogenous hormone, explant failed to induce callus. Two types of explants (cotyledon and midrib) were used from the two cultivars of eggplant. Explants were cultured on MS medium supplemented with different concentrations of 2,4-D, NAA, IAA and in combinations with BAP in order to find out the most suitable culture media formulation to induce maximum callus from cotyledon and midrib of the two cultivars.

Among the three auxins (2,4-D, NAA and IAA), NAA was found to be the best for friable greenish (FG) callus induction and callus growth in the two cultivars of eggplant. Among the seven concentrations of NAA, 2.0mgl^{-1} performed the best for callus induction. In this concentration, highest rate of callusing were found to be 99.00% for cotyledon and 99.73% for midrib recorded in cv. China. In cv. Loda highest rate of callusing were found to be 98.60% for cotyledon and 98.50% for midrib recorded in the same concentration of NAA. Besides NAA, 2,4-D also gave better result for callus induction but the induced callus is spongy-whiter (SW) in nature, which is not favorable for somatic embryogenesis. Another treatment of IAA was also conducted for callus induction for the two cultivars, but its effect on callus induction was not satisfactory. Among the three auxins, NAA was found to be the best for embryogenic callus induction in eggplant when they were used singly. IAA was found to be ineffective for functional callus induction in the two cultivars of eggplant. According to the experimental results, different concentrations and

combinations of auxins with cytokinin (BAP) were also found to induce callus from cotyledon and midrib explants of the two eggplant cultivars.

Three different concentrations of auxins (1.0, 2.0 and 3.0 mgl^{-1}) were used in combination with three different concentrations of BAP (0.05, 0.10 and 0.50 mgl^{-1}) to evaluate the effect of auxins influenced by BAP for callus induction in eggplant. Among the different treatments, 2.0 mgl^{-1}NAA +0.05 mgl^{-1}BAP was found to be the best for callus induction and callus growth in the two cultivars of eggplant. In this concentration, highest rate of callusing were found to be 85.43% for cotyledon and 83.30% for midrib recorded for cv. China. In cv. Loda highest rate of callusing were found to be 82.50% for cotyledon and 84.50% for midrib recorded in the same treatment. In this case, the nature of the calli for both of the cultivars were found as friable-greenish (FG) and the friable-greenish (FG) callus is favorable for somatic embryogenesis. This result on callus induction was closely followed by 2.0 mgl^{-1} 2,4-D+0.05 mgl^{-1}BAP, but the induced callus was spongy-greenish which is not favorable for somatic embryogenesis. This result is in agreement with those reported by Kamat and Rao (1978), but this is contrast with that of findings of Anisuzzaman (1992). The treatment of IAA+BAP was found to be ineffective for functional callus induction in the two cultivars of eggplant. Here high concentration of BAP with auxin was found to be ineffective on callus induction for the two cultivars.

This result was similar to Uddin *et al.* (2004) in the cv. Islampuri of eggplant. Khatun (2004) also found same result when using 1.0 mgl^{-1} NAA + 1.0 mgl^{-1}BAP in potato (a member of the same family, Solanaceae) for callus induction. Similar results were reported by Kathari and Chandra (1984) in African marigold. Therefore in the present investigation, it was clearly focused that MS medium supplemented with 2.0 mgl^{-1} NAA + 0.05 mgl^{-1} BAP was the best media formulation for callus induction in eggplant. Many workers observed NAA as the best auxin for callus induction as common as in dicot and even in monocot (Evans *et al.* 1981, Lu *et al.* 1982, Ho and Vasil 1983, Jaiswal and Narayan 1985 and Chee 1990). However, Mamun *et al.* (1996) reported that NAA was proved to be as less effective when used alone for other plant species. Sultana (2001) used NAA alone for callus induction for internode and leaf explants of potato.

Calli derived from the two explants were subcultured for somatic embryogenesis and shoot regeneration in MS medium with different hormonal composition such as 2,4-D, NAA, GA_3, BAP and Zeatin in different concentrations and combinations. After subculture of callus, shoot regeneration started within 11-15 days of culture. The *in vitro* grown best calli were subcultured onto agar solidified MS media supplemented with different concentrations and combinations of growth regulators to observe somatic embryogenesis and shoot regeneration. Calli derived from the preculture of 2,4-D + BAP were subcultured on semisolid MS media supplemented with 2.0 mgl^{-1}2,4-D+0.05 mgl^{-1}BAP and 0.05 $mgl^{-1}GA_3$ +1.0 mgl^{-1}BAP. In these two treatments of subculture, only callus growth was observed but no embryogenesis or shoot regeneration was took place. Calli harvested from the preculture of NAA + BAP were subcultured onto agar solidified MS media supplemented with 2.0 mgl^{-1}NAA +0.05 mgl^{-1}BAP and 0.05 $mgl^{-1}GA_3$ +1.0 mgl^{-1}BAP. In these two treatments of subculture, callus harvested from the preculture 2.0 mgl^{-1}NAA +0.05 mgl^{-1}BAP and 1.0 mgl^{-1}NAA +0.05 mgl^{-1}BAP showed somatic embryogenesis and shoot regeneration. This finding is in agreement with those reported by Mroginski and Kartha (1981). This result is also in agreement with those reported by Vasil *et al.* (1984).

Wareh *et al.* (1989) found that primary callus from leaf explants of the North American cultivars Viking and Norgold-M formed stolon like structures. In the present investigation, different combinations of cytokinin with auxin were used to see the response of shoot elongation from cotyledon and midrib explants derived callus from the two cultivars (cv. China and cv. Loda) of eggplant. The media with different concentrations and combinations of BAP with NAA, BAP with GA_3 and BAP with Zeatin were found effective for shoot regeneration of two eggplant cultivars for cotyledon and midrib derived callus. Similar experiment for shoot elongation was reported by many earlier researchers (Conover and Litz 1978; Ltiz and Conover 1981).

Among the three combinations used, BAP + Zeatin was proved to be more effective than BAP + NAA and BAP + GA_3 for maximum shoot induction from pre-established callus of eggplant cultivars. Among the different combinations, media fortified with 2.0

mgl^{-1} Zeatin+1.0 mgl^{-1} BAP were found optimum for maximum proliferation of shoots. In this subcultural treatment, best result was obtained for the calli harvested from the preculture of 2.0 mgl^{-1}NAA +0.05 mgl^{-1}BAP. In cv. China shoot regeneration rate were 87.33% for cotyledon and 82.15% for midrib as well as in cv. Loda shoot regeneration rate were 86.50% for cotyledon and 79.88% for midrib. This result is in agreement with those reported by Skoog and Tusi (1948); Miller and Shook (1953); Torrey (1958); Engelke, *et al.* (1973); Narayanswamy (1977); Beck and Coponetti (1983) and Evans, *et al.* (1984). Alam *et al.* (2002) also demonstrated similar result in tomato, a member of Solanaceae. Keiji *et al.* (2002) tested the embyogenesity of the *in vitro* grown calli on MS medium with 1mgl^{-1}IAA and 1mgl^{-1}Zeatin in *Panax ginseng*.

Akhtar (2005) also found the same results in eggplant. Hoque (1996) used BA with NAA for multiple shoots induction and obtained maximum shoots when cytokinin and auxin were used in MS medium in *Chrysanthemum morifolium*. The effect of BA with NAA fortified MS medium on shoot formation has also been demonstrated by Malaure *et al.* (1991) in *Chrysanthemum morifolium*. These results are similar to Nasrin *et al.* (2003). Sultana (2001) used KIN with NAA in MS medium for shoot regeneration from callus in *Solanum tuberosum*. Sah and Park (1986) recorded that KIN with NAA in MS medium also stimulated proliferation and elongation of shoots in garlic (*Allium sativa* L.).

Ratio of cytokinin and auxin seems to play an important role in the morphogenic differentiation of cultured explants (calli), as suggested by Murashige and Skoog (1962), Steward *et al.* (1969), Thomas and Street (1970), Pareek and Chandra (1981), Beck and Coponetti (1983) and Haider (1992). This differential response with regard to morphogenic response of *Solanum* explants may be due to the genotypic differences of the plant material used in the present investigation. Khatun (2004) also consented for morphogenic responses from callus of potato.

In the other experiment, cell culture was carried out in liquid MS medium supplemented with 2.0 mgl^{-1} NAA+0.05 mgl^{-1} BAP to observe the growth of cells of the two cultivars of eggplant. For this purpose, friable greenish (FG) and embryogenic calli containing

rapidly dividing cells were harvested from the pre-established *in vitro* culture in semisolid MS medium having 2.0 mgl^{-1} NAA+0.05 mgl^{-1} BAP. It is prerequisite to prepare liquid media for the isolation of single cells from callus tissue. Conical flasks measuring 250 ml were used for cell suspension culture. Around 1gm of the harvested calli was cultured in conical flask containing 75 ml of MS liquid medium supplemented with 2.0 mgl^{-1} NAA+0.05 mgl^{-1} BAP to initiate cell suspension. Data were recorded on cell weight per 10ml cell suspension after every 2 days of culture. The grand mean weights of cells/10ml suspension were found to be 0.205 gm for cotyledon and 0.198 gm for midrib of the cv. China as well as it was 0.202 gm for cotyledon and 0.214 gm for midrib of cv. Loda. This revealed that the midrib of cv. Loda performed better for cell division than that of cv. China, but cell division performance of cotyledon for both the cultivars was more or less similar. The cells of suspension continued to grow until 13th day of incubation and then the cell division was ceased and the peak period of cell division was found within 5th day to 7th day of culture. The experimental observation revealed that the cells grew gradually in the suspension culture.

Single cells were isolated from the cell suspension and cultured onto agarified MS media having 7 different treatments of NAA+ BAP for callus induction. Among the 7 treatments, 2.0 mgl^{-1} NAA+0.05 mgl^{-1} BAP was found to be best for callus induction from the isolated single cells. Then the single cell derived calli were harvested and subcultured in agarified MS media having required growth regulators for morphological differentiation. It has been pre-established in the previous section (**3.3.3.2.**) that 2.0 mgl^{-1} NAA+0.05 mgl^{-1}BAP and 2.0 mgl^{-1} Zeatin+1.0 mgl^{-1}BAP were the suitable formulation for somatic embryogenesis and shoot regeneration. So these two combinations were used in the MS media to subculture the single cell derived calli. Calli derived from the isolated single cells gave best morphogenic responses on MS containing 2.0 mgl^{-1} Zeatin +1.0 mgl^{-1}BAP. On the other hand, 2.0 mgl^{-1} NAA+0.05 mgl^{-1}BAP also gave satisfactory result for somatic embryogenesis and shoot regeneration. It was observed that the efficiency of somatic embryogenesis from the single cell derived calli was similar to that of explant derived callus

tissue. The regenerated shoots obtained from the somatic embryogenesis of single cell derived callus were subcultured in MS having 3.0mgl^{-1}IBA for root induction.

Makunga *et al.* (2003) conducted an experiment on cell culture using 2,4-D and got similar result in *Thapsia garganica*. Fki *et al.* (2003) used 2,4-D for cell culture in date palm (*Phoenix dactylifera* L.) and obtained similar result for cell growth. Choi *et al.* (2002) also conducted cell culture in Siberian Ginseng plantlets using 2,4-D.

In the present investigations, somatic embryos originated directly from the superficial surface of the callus and the embryos regenerated into shoots. Root initiation and their healthy growth are essential for their successful establishment to the field. For this purpose, the *in vitro* callus derived shoots were subcultured on MS semisolid medium supplemented with different concentrations of IBA, NAA and IAA for further shoot elongation and root induction. Percentage of root formation and mean number of roots per shoot were highly influenced by the concentrations of IBA. Among different concentrations of auxin, 3.0 mgl^{-1} IBA was found to be the best for root induction and its percentage (%) of shoots induced roots was 93.30% for both of the cultivars. This experiment revealed that IAA was less effective and NAA was ineffective for root induction in eggplant. Similar result was also reported by Sarker *et al.* (2002) and they conducted an experiment for root induction in eggplant on half MS or full MS without hormonal supplements or on MS supplemented with 0.1gml^{-1} IBA, 0.1gml^{-1} NAA and 0.1gml^{-1} IAA and they found that about 75% of the shoots produced roots in hormone free MS medium. Efficient effect of IBA on root induction was also observed in Chickpea (Haque *et al.* 1984), grape (Chakravorty 1986) and bitter gourd (Rahman 1998). Rao and Lee (1986) and Mante *et al.* (1989) also reported faster growth of root in *Calotropis gigantean* and *Prunus sp.*, respectively.

