Chapter 1

INTRODUCTION
About 212.5 million plants including 157 million ornamental plants amounting to 78% of the total production have been recorded so far (Pierik, 1991a, b). As many as 156 ornamental genera are propagated through tissue culture in different commercial laboratories worldwide. Among the major producers are The Netherlands (33%), Japan (24%), Italy (11%), USA (12%), Thailand (10%) and others (14%). The major exporting countries are The Netherlands (59%), Colombia (10%), Italy (16%), Israel (4%), Spain (2%), Kenya (1%) and others (18%). The four leading exporters (The Netherlands, Colombia, Italy and Israel) constitute about 80% of the world market. The share of the developing countries of Africa, Asia and Latin America is less than 20% (Rajagopalan, 2000; Schiva, 2000). Planting material of ornamental plants is in great demand for commercial production as well as for domestic gardens and landscaping. The better quality planting material is a basic need of growers for boosting productivity.

*In vitro* culture is one of the key tools of plant biotechnology that exploits the totipotency nature of plant cells, a concept proposed by Haberlandt (1902) and unequivocally demonstrated, for the first time, by Steward et al. (1958). Tissue culture is alternatively called the cell, tissue and organ culture through *in vitro* condition (Debergh and Read, 1991). It can be employed for large-scale propagation of disease free clones and gene pool conservation. Ornamental industry has applied *in vitro* propagation approach immensely for large-scale plant multiplication of elite superior varieties.

Commercial application of tissue culture began in the USA with micropropagation of a number of herbaceous medicinal or ornamental plants. Commercial approach of micropropagation of ornamental plants has set up multibillion-dollar industry in recent years in several developed countries. The rapid *in vitro* clonal propagation of many ornamental plants like orchids, carnation, lilies, roses, and several leafy ornamentals is one of the major successes of commercial application of plant tissue culture. It is practised in thousands of nurseries and biotechnological laboratories throughout the world.
Application of plant tissue culture

In recent years, plant, cell and tissue culture technology has made tremendous progress in plant regeneration from a wide range of plants such as cereals, ornamentals, fruits and forest trees. There are many techniques available for the conservation of plant genetic resources of rare and endangered species. These include micropropagation, seed germination, regeneration via callus, embryo rescue, micrografting and cryopreservation (Nitzsche, 1983, Rick, 1984; Stanilova et al., 1994). Micropropagation of bulb plants is an alternative to the conventional methods for vegetative propagation. It increases the multiplication level many times (Novak and Petru, 1981; Van Aartijk and Blom-Barnhoorn, 1981; Takayama and Misawa, 1982; Takayama and Misawa, 1983; Van Aartijk et al., 1990; Wickremesinhe et al., 1994) and enables materials to remain free from viruses or similar other diseases.

Of the various methods used to micropropagate plants, somatic embryogenesis and enhanced axillary branching, have emerged as the principal method of multiplication (Junaid et al., 2004; Andrade et al., 2005). Long-term benefits of this enterprise lie in the production of clonally uniform plants. Nearly all the important genera have been successfully multiplied through in vitro methods. Beside mass propagation of elite plants, applications of plant tissue culture are possible in the following areas:

- Production of pharmaceuticals and other natural products.
- The genetic improvement of crops.
- The recovery of disease free clones and preservation of valuable germplasm and
- Rapid clonal multiplication of selected F₁ varieties.

Role of tissue culture in floriculture industry

Floriculture has changed from a backyard, farm-oriented activity to a market-oriented activity. Increasing demand of flower crops and increased awareness make many innovative and progressive growers and entrepreneurs to take floriculture as a commercial enterprise. At present, floriculture has emerged as a valuable agriculture business in our country. The major components of floriculture industry are cut
flowers, ornamental plants, seeds, floral extracts etc. The Indian cut flower exports include rose, jasmine, crossandra, chrysanthemum, golden rod, orchid, lily and jasmine etc. Tissue culture has a multidimensional role in floriculture. It provides an important biotechnological tool that can be used for the promotion of floriculture industry.