After sufficient development of root the plantlets obtained from the two eggplant cultivars were successfully transplanted into small plastic pots containing soil. Following proper acclimatization the plantlets were transferred to the natural condition. The objective of this investigation was to establish an efficient and reproducible m
in vitro plant regeneration. Callus induction and somatic embryogenesis techniq

described in this dissertation, have potential use for clonal propagation of eggplant on commercial use. It would be helpful for eggplant improvement program. Information from this study could be used as an alternative path to induce genetic and epigenetic changes in regenerated plants, which eventually can be used for crop improvement program in eggplant. Moreover, callus induction and its subsequent plant regeneration protocol hold great promise for production of transgenic eggplants of desirable traits by genetic transformation techniques.

3.5. SUMMARY

The objective of the present investigation was to establish a protocol for *in vitro* regeneration through callus induction, cell culture and somatic embryogenesis using cotyledon and midrib explants in eggplant (*Solanum melongena* L.). The present work included two genotypes of eggplant, viz. cv. China and cv. Loda.

For the above purpose, cotyledon pair and midrib were isolated from *in vitro* grown plants. Seven different concentrations of auxins (2,4-D, NAA and IAA) were used singly or in combination with BAP in semisolid MS media for callus induction. Among the different treatments of single auxins, 2.0 mgl^{-1} 2,4-D was proved to be the best formulation for callus induction. In this case, the cv. China gave the highest callus induction frequency 99.05% for cotyledon and 99.73% for midrib; on the contrary, the cv. Loda gave 98.60% for cotyledon and 98.5% for midrib. Among the different treatments of auxins-cytokinin combinations, 2.0 mgl^{-1} NAA + 0.05 mgl^{-1} BAP was proved to be the most suitable medium formulation for callus induction. In this case, the cv. China gave the highest callus induction frequency 85.43% for cotyledon and 83.30% for midrib; on the other hand, cv. Loda gave 82.50% for cotyledon and 84.50% for midrib. Auxin-cytokinin combinations were proved more potent for embryogenic callus (friable-greenish) induction as well as callus growth than that of auxin tried singly in the media. Best calli (friable-greenish) were subcultured in MS media supplemented with different concentrations and combinations of auxins (2,4-D and NAA), GA_3 and cytokinin (BAP and Zeatin). Among the different treatments of subculture, 2.0 mgl^{-1} Zeatin+1.0 mgl^{-1} BAP gave the highest rate (87.33%) of shoot regeneration from cotyledon derived callus harvested from 2.0 mgl^{-1} NAA + 0.05 mgl^{-1} BAP.

For cell culture, best calli harvested from 2.0 mgl^{-1} NAA + 0.05 mgl^{-1} BAP were subcultured in liquid MS medium supplemented with the same hormonal formulation (2.0 mgl^{-1} NAA +0.05 mgl^{-1} BAP) and kept on orbitary shaker (200 rpm). The cell culture performed highest 0.214 gm cells (per 10ml cell suspension) for midrib of cv. Loda followed by 0.205 gm cells (per 10ml cell suspension) for cotyledon of cv. China. The

single cells were isolated from the cell suspension and subcultured in liquid MS media supplemented with seven different combinations of NAA + BAP for single cell derived callus induction (keeping without shaking). Among the different combinations of NAA + BAP, 2.0 mgl^{-1} NAA + 0.05 mgl^{-1} BAP was proved to be the best formulation for callus induction from the isolated single cells. The single cell derived callus mass was rescued from the liquid media and subcultured in agarified MS media supplemented with 2.0 mgl^{-1} NAA + 0.05 mgl^{-1} BAP and 2.0 mgl^{-1} Zeatin + 1.0 mgl^{-1} BAP. Between the two treatments, 2.0 mgl^{-1} Zeatin + 1.0 mgl^{-1} BAP was proved to be more suitable formulation for somatic embryogenesis and shoot regeneration.

The somatic embryo regenerated shoots, either through callus culture or cell culture, were subcultured onto semisolid MS media supplemented with different concentrations of IBA, NAA and IAA for root induction, and 3.0 mgl^{-1}IBA was proved to be the best (93.3%) for root induction in eggplant.

CHAPTER FOUR

Artificial Seed Production in Eggplant

CHAPTER IV

4. ARTIFICIAL SEED PRODUCTION IN EGGPLANT

4.1. INTRODUCTION

The eggplant (*Solanum melongena* L.), belonging to the family of Solanaceae, is highly productive and usually finds its place as the "poor man's crop" (Chowdhury 1966), which is an important article of diet consumed in a great variety of ways. It is cultivated as a vegetable throughout the tropics and as a summer annual in the worm subtropics. It is an important vegetable for not only its food value but also its considerable medicinal uses. The eggplant contains considerable food values because its edible portion contains considerable amount of protein, carbohydrate, fat, minerals and vitamins (Bose *et al.* 1993). Kirtikar and Basu (1933) have mentioned its use in "Ayurvedya" as appetizer, aphroisiac, cardiotonic etc. In many developing countries like Bangladesh, population explosion is the main problem. Food and nutritional requirements for the majority of the population in those countries are not adequate yet. The lack is widening both in qualitative and quantitative term. This lack can be shorten by producing this type of crop in a large scale because it is a high productive vegetable and can be cultivated in our natural environment with some care. But damages of eggplant due to insect attack are the burning problem, because there are various types of insect pests among which a fruit fly (*Leucinodes orbonalis*) damaged upto 30% of total yield (Shukla and Upadhyay 2000).

This is why, it is essential to develop insect resistance and high yielding varieties of eggplant to balance the population food requirement. But in this case maintenance of resultant genotype (e.g., hybrid or genetically engineered genotypes) cannot be possible, because most of the resultant genotypes are meiotically unstable. To overcome such type of problem, this research work was undertaken and it would be helpful to maintain the proper genotype of the resultant plant and its preservation.

4.2. MATERIALS AND METHODS

4.2.1. MATERIALS

4.2.1.1. Plant materials

In this investigation, the two local cultivars of eggplant (*Solanum melongena* L.), namely China and Loda were selected as experimental materials. Nodal segments collected from *in vitro* grown seedlings and somatic embryos obtained from *in vitro* somatic embryogenesis were used as explants for artificial seed production.

4.2.1.2. Chemicals

All chemical compounds including micro and macro nutrients, sugar, agar, sodium alginate, $CaCl_2$, $HgCl_2$, alcohol etc., used in the present study were the reagent grade product of Carolina (America) and Fluka (Germany). The vitamins, amino acids and growth regulators were the product of Carollina Biological Supply Company of U.S.A.

4.2.1.3. Other Materials

Culture vessels such as conical flask, test tube, separating funnel, pipette, forceps, cotton, fire box, marker pen, spirit lamp, needle, sharp blade, electronic balance, autoclave, laminar air flow machine etc., were also used in the present experiment.

4.2.2. Method

Sodium alginate beads were produced by encapsulation according to the method of Kinoshita and Satio (1990). The method involved in this investigation can be described under the following heads:

4.2.2.1. Media Preparation

Culture media were prepared following MS media preparation technique and agar was added at the rate of 8 gml^{-1} and then autoclaved for 21 minutes under 121^0C temperature.

Following steps were carried out for the preparation of solution for explants encapsulation:

i) Preparation of 200 MS solution: With the MS nutrients, 200 ml MS media was prepared. Then 6 gm sucrose and required growth regulators in different concentration and combination were added to 150 ml MS solution out of the 200 ml and mixed well. The final volume was made upto 200 ml by further addition of MS solution.

ii) Alginate solution: 20 ml of the above mentioned MS solution was taken in a small beaker (50 ml beaker) and 0.8gm (800mg) sodium alginate was added to it. With a small glass-rod (16cm) efforts were made to mix the alginate in solution. Alginate was partially dissolved and it was then kept aside. During autoclaving alginate was completely dissolved.

iii) $CaCl_2$ solution: 50 ml of the above mentioned solution (200 ml MS) was taken in a small beaker. 0.7gm (70 mg) $CaCl_2$ was added to it and dissolved. It to be mentioned here that out of 200 ml, 70ml (50+20) was used during the preparation of alginate and $CaCl_2$ solution. Another 130 ml was remained as reserved. After autoclaving it was used during washing the encapsulated beads. These prepared solutions were autoclaved at 15 lb/sq inch's pressure and at 120°C-121°C temperature for 21 minutes for sterilization.

4.2.2.2. Encapsulation of Explants

The nodal segments with active buds and somatic embryos were collected from *in vitro* cultured sources. Though the procedures of encapsulating both of the explants are more or less similar, they are stated in separate heads.

i) Nodal segments: The nodal segments with active buds were collected from *in vitro* grown seedlings of 21-25 days old and taken into a beaker containing alginate solution. The nodal segments were dipped into alginate solution. The explants were kept in the alginate solution for about 30 minutes. Then the rolling explant were taken out by a forcep and dropped into $CaCl_2$ solution of another beaker. During picking up the explants, some addition alginate solution together/along with the explant was also took by the forcep. Then the nodal segment became a hardball encoating with alginate. The dropping process was repeated for each of the nodal segments to encapsulate it producing artificial seeds. After 30 minutes in $CaCl_2$ solution, each explant became a hardball encoated by alginate.

ii) **Somatic embryo:** Somatic embryos formed within 7-28 days of *in vitro* callus culture were used as explants for constructing synthetic/artificial seed. The somatic embryos were isolated aseptically by a sterile forcep. The alginate solution was taken into a beaker and the isolated somatic embryos were dipped into the alginate solution. The somatic embryos were kept in the alginate solution for about 30 minutes. With the aid of a forcep, a single embryo was taken out with some alginate solution from the dipping somatic embryos and was dropped into a beaker containing $CaCl_2$ solution. Then the embryo became a hardball encoating with alginate. The dropping process was repeated for each embryo to encapsulate it producing artificial seeds.

4.2.2.3. Inoculation of Encapsulated Explants

The encapsulated explants or synthetic seeds were washed with autoclaved MS liquid medium (remaining 130 ml above mentioned medium). After washing the synthetic seed were inoculated in conical flask containing appropriate culture media. Normally MS_0 medium was used for inoculation of the artificial seed.

4.2.2.4. Storage of Artificial Seed

To evaluate the storage influence of the artificial seeds, the produced seeds were kept into a sterilized glass vessel and it was sealed with alluminium foil. Then the glass vessel containing the artificial seed was stored at 4^0c and 0^0c temperature in the refrigerator for varying period of 15, 30, 45, 60, 90 and 120 days. To determine the viability of artificial seeds after storage, seeds were brought out from refrigerator and cultured on MS_0 media in controlled environment of growth chamber. A regular observation was performed daily to record the response of germination.

4.2.2.5. Culture Incubation

The produced seeds were cultured in conical flask, each of which contained 8-10 artificial seeds. Inoculated glass vessels were incubated in growth chamber providing a special culture environment having 25±2°C under a 16 hours photoperiod (cool-white fluorescent tube supplying). A regular observation was performed daily to record the response of germination.

4.2.3. DATA COLLECTION

To evaluate the artificial seed performances, a regular observation was performed daily to record the response to germination during incubation and data were collected on the following parameters:

4.2.3.1. Germination Period

The required days to seed germination is one of the important factors to evaluate the viability of artificial seeds. After inoculation the culture vessels were checked daily to note the required days within which the inoculated seeds were seed germinated.

4.2.3.2. Percentage of germination

Percentage of germination of synthetic seed was computed applying the following formula:

$$\text{Percentage (\%) of germination} = \frac{\text{No. of germinated seeds}}{\text{Total No.of seeds inoculated}} \times 100$$

4.2.3.3. Shoot Length

Length of artificial seed derived shoots was measured by a centimeter scale in cm. For each of treatments, average length of shoots and its standard error were calculated using the following formula:

$$\text{Average length of shoot } (\bar{X}) = \frac{\text{Total length of shoots}}{\text{No.of shoots}}$$

$$\text{Standard error (SE)} = \frac{SD}{\sqrt{n}}$$

$$\text{Standard deviation (SD)} = \frac{\sqrt{\Sigma x^2 - (\Sigma x)^2 / n}}{n-1}$$

4.3. RESULTS

Different hormonal supplements namely, Auxin (IBA, IAA and NAA) and cytokinin (BAP and KIN) with their various concentrations and combinations were used in artificial seed bead for the evaluation of hormonal effect on artificial seed germination. The encapsulated nodal segments and somatic embryos are shown in **Plate 4.1 (A-D)**. Details of the results obtained from each of the experiments are described under following heads:

4.3.1. Effect of Hormonal Treatment on Artificial Seed Germination

Different concentrations of cytokinin (BAP and KIN), auxin (NAA and IBA) and GA_3 were used singly or in combinations in seed bead to investigate the germination rate of artificial seeds and its subsequent growth. Encapsulated synthetic seeds were cultured on hormone free MS media. Three parameters such as days to germination, germination frequency (%) and shoot length (cm) were recorded. Artificial seed construction and the hormonal effect on their germination are shown in **Plate 4.2(A-F), Plate 4.3 (A-F)** and **Plate 4.4 (A-D)**. The hormonal effect, singly and their combination are described separately as follows:

4.3.1.1. Effect of BAP

To observe the effect of BAP on artificial seed germination, five different concentrations (0.1, 0.5, 1.0, 1.5 and 2.0 mgl^{-1}) were used in artificial seed bead and a controlled treatment (without growth regulator) was also conducted for artificial seed production. No good result for germination of artificial seed was observed in MS_0 and the experiment proved that the growth regulator played an important role in germination. The obtained results have been presented in **Table 4.1**.

When node was used as explant for artificial seed production, best rate of germination was observed in 0.5 mgl^{-1} BAP. In this case, the germination rate were 60% for cv. China and 65% for cv. Loda and its required days to germination were 6-9 and 6-10, respectively. In the same concentration, the highest length of the germinated shoot were 2.20 cm and 2.50 cm, respectively. When somatic embryos were used as explant for artificial seed production, best result was observed in 1.0 mgl^{-1} BAP and the highest regeneration frequency were 50% for cv. China and 70% for cv. Loda and its required days to

germination were 7-14 and 6-10, respectively. In the same concentration, the highest length of the germinated shoots were 1.03 cm and 1.88 cm, respectively. When the concentration of growth regulators was decreased or increased, the germination percentage was also decreased. When low concentration (<1.0 mgl^{-1}) of BAP was used, the germination percentage was decreased and required days to germination was also longer. Only 10% seed of cv. Loda was germinated when somatic embryo containing seeds were cultured on hormone free MS medium and it took 15-20 days for seed germination. When the concentrations of BAP was increased above 1.0 mgl^{-1} the synthetic seeds of node and somatic embryo under went callus formation rather than shoot initiation (**Plate 4.4 A & C**).

4.3.1.2. Effect of BAP with NAA

Since the belter result of synthetic seed germination was observed in 0.5 mgl^{-1} and 1.0 mgl^{-1} of BAP, three concentrations of NAA (0.01, 0.05 and 0.1 mgl^{-1}) were used in combination with the two concentrations of BAP to enhance the germination of the synthetic seeds (**Table-4.1**).

Among the different treatments of BAP and NAA, best result for seed germination was observed in 1.0mgl^{-1}BAP+0.05mgl^{-1}NAA for both of the cultivars. In case of node, the germination rate were 75% for cv. China and 80% for cv. Loda and its required days to germination was 5-10 for both the cultivars. In the same concentration, the highest length of the germinated shoot were 2.50 cm and 2.65 cm, respectively. When somatic embryos were used as explant for artificial seed production, the best result for germination frequency were 55% for cv. China and 60% for cv. Loda and its required days to germination were 5-11 and 5-10, respectively. In the same concentration, the highest length of the germinated shoots were 1.73 cm and 2.03 cm, respectively. This result was closely followed by the treatment of 1.0 mgl^{-1}BAP+0.01 mgl^{-1}NAA. In this case the cv. China showed germination frequency 70% for node and 50% for somatic embryos with required days of germination 5-10 and 7-16, respectively as well as the average length of the germinated shoots were 2.45 cm and 1.65 cm, respectively. In this case the cv. Loda showed germination frequency 75% for node and 55% for somatic embryos with required days of germination 6-11 and 5-12, respectively as well

as the average length of the germinated shoots were 2.65 cm and 2.08 cm, respectively. Among the different treatments, 0.5 mgl^{-1}BAP+0.01 mgl^{-1}NAA showed the lowest result for seed germination. In case of node, the germination rate was 60% for both of the cultivars and its required days to germination was 5-10 and 5-9, respectively. In the same concentration, the average length of the germinated shoot were 1.43 cm for cv China and 1.60 cm for cv. Loda. In case of somatic embryos, the germination frequency were 35% for cv. China and 40% for cv. Loda and its required days to germination were 11-22 and 6-13, respectively. In this case, the average length of the germinated shoots were 1.13cm and 1.48cm, respectively.

4.3.1.3. Effect of BAP with IBA

Three different concentrations of IBA (0.01, 0.05 and 0.1 mgl^{-1}) were used in combination with 0.5 mgl^{-1} and 1.0 mgl^{-1} BAP (**Table 4.1**).

When node was used as explant for artificial seed production, best result of seed germination was observed in 1.0 mgl^{-1}BAP+0.05 mgl^{-1}IBA. The best germination rate were 75% for cv. China and 80% for cv. Loda and its required days to germination were 5-8 and 5-10, respectively. In the same concentration, the highest length of the germinated shoot were 2.13 cm and 2.08cm, respectively. The same treatment also showed the best result for seed germination when somatic embryos were used as explant for artificial seed production and the best frequency were 65% for cv. China and 75% for cv. Loda. Its required days to germination were 6-10 and 6-12, respectively and the highest length of the germinated shoots were 1.88 cm and 2.30 cm, respectively. The second highest result for seed germination was obtained in the treatment of 1.0 mgl^{-1}BAP+0.01 mgl^{-1}IBA. In this case the cv. China showed germination frequency 75% for node and 55% for somatic embryos with required days of germination 5-10 and 9-17, respectively as well as the average length of the germinated shoots were 2.08cm and 1.55 cm, respectively. In this case the cv. Loda showed germination frequency 75% for node and 70% for somatic embryos with required days of germination 5-10 and 5-13, respectively as well as the average length of the germinated shoots were 2.0cm for both of the explants. Among the different treatments, 1.0mgl^{-1}BAP+0.10mgl^{-1}IBA showed the

lowest result for seed germination. In this case, the lowest germination rate obtained in cv. China were 55% for node and 40% for somatic embryos with required days of germination 5-9 and 6-11, respectively as well as the average length of the germinated shoots were 1.70cm and 1.85cm, respectively. In this case the cv. Loda showed lowest germination frequency 60% for both of the explants with required days of germination 5-10 for node and 5-12 for somatic embryos as well as the average length of the germinated shoots were 1.93cm and 2.0cm, respectively. When the concentration of growth regulators was decreased or increased, the germination percentage was decreased.

The average effect of BAP singly or in combination with auxin (NAA and IBA) on synthetic seed germination for nodal segments and somatic embryos of the two cultivars are shown in **Fig 4.1**.

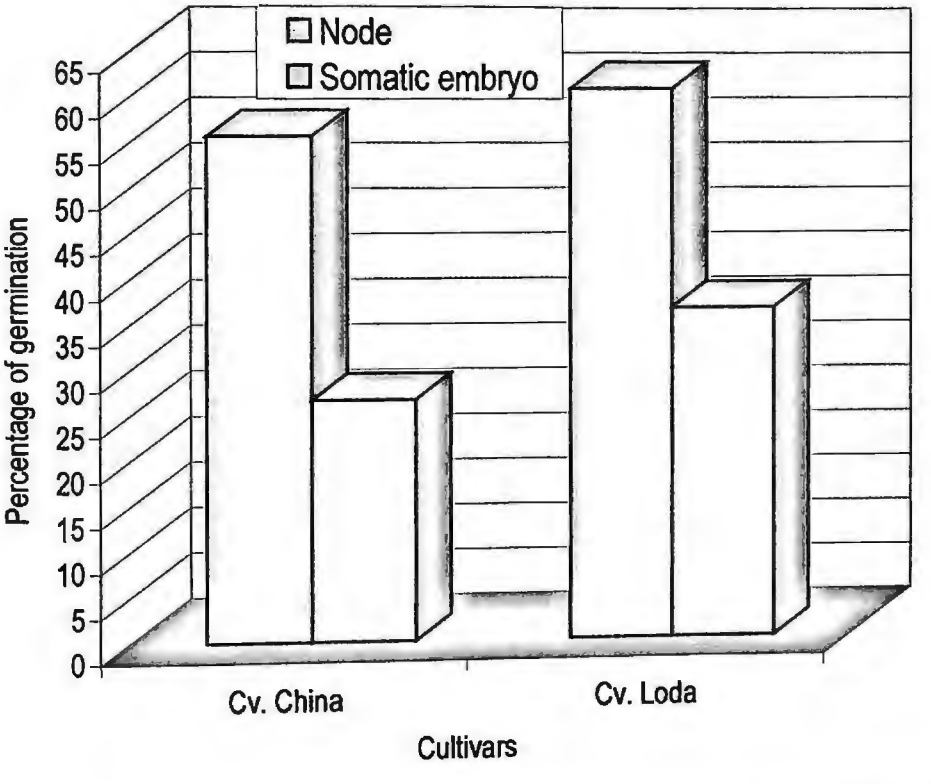

Fig. 4.1: Graph showing the average effect of BAP singly or in combination with auxin (NAA and IBA) on synthetic seed germination of the two cultivars of eggplant.

Table 4.1. Effect of different concentrations of BAP singly or in combination with auxin (NAA and IBA) on synthetic seed germination. Each treatment consisted of 20 explants and data were recorded from 2 day of inoculation to 28 day of culture.

Medium with supplements	China						Loda					
	Node			Somatic embryo			Node			Somatic embryo		
	Days to germination	% of germination	Shoot length in cm (M±SE)	Days to germination	% of germination	Shoot length in cm (M±SE)	Days to germination	% of germination	Shoot length in cm (M±SE)	Days to germination	% of germination	Shoot length in cm (M±SE)
MS_0	-	-	-	-	-	-	-	-	-	15-20	10	0.68±0.09
BAP												
0.10	5-12	50	0.98±0.14	15-20	5	0.63±0.13	7-11	55	1.08±0.14	8-15	10	1.00±0.11
0.50	**6-9**	**60**	**2.20±0.29**	**10-21**	**45**	**1.03±0.17**	**6-10**	**65**	**2.50±0.21**	**6-11**	**60**	**1.35±0.21**
1.00	5-11	55	1.55±0.19	7-14	50	1.53±0.24	6-10	55	1.88±0.05	6-10	70	1.68±0.13
1.50	-	-	-	-	-	-	-	-	-	-	-	-
2.00	-	-	-	-	-	-	-	-	-	-	-	-
BAP+NAA												
0.5+0.01	5-10	65	1.43±0.25	11-22	35	1.13±0.36	5-9	60	1.60±0.15	6-13	40	1.48±0.23
0.50+0.05	7-11	60	2.13±0.27	9-22	40	1.48±0.37	5-10	65	2.28±0.13	5-12	60	1.60±0.21
0.50+0.10	5-13	60	2.13±0.25	8-21	50	1.20±0.25	5-11	65	2.48±0.24	10-23	60	1.55±0.24
1.00+0.01	5-10	70	2.45±0.34	7-16	50	1.65±0.30	6-11	75	2.65±0.25	5-12	55	2.08±0.36
1.00+0.05	**5-10**	**75**	**2.50±0.47**	**5-11**	**55**	**1.73±0.28**	**5-10**	**80**	**2.65±0.22**	**5-10**	**60**	**2.03±0.25**
1.00+0.10	5-12	60	1.95±0.32	12-21	40	1.68±0.32	4-10	60	2.05±0.24	9-17	45	1.83±0.14
BAP+IBA												
0.50+0.01	7-15	65	1.65±0.32	8-17	55	1.43±0.24	7-12	70	1.73±0.38	7-12	65	1.45±0.27
0.50+0.05	7-14	70	1.93±0.36	11-18	60	1.38±0.21	5-10	70	1.88±0.27	6-12	70	1.58±0.34
0.50+0.10	5-13	70	1.83±0.31	12-20	45	1.45±0.25	7-12	75	1.88±0.33	7-11	55	1.53±0.34
1.00+0.01	5-10	75	2.08±0.30	9-17	55	1.55±0.31	5-10	75	2.00±0.39	5-13	70	2.00±0.27
1.00+0.05	**5-8**	**75**	**2.13±0.22**	**6-10**	**65**	**1.88±0.29**	**5-10**	**80**	**2.08±0.32**	**6-12**	**75**	**2.30±0.20**
1.00+0.10	5-9	55	1.70±0.07	6-11	40	1.85±0.27	5-10	60	1.93±0.27	5-12	60	2.00±0.25