Plant tissue culture technology helps in rapid large-scale multiplication from tiny stem cuttings, axillary buds or from somatic embryos. Micropropagation technique has been standardized in different ornamental crops in different countries as per requirement for commercial exploitation. Regeneration potentiality has been investigated by several workers (Bapat and Narayanaswamy, 1976; Seabrook and Cumming, 1976; Lin and Monette, 1987; O’ Rourke, 1991; O’ Rourke et al., 1991a, b; Misra and Datta, 2001a; Datta et al., 2002; Soomro et al., 2003).

Micropropagation

Micropropagation is a method of propagating plants from very small parts grown in sterile culture. It is a relatively complicated process that involves many steps or stages. Murashige divided the process into four distinct stages, each with specific requirements and considerations (Murashige, 1974; 1978a; b); stages I-III occur in vitro, whereas stage IV generally occurs in a greenhouse environment. An additional separate stage, called stage 0, has been proposed for description of micropropagation system (Debergh and Maene, 1981).

Stages of micropropagation

Stage 0: Preparation of mother plant

In this stage, the stock plants used for culture initiation are grown for at least 3 months under carefully monitored conditions. The stock plant conditioning stage includes precautions to reduce the level of bacterial and fungal contaminants, both surface and systemic, associated with plants.

Procedures to monitor the stock plants free from bacteria and fungi are being implemented in commercial micropropagation of many ornamental species e.g., foliage plants, carnations, chrysanthemums and geraniums (Knauss, 1976).
Stage I: Establishment of aseptic culture

Murashige (1974) defined stage I as the establishment of an aseptic culture or the initiation stage. Cultures can be initiated from several explants but in case of micropropagation, shoot tips and axillary buds are most often utilized. Procedures to surface-sterilize the explants and induce a healthy growth on the defined culture medium must be established for each species. Stage I usually requires 3 to as long as 24 months, and at least four subcultures.

Stage II: Multiplication of propagules

In this stage propagules, which may be shoots, embryos, meristematic nodules or calli, are multiplied. During this stage, proper use of plant growth regulators and their concentrations is critical.
Depending on the crop, stage II generally lasts 10-36 months, with almost the same number of passages.

Stage III: Pretreatment for transfer to soil

When the shoots or plantlets are prepared for soil, it may be necessary to evaluate several factors such as dividing the shoots and rooting them individually, hardening the shoots to increase their resistance to moisture and disease, rendering plants to autotrophic development in contrast to the heterotrophic state induced by culture and fulfilling the dormancy required for bulb crops.
Stage III requires 1-6 weeks.
Stage IV: Transfer to soil

The plantlets have to be maintained at high relative humidity or they will wilt and desiccate rapidly. Depending on the crop, several methods are used the acclimation process; these include an artificial acclimation room with controlled humidity, light and temperature, plastic covered incubators, plastic tents and a dense, fine particle fog system in the greenhouse. Complete plants can be established in artificial growing medium such as soil less mixes, rock wool, plugs or even sponges. For removal of dormancy, trees and bulb crops often need chilling once in greenhouse in order to grow properly.

Stage IV requires 4-16 weeks.

Methods of micropropagation

In micropropagation, the in vitro process utilizes three different pathways:

- Shoot tip culture
- Nodal stem culture
- Regeneration via callus

Shoot tip culture is very common in micropropagation especially in floral groups (Wang and Hu, 1980; Debergh and Zimmerman, 1990; Mujib and Pal, 1995; Sobhakumari and Lalithakumari, 2003) while nodal segment is also being regularly employed (Hazarika et al., 1995; Patnaik and Debata, 1996) and shoot multiplication is improved manifold only upon repeated culture on cytokinin. Adventitious buds developed directly on explants also minimize variation chances remarkably. Somatic embryogenesis is another efficient method for in vitro mass propagation of plants (Durzan and Durzan, 1991; Parrot et al., 1991; Junaid et al., 2006). The in vitro morphogenic processes are usually affected by physical environment such as growth regulators (Sul and Korban, 1998; Tsudo et al., 1999), carbon source (Biahoua and Booneau, 1999; Fuentes et al., 2000), gelling agent (Ladyman and Girard, 1992; Klimaszewska et al., 2000) and the explant type and origin (Sharma and Rajan, 1995; Peterson et al., 1999; Dhar and Joshi, 2005).
Induced mutations in improving crops

Genetic variation is an essential component of plant breeding; it can be induced by tissue culture, or by using physical, chemical and biological mutagens. Both somaclonal variation and induced mutations can generate a wide range of genetically stable useful variants or mutants (Skirven et al., 1993; Jain et al., 2001; Maluszynski et al., 2001). Induced mutations with radiations and chemical mutagens are random changes in the nuclear DNA or cytoplasmic organelles, resulting in chromosomal or genomic mutations that enable plant breeders to select useful mutants such as high yield, flower colour, disease resistance and early maturity in plants including the ornamentals (Micke et al., 1990; Crino et al., 1994).