PLATE 4.1

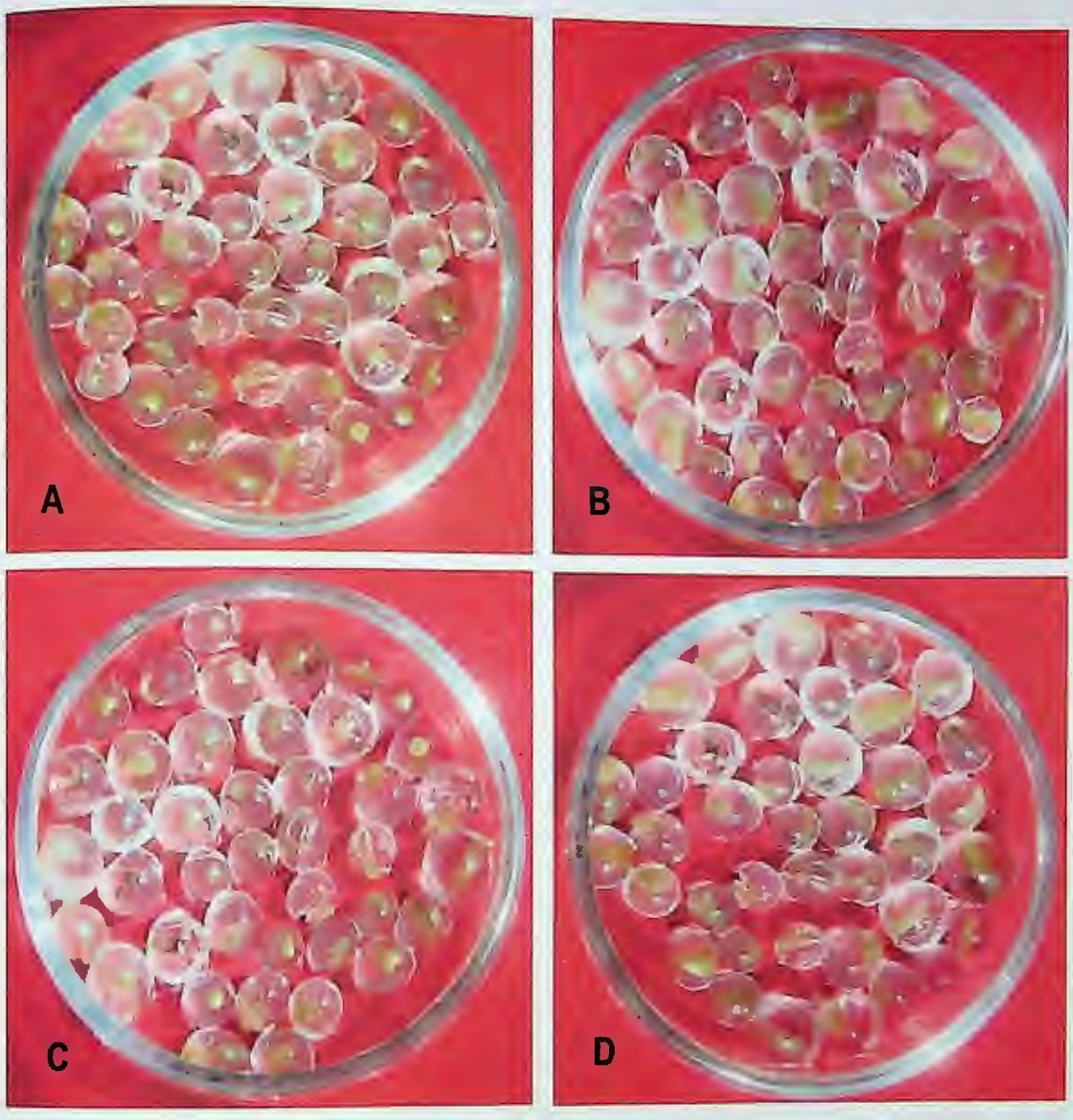

Plate 4.1

Production of artificial seeds by encapsulating the explants with sodium alginate:

A. Encapsulated somatic embryos of cv. China.

B. Encapsulated nodal segments of cv. China.

C. Encapsulated somatic embryos of cv. Loda.

D. Encapsulated nodal segments of cv. Loda.

4.3.1.4. Effect of KIN

Five different concentrations of KIN (0.1, 0.5, 1.0, 1.5 and 2.0 mgl^{-1}) were used in artificial seed bead to investigate the effect of BAP on artificial seed germination and the obtained results have been presented in **Table 4.2.**

When node was used as explant for artificial seed production, best germination frequency was observed in 1.0 mgl^{-1} KIN. In this case, the germination frequency were 60% for cv. China and 65% for cv. Loda and its required days to germination were 7-11 and 6-10, respectively. In this case, the highest length of the germinated shoots were 1.25 cm and 1.35cm, respectively. When somatic embryos were used as explant for artificial seed production, the same concentration showed the best frequency 40% for both of the cultivars and the required days to germination were 8-19 for cv. China and 10-20 for cv. Loda. In this case, the highest length of the germinated shoots were 1.23 cm and 1.10 cm, respectively. This highest result was closely followed by 0.5mgl^{-1} as shown in Table 4.2. When low concentration (<1.0 mgl^{-1}) of KIN was used, the germination percentage was decreased and required days to germination was also longer. The germination percentage was decreased with the decrease of KIN (<1.0mgl^{-1}) and when the concentration of KIN was increased above 1.0 mgl^{-1} the synthetic seeds of node and somatic embryo under went callus formation rather than shoot initiation **(Plate 4.4 B & D).**

4.3.1.5. Effect of KIN with NAA

Among the different concentrations of KIN, 0.5 mgl^{-1} and 1.0 mgl^{-1} gave better performance for seed germination, so three concentrations of NAA (0.01, 0.05 and 0.1 mgl^{-1}) were treated with the two concentrations of KIN to enhance the germination and the results are presented in **Table 4.2.**

Among the different treatments of KIN and NAA, best result for seed germination was observed in 0.50mgl^{-1}KIN+0.10mgl^{-1}NAA for both of the cultivars. In case of node, the germination frequency were 75% for cv. China and 85% for cv. Loda and its required days to germination was 4-11 and 5-10, respectively as well as the highest length of the

germinated shoot were 3.03 cm and 3.10 cm, respectively. In case of somatic embryos, the best result for germination frequency were 40% for cv. China and 55% for cv. Loda and its required days to germination were 9-16 and 10-16, respectively. In this case, the average length of the germinated shoots were 1.40 cm and 1.45 cm, respectively. This result was closely followed by the treatment of 1.0 mgl^{-1}KIN+0.01 mgl^{-1}NAA and in this case the cv. China showed germination frequency 75% for node and 40% for somatic embryos with required days of germination 7-14 and 11-17, respectively as well as the average length of the germinated shoots were 2.68 cm and 1.53 cm, respectively. In this case the cv. Loda showed germination frequency 80% for node and 50% for somatic embryos with required days of germination 5-16 and 9-17, respectively as well as the average length of the germinated shoots were 2.63cm and 1.40cm, respectively. Among the different treatments, 1.0 mgl^{-1}KIN+0.10 mgl^{-1}NAA showed the lowest result for seed germination. In case of node, the germination frequency were 60% for cv. China and 70% for cv. Loda and its required days to germination was 9-19 and 9-18, respectively. In the same concentration, the average length of the germinated shoot were 2.53 cm for cv China and 2.85 cm for cv. Loda. In case of somatic embryos, the germination frequency were 20% for cv. China and 35% for cv. Loda and its required days to germination were 11-25 and 11-22, respectively. In this case, the average length of the germinated shoots were 1.13 cm and 1.33 cm, respectively.

4.3.1.6. Effect of KIN with IBA

Three different concentrations of IBA (0.01, 0.05 and 0.1 mgl^{-1}) were used in combination with 0.5 mgl^{-1} and 1.0 mgl^{-1}KIN (**Table-4.2**).

In case of node explants, best result of seed germination was observed in 1.0mgl^{-1}KIN+0.01mgl^{-1}IBA (**Plate 4.2 C & E** and **Plate 4.3 C & E**). The best germination frequency were 75% for cv. China and 80% for cv. Loda and its required days to germination were 5-10 for both of the cultivars. In this case, the highest length of the germinated shoots were 2.53 cm and 2.65 cm, respectively. The same treatment also showed the best result for seed germination when somatic embryos were used as explant for artificial seed production and the best frequency were 40% for cv. China and

55% for cv. Loda. Its required days to germination were 8-18 and 10-18, respectively and the highest length of the germinated shoots were 1.13 cm and 1.20 cm, respectively. The second highest result for seed germination was obtained in the treatment of 0.5mgl^{-1}KIN+0.10 mgl^{-1}IBA. In this case the cv. China showed germination frequency 70% for node and 35% for somatic embryos with required days of germination 8-12 and 9-15, respectively as well as the average length of the germinated shoots were 1.60cm and 1.33cm, respectively. In this case the cv. Loda showed germination frequency 75% for node and 50% for somatic embryos with required days of germination 8-19 and 10-18, respectively as well as the average length of the germinated shoots were 1.30 cm for both of the explants. Among the different treatments, 1.0mgl^{-1}KIN+0.10mgl^{-1}IBA showed the lowest result for seed germination. In this case, the lowest germination rate obtained in cv. China were 45% for node and 20% for somatic embryos with required days of germination 10-24 and 10-25, respectively as well as the average length of the germinated shoots were 1.35 cm and 0.97 cm, respectively. In this case the cv. Loda showed lowest germination frequency 55% for node and 30% for somatic embryo, with required days of germination 11-24 for node and 11-25 for somatic embryos as well as the average length of the germinated shoots were 1.10 cm for both of the explants.

The average effect of KIN singly or in combination with auxin (NAA and IBA) on synthetic seed germination for nodal segments and somatic embryos of the two cultivars are shown in **Fig 4.2.**

Table 4.2. Effect of different concentrations of KIN singly or in combination with auxin (NAA and IBA) on synthetic seed germination. Each treatment consisted of 20 explants and data were recorded from 2 day of inoculation to 28 day of culture.

Medium with supplements	China						Loda					
	Node			Somatic embryo			Node			Somatic embryo		
	Days to germination	% of germination	Shoot length in CM (M±SE)	Days to germination	% of germination	Shoot length in CM (M±SE)	Days to germination	% of germination	Shoot length in CM (M±SE)	Days to germination	% of germination	Shoot length in CM (M±SE)
KIN												
0.10	8-17	45	1.15±0.13	-	-	-	8-15	50	1.42±0.24	10-25	30	1.15±0.23
0.50	9-17	50	1.18±0.18	15-25	35	0.90±0.04	8-15	55	1.45±0.19	13-22	40	1.22±0.25
1.00	**7-11**	**60**	**1.25±0.10**	**8-19**	**40**	**1.23±0.22**	**6-10**	**65**	**1.35±0.26**	**10-20**	**40**	**1.10±0.13**
1.50	-	-	-	-	-	-	-	-	-	-	-	-
2.00	-	-	-	-	-	-	-	-	-	-	-	-
KIN+NAA												
0.50+0.01	7-21	70	2.50±0.25	10-20	40	1.05±0.25	7-19	75	2.50±0.30	10-19	50	0.98±0.14
0.50+0.05	8-18	70	2.85±0.10	10-20	30	1.60±0.25	8-14	75	3.03±0.37	10-20	40	1.73±0.13
0.50+0.10	**4-11**	75	**3.03±0.30**	**9-16**	**40**	**1.40±0.23**	**5-10**	**85**	**3.10±0.16**	**10-16**	**55**	**1.45±0.26**
1.00+0.01	7-18	75	2.68±0.33	11-17	40	1.53±0.17	5-16	80	2.63±0.19	9-17	50	1.40±0.13
1.00+0.05	8-15	65	2.65±0.25	12-20	25	0.95±0.20	5-12	70	2.78±0.19	10-20	35	1.13±0.16
1.00+0.10	9-19	60	2.53±0.35	11-25	20	1.13±0.20	9-18	70	2.85±0.24	11-22	35	1.33±0.27
KIN+IBA												
0.50+0.01	8-19	65	1.10±0.09	13-23	30	1.20±0.20	8-20	60	1.68±0.11	9-23	40	1.45±0.21
0.50+0.05	8-18	65	1.30±0.21	12-22	30	1.23±0.16	9-16	70	1.40±0.22	9-21	45	1.50±0.25
0.50+0.10	8-12	70	1.60±0.11	9-15	35	1.33±0.21	8-19	75	1.30±0.14	10-18	50	1.30±0.09
1.00+0.01	**5-10**	75	**2.53±0.18**	**8-18**	**40**	**1.13±0.10**	**5-11**	**80**	**2.65±0.22**	**10-18**	**55**	**1.20±0.22**
1.00+0.05	7-15	60	2.40±0.32	10-25	30	0.88±0.19	7-14	75	2.50±0.41	10-21	30	1.10±0.24
1.00+0.10	10-24	45	1.35±0.13	10-25	20	0.97±0.16	11-24	55	1.10±0.25	11-25	30	1.10±0.09

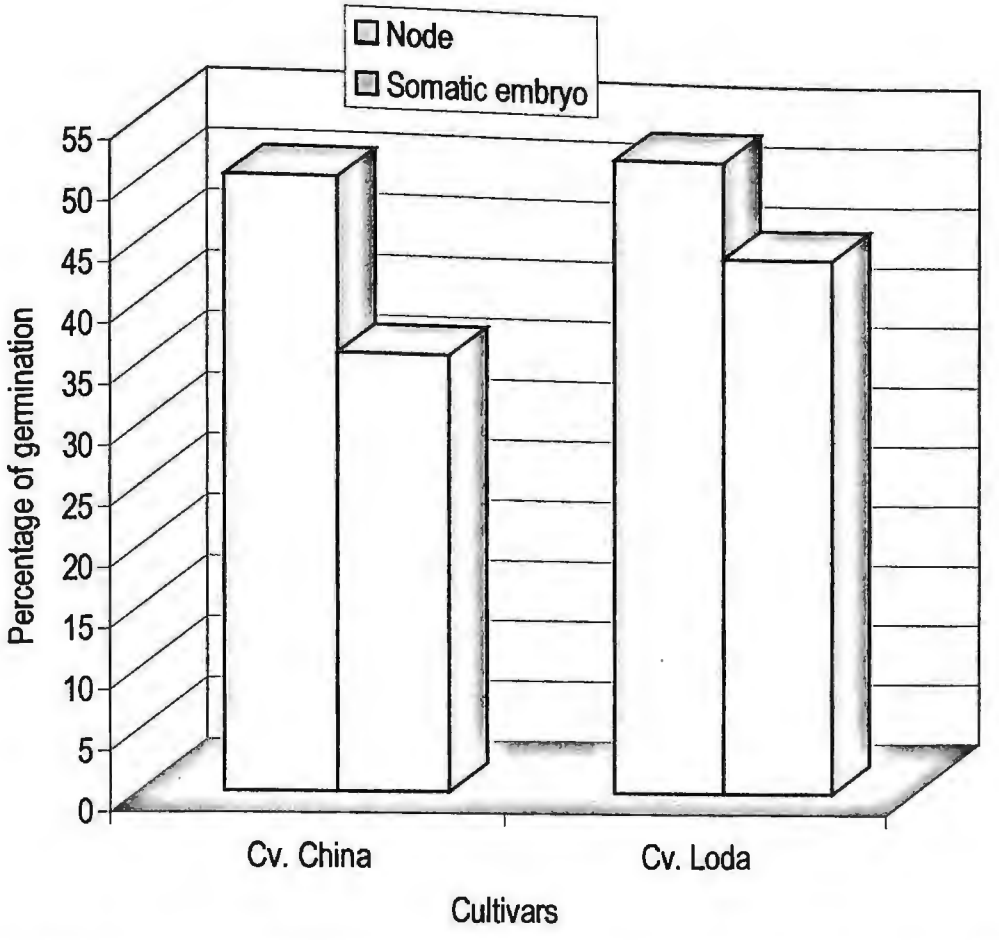

Fig. 4.2: Graph showing the average effect of KIN singly or in combination with auxin (NAA and IBA) on synthetic seed germination of the two cultivars of eggplant.

4.3.1.7. Effect of GA_3 Alone and in Combination with Cytokinin

Three concentrations (0.10, 0.50 and 1.0 mgl^{-1}) were used singly and in combination with BAP (0.5 and 1.0mgl^{-1}) and KIN (1.0mgl^{-1}) for synthetic seed production to observe the effect of GA_3 on synthetic germination (**Table 4.3**).

Among the single treatments of GA_3, 1.0mgl^{-1} gave the best seed germination frequency for both the cultivars. In case of cv. China, the germination rate were 80% for node and 60% for somatic embryo and its required days to germination were 4-8 and 5-10, respectively. In the same concentration, the highest length of the germinated shoots were 2.05 cm and 1.03 cm, respectively. In case of cv. Loda, the germination rate were 85% for node and 65% for somatic embryo and its required days to germination were 4-10 and 5-14, respectively. In

the same concentration, the highest length of the germinated shoots were 2.38 cm and 1.25 cm, respectively. In this investigation, it was observed that GA_3 is the most effective hormonal supplement for synthetic seed germination. When the concentration of GA_3 was increased the germination rate of seeds was also increased.

Among the combined effect of GA_3 with BAP and KIN, 0.1mgl^{-1} GA_3+0.50 mgl^{-1}BAP showed the best result for seed germination for both the cultivars (**Plate 4.2 B & D** and **Plate 4.3 B & D**). In case of cv. China, the best germination frequency were 90% for node and 65% for somatic embryo and its required days to germination were 4-9 and 6-9, respectively. In this concentration, the highest length of the germinated shoots were 2.60 cm and 2.10 cm, respectively. In case of cv. Loda, the germination rate were 90% for node and 70% for somatic embryo and its required days to germination were 4-8 and 6-10, respectively. In the same concentration, the highest length of the germinated shoots were 2.88 cm and 2.53cm, respectively.

Among the combine effects, the second highest result was obtained in 0.10mgl^{-1} GA_3+0.10 mgl^{-1}BAP for both of the cultivars. The cv. China showed 85% for node and 70% for somatic embryo with required days to germination were 4-8 and 5-10, respectively. The average length of the regenerated shoots were1.75 cm and 1.23 cm, respectively.

In case of cv. Loda, the second highest germination frequencies were 85% for node and 65% for somatic embryo with required days to germination 4-9 and 5-10, respectively. The average length of the regenerated shoots were1.75 cm and 1.23 cm, respectively. The average effect of GA_3 singly or in combination with auxin (NAA and IBA) on synthetic seed germination for nodal segments and somatic embryos of the two cultivars are shown in **Fig 4.3**.

Table 4.3. Effect of different concentrations of GA_3 singly or in combination with auxin (NAA and IBA) on synthetic seed germination. Each treatment consisted of 20 explants and data were recorded from 2 day of inoculation to 28 day of culture.

Medium with supplements	China						Loda					
	Node			Somatic embryo			Node			Somatic embryo		
	Days to germination	% of germination	Shoot length in cm (M±SE)	Days to germination	% of germination	Shoot length in cm (M±SE)	Days to germination	% of germination	Shoot length in cm (M±SE)	Days to germination	% of germination	Shoot length in cm (M±SE)
GA_3												
0.1	5-11	60	1.65±0.10	6-11	45	0.88±0.11	5-9	65	1.93±0.10	6-16	45	1.05±0.17
0.5	5-10	75	1.88±0.13	5-10	45	1.00±0.09	5-10	80	2.15±0.14	6-16	50	1.10±0.26
1.0	4-8	80	2.05±0.27	5-10	60	1.03±0.08	4-10	85	2.38±0.18	5-14	65	1.25±0.20
GA_3+BAP												
0.10+0.50	4-9	90	**2.60±0.22**	6-9	65	**2.10±0.15**	4-8	90	**2.88±0.09**	6-10	70	**2.53±0.11**
0.10+1.0	4-9	85	1.75±0.07	5-10	70	1.23±0.06	4-9	85	2.08±0.16	6-10	65	1.43±0.09
GA_3+KIN												
0.10+1.0	4-10	80	2.50±0.15	8-17	55	1.38±0.16	4-9	85	2.58±0.18	7-13	50	1.68±0.16

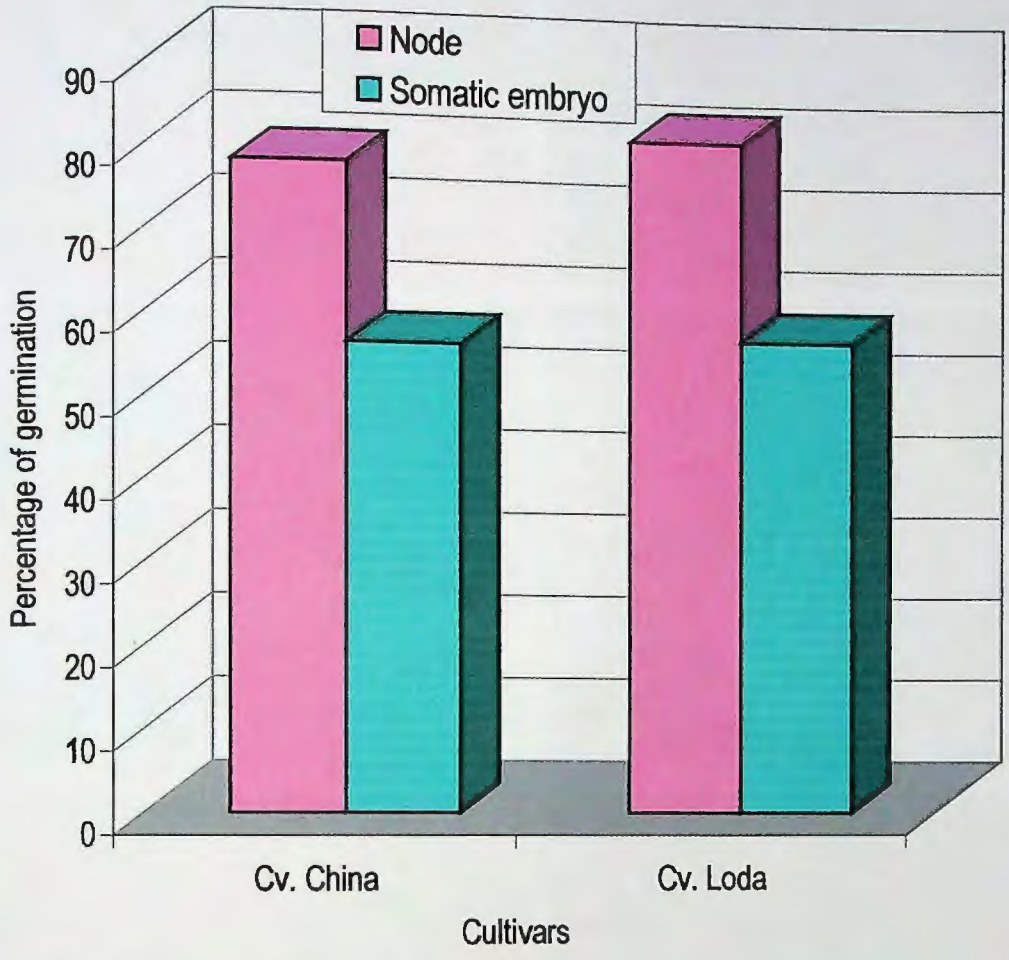

Fig. 4.3: Graph showing the average effect of GA_3 singly or in combination with auxin (NAA and IBA) on synthetic seed germination of the two cultivars of eggplant.

PLATE 4.2

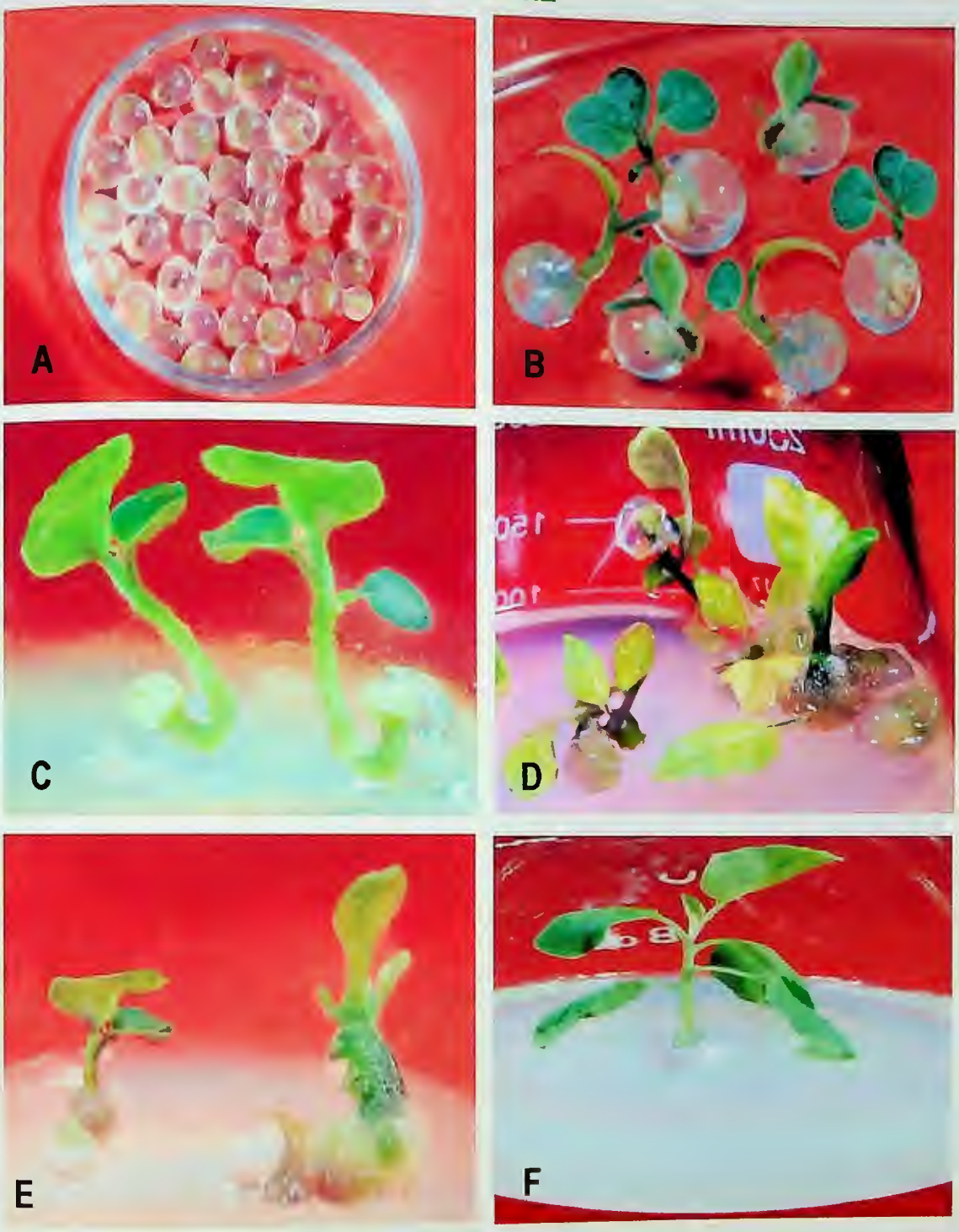

Plate 4.2 (A-F)