To induce mutations, the most common mutagens are:

- Chemical (alkylating agents): ethylnitroso-urea (ENH), methyl nitroso-urea (MNH), ethylmethyl-sulphonate (EMS), and colchicines.
- Physical agents: X-rays, gamma rays, UV rays and laser beam (Jain 2002b).

More than 2250 improved varieties of major crops derived through induced mutations have been officially released worldwide (Maluszynski et al., 2000; Maluszynski, 2001). Of these, 605 were released in China, 259 in India, 210 in the former Soviet Union, 176 in The Netherlands, 128 in the USA, and 120 in Japan.

The achievement of mutation breeding in the ornamentals and horticultural crops is particularly impressive in India. In *Chrysanthemum* alone, 46 mutants are commercially released.
Table 1: Some of the released mutant varieties of the ornamentals (India)

<table>
<thead>
<tr>
<th>Latin Name</th>
<th>Common Name</th>
<th>Number of Varieties</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bougainvillea spectabilis</td>
<td>Bougainvillea</td>
<td>10</td>
</tr>
<tr>
<td>Chrysanthemum sp.</td>
<td>Chrysanthemum</td>
<td>46</td>
</tr>
<tr>
<td>Dahlia sp.</td>
<td>Dahlia</td>
<td>11</td>
</tr>
<tr>
<td>Gladiolus L.</td>
<td>Gladiolus</td>
<td>2</td>
</tr>
<tr>
<td>Hibiscus sinensis L.</td>
<td>Hibiscus</td>
<td>2</td>
</tr>
<tr>
<td>Polyanthus tuberosa L.</td>
<td>Polyanthus</td>
<td>2</td>
</tr>
<tr>
<td>Rosa sp.</td>
<td>Rose</td>
<td>16</td>
</tr>
<tr>
<td>Portulaca grandiflora L.</td>
<td>Portulaca</td>
<td>11</td>
</tr>
<tr>
<td>Lantana depressa L.</td>
<td>Wild Sage</td>
<td>3</td>
</tr>
</tbody>
</table>

A few more advantages of in vitro mutagenesis are:

- mutagen treatment can be given to large cell / protoplast populations or somatic embryos directly
- fast multiplication of mutated plant material
- in vitro selection of mutant

Secondary metabolite production in plant cell cultures

Plants are a tremendous source for the discovery of new products of medicinal value for drug development. Plant cell culture technologies were introduced at the end of the 1960’s for both studying and producing plant secondary metabolites. Cell suspension culture systems could be used for large scale culturing of plant cells and secondary metabolites could be extracted therefrom.

The major advantages of cell culture system over the conventional cultivation of whole plants are:

- Useful compounds can be produced under controlled conditions.
- Cultured cells would be free from microbes and insects.
- The cells of any plants (tropical or alpine) could also be multiplied to yield their specific metabolites.
- Automated control of cell growth and rational regulation of metabolite processes would reduce labour costs and improve productivity.
Organic substances are extractable from callus cultures.

Some of the medicinal compounds localized in morphologically specialized tissues or organs have been produced in culture systems not only by specific organized cultures but also from undifferentiated cell cultures. The possible use of plant cell cultures for the specific biotransformations of natural compounds has also been demonstrated in literature (Cheetam, 1995; Scragg 1997; Kringis and Berger 1998; Ramachandra Rao and Ravishankar, 2000). Because of advancement in technology, research in the area of production of phytochemicals using tissue culture has bloomed beyond expectations. Suspension cultures are more suitable, as compared with solid callus culture, for the production of secondary metabolites. They may be obtained either by batch culture or by continuous culture processes.