Different stages of nodal segments producing artificial seed germination:

A. Artificial seeds of nodal segments encapsulated by sodium alginate.
B. Germinated artificial seeds of cv. China with the treatments of 0.10mgl^{-1} GA_3 + 0.50 mgl^{-1} BAP.
C. Germinated artificial seeds of cv. China with the treatments of 1.0mgl^{-1}KIN + 0.01 mgl^{-1} IBA.
D. Germinated artificial seeds of cv. Loda with the treatments of 0.10 mgl^{-1} GA_3 + 0.50 mgl^{-1}BAP.
E. Germinated artificial seeds of cv. Loda with the treatments of 1.0 mgl^{-1} KIN + 0.01 mgl^{-1}IBA.
F. Artificial seed derived plant of cv. Loda.

PLATE 4.3

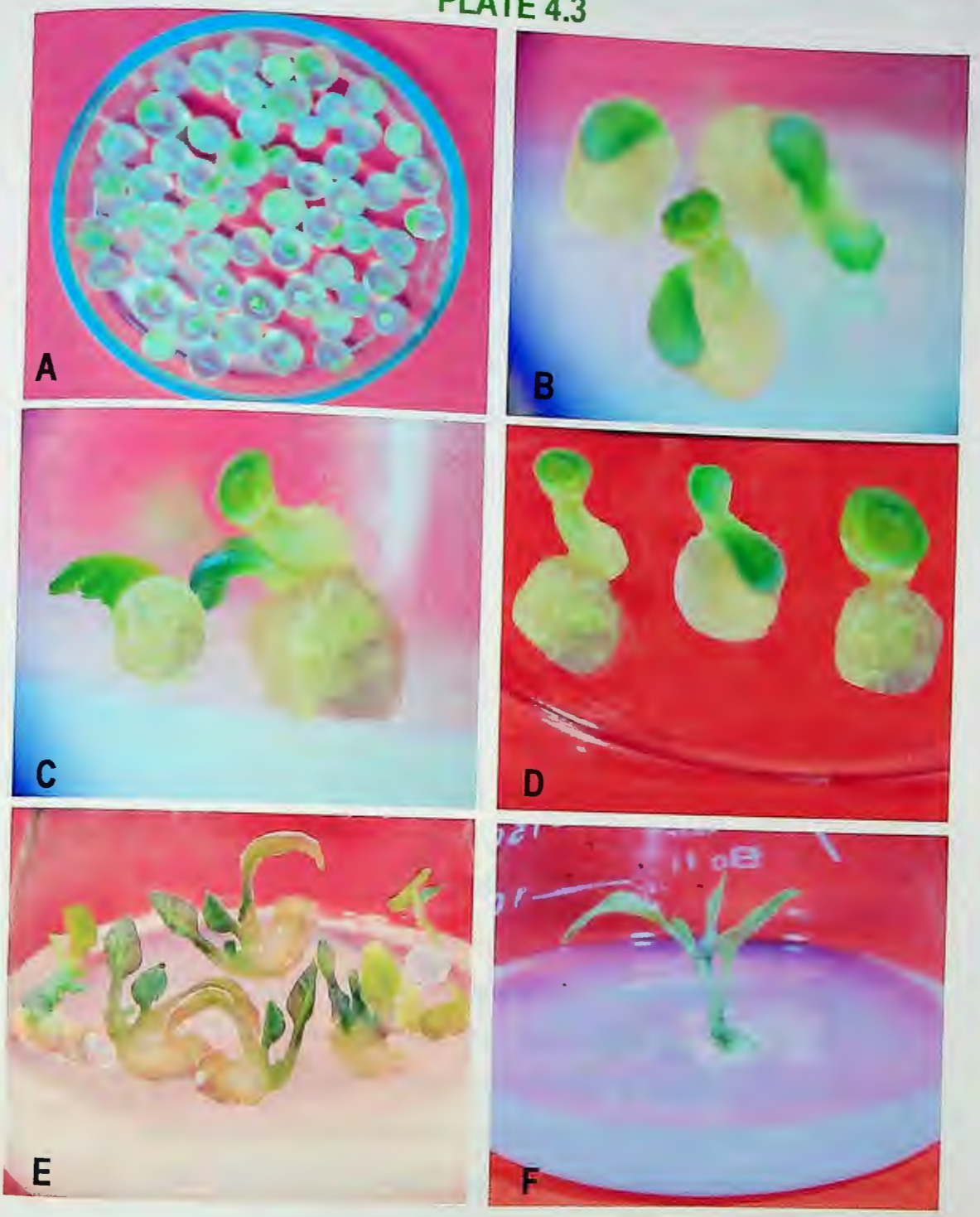

Plate 4.3 (A-F)

Different stages of somatic embryo producing artificial seed germination:

A. Artificial seeds of somatic embryo encapsulated by sodium alginate.

B. Germinated artificial seeds of cv. China with the treatment of 0.10mgl^{-1} GA_3 + 0.50 mgl^{-1}BAP.

C. Germinated artificial seeds of cv. China with the treatment of 1.0mgl^{-1} KIN + 0.01 mgl^{-1} IBA.

D. Germinated artificial seeds of cv. Loda with the treatment of 0.10mgl^{-1}GA_3 + 0.50 mgl^{-1} BAP.

E. Germinated artificial seeds of cv. Loda with the treatment of 1.0mgl^{-1} KIN + 0.01 mgl^{-1}IBA.

F. Artificial seed derived plant of cv. China.

PLATE 4.4

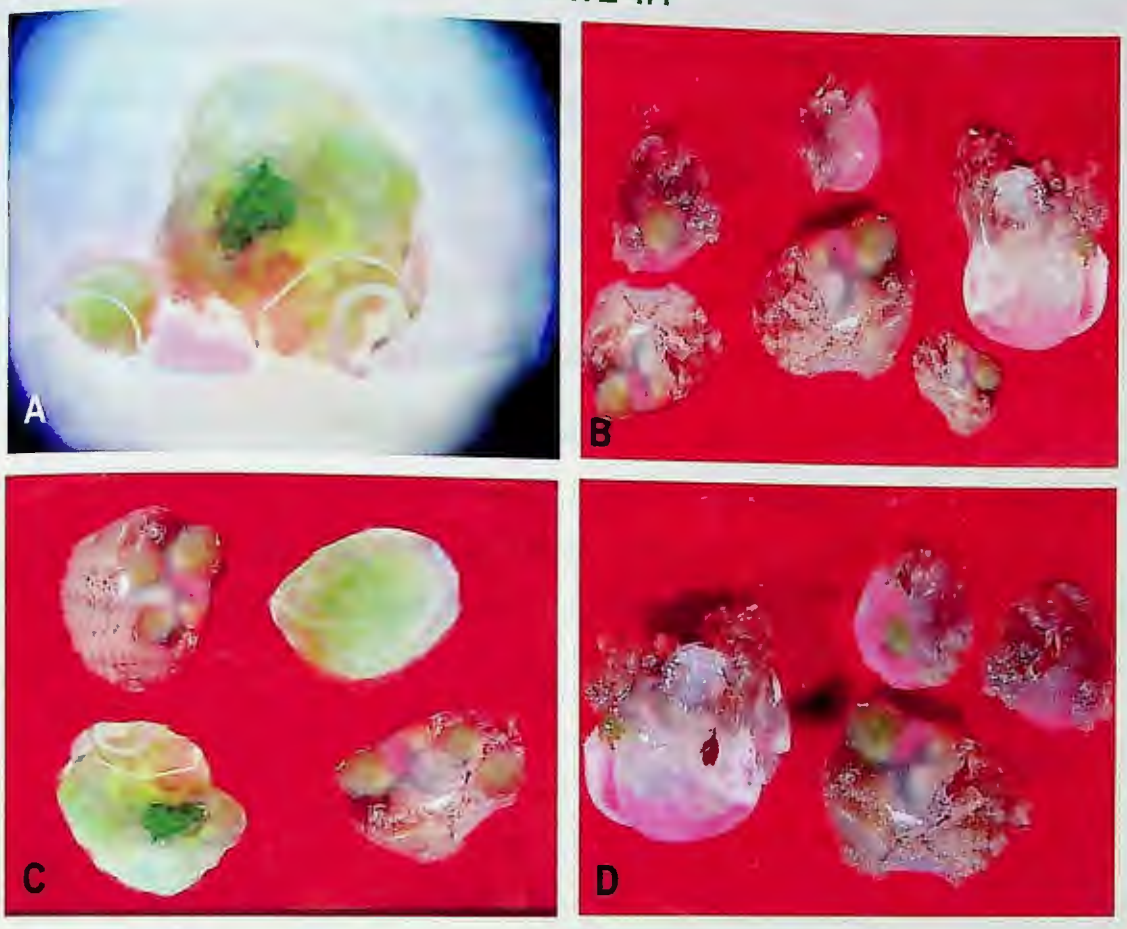

Plate 4.4(A-D)

Adverse case in artificial seed germination:

A. Callus was induced instead of germination in node containing artificial seed of cv. China with the treatment of 1.5mgl^{-1}BAP.

B. Callus was induced instead of germination in somatic embryo containing artificial seed of cv. China with the treatment of 1.5mgl^{-1} KIN.

C. Callus was induced instead of germination in node containing artificial seed of cv. Loda with the treatment of 1.5mgl^{-1}BAP.

D. Callus was induced instead of germination in somatic embryo containing artificial seed of cv. Loda with the treatment of 1.5mgl^{-1} KIN.

4.3.2. Effect of Culture Media

MS (Murashige and Skoog) culture media supplemented with different concentrations of hormone and different levels of carbon source were used to observe the effect of culture media on synthetic seed germination. The results are presented in **Table 4.4.**

In this investigation, it was found that there is no effect of culture media on seed germination. Its effect was observed in the subsequent growth of the germinated plantlets. Among the different culture media, $\frac{1}{2}$MS and hormone free MS media (MS_0) were found best for synthetic seed germination and subsequent development. Low level of carbon source in the media (2% or 1%) showed better results. But when low concentration of GA_3 was used in the culture media, longest shoot length was obtained. But addition of BAP to the media did not show any satisfactory results.

Table 4.4. Effect of different concentrations of culture media (MS) of various concentrations of sucrose with BAP and GA_3 on synthetic seed germination. Each treatment consisted of 20 explants and data were recorded after 30 day of culture.

Strength of MS nutrient	Sucrose conc. (%)	Concentration of GR (mgl^{-1})	Cv. China						Cv. Loda					
			Node			Somatic embryo			Node			Somatic embryo		
			Germination rate (%)	Nature of response	Shoot length in cm (M±SE)	Germination rate (%)	Nature of response	Shoot length in cm (M±SE)	Germination rate (%)	Nature of response	Shoot length in CM (M±SE)	Germination rate (%)	Nature of response	Shoot length in cm (M±SE)
Water	2	BAP 0.5	-	-	-	-	-	-	-	-	-	-	-	-
	0	BAP 0.5	-	-	-	-	-	-	-	-	-	-	-	-
	2	0	-	-	-	-	-	-	-	-	-	-	-	-
1/2MS	0	0	-	-	-	-	-	-	-	-	-	-	-	-
	2	0	90	S	1.78±0.09	85	S	1.50±0.04	90	S	1.85±0.10	90	S	1.55±0.03
	0	BAP 0.5	50	S	2.10±0.19	40	S	1.90±0.08	55	S	2.18±0.17	45	S	1.93±0.08
	3	0	95	S	1.53±0.14	85	S	1.20±0.04	95	S	1.63±0.13	90	S	1.25±0.03
Full MS	2	0	95	S	1.58±0.14	80	S	1.33±0.09	95	S	1.65±0.12	80	S	1.35±0.07
	3	0	90	S	1.53±0.13	80	S	1.10±0.11	95	S	1.60±0.13	85	S	1.13±0.09
	4	0	95	S	1.23±0.06	85	S	1.05±0.09	90	S	1.30±0.09	80	S	1.10±0.11
	3	BAP 0.5	90	S+C	1.98±0.18	90	S+C	1.70±0.09	95	S+C	2.08±0.13	90	S+C	1.73±0.09
	3	BAP 1.0	85	C	0	80	C	0	90	C	0	85	C	0
	3	GA3 0.5	95	S	2.43±0.08	85	S	2.13±0.14	95	S	2.50±0.04	85	S	2.15±0.12
	3	GA3 1.0	90	S	2.80±0.07	85	S	2.30±0.09	90	S	2.88±0.09	80	S	2.33±0.10
	3	GA3 3.0	85	C	0	90	C	0	90	C	0	90	C	0

4.3.3. Effect of Carbon Source on Artificial Seed Germination

To find out the effect of different carbon source on synthetic seed germination, three types carbon sources *viz.* sucrose, mannitol and sorbitol were used singly and in combination (Sucrose+Manitol, Sucrose+Sorbitol and Manitol+Sorbitol) as shown in Table 4.5. In this experiment 0.1mgl^{-1}GA$_3$ + 0.5 mgl^{-1}BAP was used as growth regulator in seed bead for artificial seed construction and hormone free MS media was used for the synthetic seed germination. For this purpose following two experiments were conducted and the results of this experiment have been presented in **Table 4.5**.

4.3.3.1. Effect of Sucrose Level on Artificial Seed Germination

In this experiment, effect of different levels of sucrose (0.5, 1.0, 2.0, 3.0, 4.0 and 5.0%) on artificial seed germination was tested.

It was evident from this experiment that among different sucrose levels treated in MS media, the media having 1% (10 mgl^{-1}) sucrose showed the optimum result for germination of synthetic seed made of nodal segment. In this sucrose level, 90% seeds of cv. China and 95% of cv. Loda were germinated. Second highest result was observed when 2% (20gml^{-1}) sucrose was used. But when the amount of sucrose was increased, the germination rate was decreased. Required days to germination was increased and shoot length was decreased also. In case of somatic embryo, the same sucrose level (1%) also showed the best germination frequency 60% for cv. China and 65% for cv. Loda with the required days to germination 5-12 and 5-10, respectively. Highest shoot length was also observed in this treatment. But when the sucrose level was increased or decreased, the germination rate was decreased.

4.3.3.2. Effect of Different Types of Carbon Source

Among the different carbon sources, when sucrose alone was used in synthetic seed production using nodal segment, best result was observed in 1% sucrose. In case of node, 90% seeds of cv. China were germinated within 5-10 days of culture and 95% seeds of cv. Loda were germinated within 4-10 days of culture. In this case the average shoot length were 2.13 cm and 2.25 cm, respectively. In case of somatic embryo, 60% seeds of cv. China were germinated within 5-12 days of culture and 65% seeds of cv.

Loda were germinated within 5-10 days of culture. In this case the average shoot length were 1.43 cm and 1.58 cm, respectively. This result was closely followed by 2% sucrose and in this concentration, 85% seed of cv. China and 90% seed of cv. Loda were germinated within 6-12 days of culture and the average length of the germinated shoots were 1.68 cm and 2.13 cm, respectively. The germination frequencies were decreased with the increase of sucrose concentrations.

Among the other carbon source, sucrose + mannitol (1:1) showed the best result for seed germination for node explants. When sucrose + mannitol was used as carbon source, 80% seeds of both of the cultivars were germinated within 4-10 days of culture. But in case of somatic embryo, best result for synthetic seed germination was obtained when sucrose + sorbitol (1:1) was used as carbon source. When sucrose + sorbitol was used as carbon source, 65% seeds of cv. China were germinated within 5-12 days of culture and 70% seeds of cv. Loda were germinated within 5-11 days of culture.

Table 4.5. Effect of different types of carbon sources in different concentrations on synthetic seed germination. Each treatment consisted of 20 explants and data were recorded from 2 day of inoculation to 28 day of culture.

Carbon source (%)	Cv. China						Cv. Loda					
	Node			Somatic embryo			Node			Somatic embryo		
	Days to germination	% of germination	Shoot length in cm (M±SE)	Days to germination	% of germination	Shoot length in cm (M±SE)	Days to germination	% of germination	Shoot length in cm (M±SE)	Days to germination	% of germination	Shoot length in cm (M±SE)
Sucrose												
0.50%	7-13	70	2.03±0.09	6-15	45	1.03±0.14	4-10	75	1.80±0.11	7-13	40	1.50±0.15
1%	**5-10**	**90**	**2.13±0.22**	**5-12**	**60**	**1.43±0.22**	**4-10**	**95**	**2.25±0.15**	**5-10**	**65**	**1.58±0.09**
2%	6-12	85	1.68±0.09	6-12	55	1.53±0.13	6-12	90	2.13±0.13	6-18	60	1.73±0.11
3%	4-9	80	1.50±0.16	5-10	45	1.53±0.09	4-8	85	1.88±0.17	5-13	60	1.88±0.09
4%	7-10	65	1.28±0.09	9-20	45	1.18±0.14	5-10	75	1.53±0.13	7-11	65	1.58±0.09
Mannitol (3%)	4-7	60	1.73±0.25	7-14	50	1.53±0.14	4-8	60	1.68±0.05	5-10	65	1.88±0.27
Sorbitol (3%)	4-9	65	1.68±0.32	11-22	50	1.43±0.14	4-9	70	1.80±0.16	4-10	65	2.00±0.18
Suc+Man (1:1)	4-9	80	1.65±0.23	4-11	60	1.50±0.11	4-10	80	1.83±0.13	5-8	65	1.78±0.13
Suc+Sor (1:1)	4-9	75	2.63±0.21	5-12	65	1.53±0.11	4-10	75	2.88±0.17	5-11	70	1.80±0.16
Man+Sor (1:1)	4-10	70	2.80±0.29	9-20	50	1.45±0.13	4-9	70	2.73±0.21	5-10	65	1.80±0.11

4.3.4. Preservation Effect on Artificial Seed Germination

In this experiment, the influence of storage on germination rate of synthetic seed was also investigated. The seeds were kept in a glass vessel aseptically and then stored at 4°C and 0°C temperature for varying time duration (15, 30, 60, 90 and 120 days). The result of this experiment has been presented in the **Table 4.6.**

After 15 days of storage at 4°C, 95% seeds of the both cultivars were germinated and after 30 days, 85% seed of cv. China and 90% seeds of cv, Loda were germinated on the culture media. But after 45 days of storage, the viability of the synthetic seeds was decreased and only 45% seeds of cv. China and 40% seeds of cv. Loda were germinated. When the seeds were cultured after 60 days of storage, no germination was observed for the both cultivars. On the other hand, all of the seeds, stored at 0°C, were failed to germinate.

Table 4.6. Effect of preservation duration on the viability of synthetic seed. Each treatment consisted of 25 explants and data were recorded after 30 day of culture.

Preservation duration (days)	Cv. China		Cv. Loda	
	Germination rate after preservation		Germination rate after preservation	
	At 4°C	At 4°C	At 4°C	At 0°C
15	95	-	95	-
30	90	-	90	-
45	45	-	45	-
60	-	-	-	-
90	-	-	-	-
120	-	-	-	-

4.3.5. *In vitro* Root Induction in Regenerated Shoot

To establish the artificial seed germinated shoots in field condition, root induction is a prerequisite. Therefore, it is necessary to induce satisfactory roots in the regenerated shoots. It has been pre-established in previous section (section 2.3.4) that IBA is the best growth regulators for root induction in eggplant. That is why, the regenerated shoots were cultured in MS media supplemented with 5 different concentrations of IBA

for root induction. Most of the cultures produced healthy root system without any basal callus formation. The obtained results are shown in the **Table 4.7** and **Plate 4.5 (A-D)**.

Among the different concentrations of IBA, 3 mgl^{-1} IBA gave the best results on root induction for both of the cultivars. In this concentration 95% shoots of the both cultivars induced roots within 7-16 day of culture and the highest length of the induced roots were 5.10cm for cv. China and 4.89cm for cv. Loda. In this case, the average number of roots/shoot were 13.3 for cv. China and 12.9 for cv. Loda. The second highest results were 80% for cv. China and 85% for cv. Loda obtained in 2 mgl^{-1} IBA. In this case the required days to root initiation were 9-21 for cv. China and 8-19 for cv. Loda and the average length of the induced roots were 3.35cm and 3.12cm, respectively. Other treatments of IBA also gave satisfactory results for root induction from the synthetic seed germinated shoots.

Table 4.7: Effect of five different concentrations IBA in MS media on root induction in the regenerated shoots from synthetic seed. Each treatment consisted of 15 explants.

Growth regulators, IBA (mgl^{-1})	Cv. China				Cv. Loda			
	Days to Root initiation	Shoots induced roots (%)	No. of root/ shoot ($\bar{x}$)	Average length of root in cm	Days to Root initiation	Shoots induced roots (%)	No. of root/ shoot ($\bar{x}$)	Average length of root (cm)
0.5	10-20	70	4.6	2.22±0.11	8-20	80	4.5	2.18±0.07
1.0	8-20	75	7.7	3.10±0.18	8-21	80	7.4	2.22±0.24
2.0	9-21	80	8.9	3.35±0.20	8-19	85	8.9	3.12±0.21
3.0	**7-15**	**95**	**13.3**	**5.10±0.20**	**8-16**	**95**	**12.9**	**4.89±0.25**
4.0	8-20	70	8.5	3.10±0.22	7-24	80	7.6	3.55±0.15

PLATE 4.5

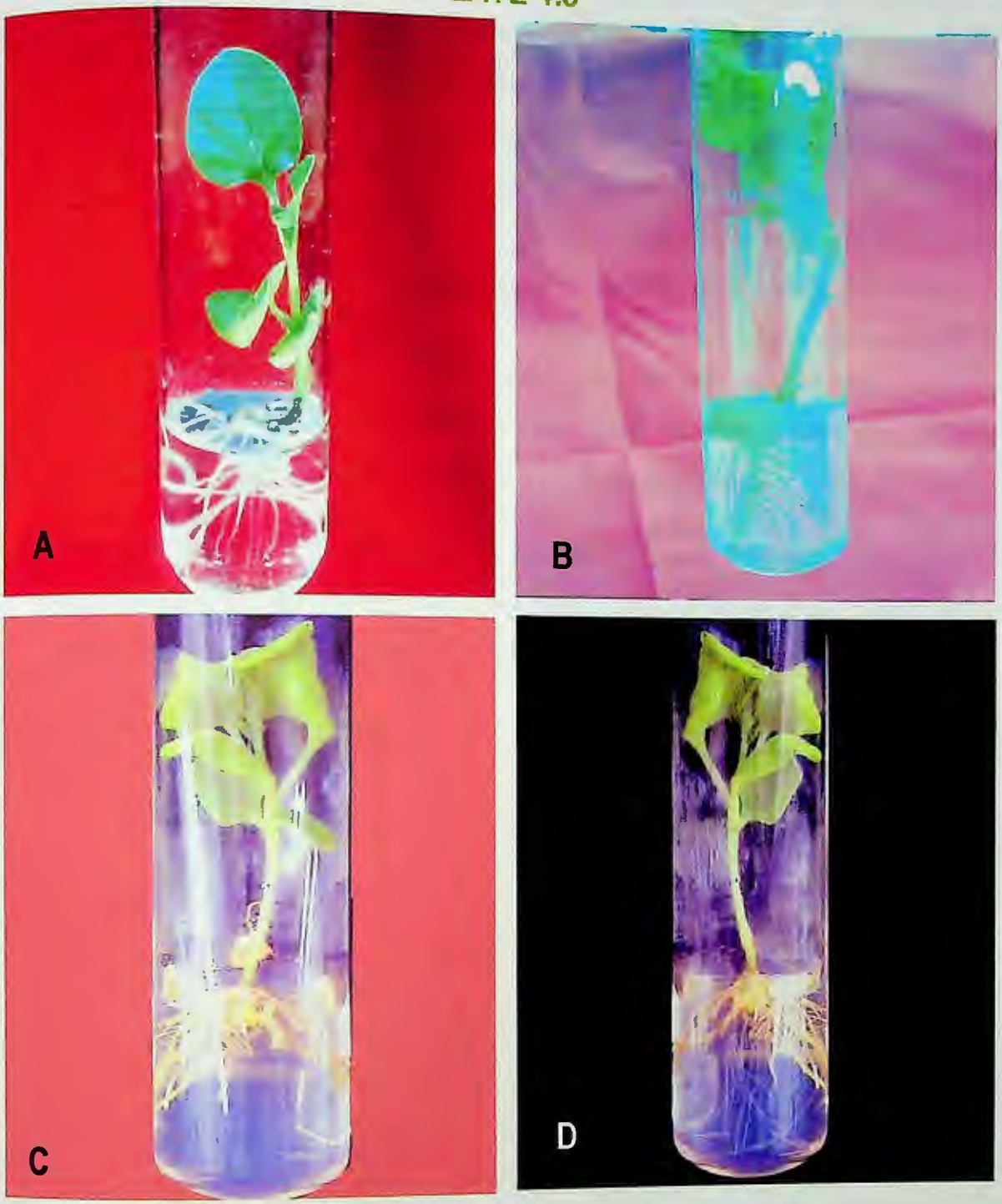

Plate 4.5 (A-D)

Root induction from artificial seed germinated shoots in MS agar gelled media:

A-B. Well rooted plantlets of cv. China in MS +3.0 mgl^{-1} IBA.

C-D. Well rooted plantlets of cv. Loda in MS + 3.0 mgl^{-1} IBA.

4.3.6. Establishment of Regenerated Plantlets to Soil

The synthetic seeds regenerated plantlets of 6-10 cm height with few leaves and suitable root system were initially transplanted in small plastic pots with a view to easy handling during transplantation to the field (**Plate 4.6 A-C**). It was found that more than 90% of the plantlets were survived during initial establishments and resumed new growth. It was observed that the prevailing atmospheric condition (mostly, temperature and humidity) of transplanting season greatly influenced the initial survivable of artificial seed derived potted plants. It was noticed that the plantlets with active growth of primary roots showed greater survival and faster initial growth as compared to the plantlets having longer and branched root system at the time of transplantation.

The performances of the both cultivars were more or less similar. Synthetic seed regenerated plants were healthy and highly vigorous than the source plant stock and no morphological modifications were observed.

PLATE 4.6

Plate 4.6 (A-C)

Acclimatization of the artificial seed regenerated plants:

A. Artificial seed regenerated plantlets of cv. China in poly bag under natural condition.
B. Artificial seed regenerated plantlets of cv. Loda in poly bag under natural condition.
C. Artificial seed regenerated plantlets under natural condition.

4.4. DISCUSSION

Today world's mankind is confronted with a number of gigantic problems in the area of agriculture, horticulture and forestry, which demand urgent attention and immediate solution. These problems are related to food, fuel, fiber and other requirements. The technique of plant cell and tissue culture has become popular and useful method in many countries, which are being applied to solve the problems in agriculture, horticulture and forestry (Rao and Lee, 1986). For this reason, this research programme was undertaken with a view to develop an efficient and reliable method of indirect regeneration through artificial seed production in eggplant. Because the development of tissue culture technique in eggplant was considered important long back. Accordingly attempts were made at times to develop tissue culture technique in this crop plant (Jain *et al.* 1984; Saxena *et al.* 1987; Jain *et al.* 1988; Rao and Singh, 1991 and Mutsubara *et al.* 1992).

In vitro developed somatic embryos can be used for production of artificial seed. Unlike the normal zygotic embryo in a seed, it has no food reserve, but suitable nutrients could be packaged by coating or encapsulating it to form same kind of artificial seed. The embryo is an organ of presentation and control of dormancy is a possibility. An alternative approach would utilize the principal of fluid drilling (Currah *et al.* 1974 and Gray, 1981) to inject plantlets developing from embryos directly in field (Evans *et al.* 1981). The result obtained in this investigation is also supported by Mariani (1992), who produced synthetic seeds using somatic embryo in eggplant.

The artificial seed technology is an exciting and rapidly growing area of research in plant cell and tissue culture and unraveling new vistas in plant biotechnology. The idea of artificial seed was first conceived by Murashige *et al.* (1978), which was subsequently developed by several investigators. Initially, the development of artificial seeds has been restricted to encapsulation of somatic embryos in a protective Jelly. It had been considered that somatic embryogenesis is the pre-requisite for the preparation of artificial seeds. But research on artificial seed has increased significantly, as demonstrated by increased attention from several laboratories, (Kim and Janick, 1987; Redembaugh *et al.* 1988) and by special

symposium at the 22nd International Horticultural Congress held in 1986 (Gray 1987). The encapsulation technique is an important application of micro propagation that improves delivery of *in vitro* derived plants to the field or to green house (Piccioni and Standardi, 1995). Artificial seeds, consisting of somatic embryos enclosed in a protective coating, have been proposed as a low-cost and high volume propagation system for several crop species (Redenbaugh *et al.* 1986).

Most of the studies on encapsulation have been carried out using somatic embryos as the encapsulated propagules (Ara *et al.* 1999). In addition to using somatic embryos, axillary buds, adventitious buds and shoot tips have also been used in the preparation of synthetic seeds. (Redenbaugh, 1993; Bapat and Rao, 1988,1990; Ganapathi *et al.* 1992, 1994). A number of encapsulating agents have been tried out, of which agar, agarose, alginate, carragenan, gelrite and polyacrylamide are important (Kitto *et al.* 1985). However, it has been suggested that most suitable encapsulating agent is sodium alginate (Bapat *et al.* 1987) due to its solubility at room temperature and its ability to form completely permeable gel with calcium chloride ($CaCl_2.2H_2O$). Our findings have revealed that this method provides an efficient mechanism for encapsulating the nodal segment and somatic embryo in eggplants. Effect of the alginate bead on growth immobilization or minimal growth storage may be attributed to a reduction in the respiration process in encapsulated cells (Brodelius 1982). Alginate is one of the most commonly used polymers for immobilization of plant cells and production of manufactured seeds because it is available in large quantities, is inert, non-toxic, cheap and easily handled (Endress 1994). However, studies on *in vitro* germplasm conservation using alginate encapsulation techniques have been reported for only a few species. Embryogenic tissue of *Samtalum album* (Bapat and Rao, 1988) and axillary buds of *Morus indica* (Bapat and Rao 1987) have been encapsulated in alginate beads.

In this investigation isolated nodal segments and somatic embryos were encapsulated in sodium alginate prepared using MS basal media. The MS basal media also used in artificial seed production of pineapple (Soneji *et al.* 2002). Different concentrations and combinations of cytokinin (BAP and KIN) and auxin (NAA and IBA) were used in

alginate bead. Five different concentrations of BAP were used in artificial seed beads. Best result was observed, when 0.5 mgl^{-1}BAP was used in synthetic seed made of nodal segment. In this case, when somatic embryos were used as explant, best result was observed in 1.0 mgl^{-1}BAP. Same concentration of BAP was also used in seed bead of Mulberry (Hiroaki 1992).

But when low concentration (0.01, 0.05 and 0.10 mgl^{-1}) of auxin (NAA and IBA) or GA_3 (0.1 mgl^{-1}) was added to cytokinin (BAP and KIN), germination percentage was increased. When nodal segment were used as explant, best result was observed in 0.5 mgl^{-1} BAP + 0.1 mgl^{-1}GA3 among the different concentrations and combinations. 1.0 mgl^{-1}BAP+0.1 $mgl^{-1}GA_3$ was the best media formation for alginate bead when somatic embryos were used as explant. 1 μ M GA_3 was also used in the treatment of artificial endosperm of *Cleopatra tangerine* zygotic embryos (Nadina *et al.* 1998).

Different type and level of carbon source (sucrose, mannitol, sorbitol) were used in alginate bead. Because a synthetic endosperm will likely be required to provide nutrients including carbon sources necessary for optimum germination and conservation (Redenbaugh *et al.* 1988). Nadina *et al.* (1998) used two types carbon source *viz.* corn starch and sucrose in the alginate seed bead of *Cleopatra tangerine* zygotic embryos. In the present investigation, among the different carbon sources, 1% sucrose showed the best performance when nodal segment was used as explant for synthetic seed germination. Among other carbon sources, when somatic embryos were used as explants sucrose + sorbitol (1:1) was found to be more efficient in germination of the synthetic seeds of eggplant and in case of somatic embryo, sucrose + mannitol (1:1) was found to be more efficient. 2% sucrose also gave satisfactory result for synthetic seed germination. Isao *et al.* (1990) observed in Japanese white birch that beads whose gel matrixes contain high concentration (5%) of sucrose, geminated more slowly. Hiroaki (1992) used 2% (20 mgl^{-1}) fructose as carbon source in artificial seed bead of mulberry and obtained satisfactory results.

The influence of storage at 4°C and 0°C temperature on germination rate was also examined. This investigation indicates that synthetic seed could be stored at 4°C for maximum 45 days without loss of viability. This type of result was also supported by the results got by Bapat and Rao 1988.

Under the present investigation, efforts have been made to develop the protocol for production of artificial seeds with two cultivars of eggplant (*Solanum melongena* L.). This is the first report in our country for production of artificial seeds. It has opened a new area of advance research for developing the conservation strategies for plant genetic resources in our country by the application of biotechnological tools like chilling and cryopreservation.

Regeneration of roots from synthetic seed regenerated shoots with vascular continuity resulted in the development of complete plantlets. Without healthy root system, plantlets cannot be transferred successfully to field conditions. Gautheret (1945) first suggested the importance of auxin for root induction and most of the plants require the presence of auxin for efficient root regeneration. Although in most of the cases regenerated shoots produced root simultaneously, for more investigation about rooting, it was necessary to culture the regenerated shoots in rooting medium for better transplantation. Five different concentrations of IBA were used in MS media for root induction in the regenerated shoots. Among the five concentrations, 3.0 mgl^{-1} IBA was proved to be the best formulation for root induction in eggplant. The best response for root induction was observed in media having 3.0 mgl^{-1}IBA for both of the cultivars.

One of the earliest recognized limitation to practical application of propagation was the high loss of plants following deflasking. Much research has now been performed on understanding the process of acclimatization with various *in vitro* and ex *vitro* (post deflasking) strategies developed (Williams 2002). Preece and Sutter (1991) concluded that it is desirable, the new leaves that form on the plantlets develop on conditions approaching those in which the plant will finally be grown. This assumes a gradual reduction of relative humidity and increase in light. *In vitro* propagated plant is not

transferred readily to an open soil environment. A weanling stage is usually used in which the plants are transplanted from *in vitro* conditions into humid conditions in green house and the humidity is then reduced gradually over three to four weeks to that of the open soil environment. In this present finding different attempts were made to establish plantlets and their survival rate was good in soil condition.

4.5. SUMMARY

The present investigation was carried out to standardize the protocol for production of artificial seed and subsequent plant regeneration in eggplant (*Solanum melongena* L.). For this purpose somatic embryos and nodal segments, obtained from *in vitro* source, were encapsulated with sodium alginate solution (4%) followed by subsequent immersion in calcium chloride solution (1.4%).

Different concentrations and combinations of NAA, IBA, GA_3, BAP and KIN were treated in alginate bead to investigate the hormonal effect on the artificial seed germination as well as hormone free MS media were used to inoculate the seeds. Among the treatments applied in alginate bead, 0.10 mgl^{-1} GA_3 + 0.50 mgl^{-1} BAP performed the best for germination and highest germination rate was 90% for nodal segment of both cultivars as well as 65% for somatic embryo of cv. China and 70% for nodal segments of cv. Loda. This result was closely followed by 0.10 mgl^{-1} GA_3 + 0.10 mgl^{-1} BAP. Different types of carbon sources (sucrose, mannitol, sorbitol) and their combinations were treated in seed bead to observe their effect on synthetic seed germination. Sucrose was found to be the best carbon source and 1% sucrose performed the best for synthetic seed germination (90-95% for node and 60-65% for somatic embryo). Among the different combinations of carbon sources, sucrose + mannitol (1:1) showed the best result for germination i.e. 80% for node and 60-65% for somatic embryo. The artificial seed regenerated shoots were cultured in MS media supplemented with 5 different concentrations of IBA for root induction and 3.0 mgl^{-1} IBA was proved to be the best concentration for root induction (95% for both the cultivars). The rooted plantlets were gradually acclimatized and successfully established to the field condition.

It would be concluded that artificial seed having 0.10 mgl^{-1} GA_3 + 0.50 mgl^{-1} BAP in the bead could be inoculated in MS medium with the hormonal supplement of 3.0 mgl^{-1} IBA for synthetic seed germination with profuse root induction in eggplant.

CHAPTER FIVE

References

CHAPTER V

5. REFERENCES

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