General description of selected plants and their importance

*Digitalis lanata* L.

*Digitalis*, also known as foxglove, belongs to the family Scrophulariaceae. The species of *Digitalis* are biennial or perennial herbs. The foliage consists of rosettes of leaves with inflorescence of about 1 metre height. *Digitalis* plants are of great importance in pharmacy due to producing cardioactive glycosides that are frequently employed in the treatment of heart diseases. These glycosides are present in several species of *Digitalis* but of them two viz., *D. lanata* and *D. purpurea*, have been thoroughly investigated. Among all the cardenolides (the term cardenolide refers to the glycosides that are used against cardiac disorders), digoxin is most important and is used frequently.

**Habitat**

The common foxglove of the woods (*Digitalis lanata*), a handsome plant, is widely distributed throughout the Europe and is common as a wild flower in Great Britain. In India, it is cultivated in Kashmir, Kishtwar, Darjeeling and Nilgiris. It flourishes best in siliceous soil and grows well in loam but is entirely absent in calcareous districts. It is a biennial or perennial herb. The inflorescence is pedicle, spike 1-4 ft, tomentose. The flowers are lanceolate to ovate, with lobed calyx and corolla, ciliated having white to pinkish purple dark spots on lower inside surface with sparsely hairy inside.
Active constituents

*Digitalis* glycoside belongs to the cardenolide type and is therefore named cardenolides. In the stereo-ring system, an unsaturated five-membered lactone ring is substituted in position 17, thus differentiating cardenolides from the bufadienolides presenting a six-membered lactone ring in this position.

Table 2: Principal cardioactive glycosides in *Digitalis* species. Overview of sugar residuals glycosidically linked to the 3-hydroxyl group.

<table>
<thead>
<tr>
<th>Aglycon</th>
<th>Glycosides</th>
<th>Sugars</th>
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<tbody>
<tr>
<td>Digitoxigenin</td>
<td>Lanatoside A</td>
<td>Gl-Acdx-Dx-Dx-Dx-Dx-</td>
</tr>
<tr>
<td></td>
<td>Glucodigifucoside</td>
<td>Gl-Fuc</td>
</tr>
<tr>
<td></td>
<td>Glucoevatrondonoside</td>
<td>Gl-Dx-Dx-Dx-Dx-Dx-Dx-</td>
</tr>
<tr>
<td></td>
<td>Purpurea glycoside A</td>
<td>Gl-Dx-Dx-Dx-Dx-Dx-Dx-</td>
</tr>
<tr>
<td></td>
<td>Digitoxin</td>
<td>Dx-Dx-Dx-Dx-Dx-Dx-Dx-</td>
</tr>
<tr>
<td>Gitoxigenin</td>
<td>Lanatoside B</td>
<td>Gl-Acdx-Dx-Dx-Dx-Dx-Dx-</td>
</tr>
<tr>
<td></td>
<td>Digitalinum verum</td>
<td>Gl-Dtl</td>
</tr>
<tr>
<td></td>
<td>Glucogitoroside</td>
<td>Gl-Dx</td>
</tr>
<tr>
<td></td>
<td>Purpurea glycoside B</td>
<td>Gl-Dx-Dx-Dx-Dx-Dx-Dx-Dx-</td>
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<td></td>
<td>Gitoxin</td>
<td>Dx-Dx-Dx-Dx-Dx-Dx-Dx-</td>
</tr>
<tr>
<td>Digoxigenin</td>
<td>Lanatoside C</td>
<td>Gl-Acdx-Dx-Dx-Dx-Dx-Dx-</td>
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<tr>
<td></td>
<td>Digoxin</td>
<td>Dx-Dx-Dx-Dx-Dx-Dx-Dx-</td>
</tr>
<tr>
<td>Diginatigenin</td>
<td>Lanatoside D</td>
<td>Gl-Acdx-Dx-Dx-Dx-Dx-Dx-</td>
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<tr>
<td></td>
<td>Diginatin</td>
<td>Dx-Dx-Dx-Dx-Dx-Dx-Dx-</td>
</tr>
<tr>
<td>Gitaloxigenin</td>
<td>Lanatoside E</td>
<td>Gl-Acdx-Dx-Dx-Dx-Dx-Dx-</td>
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<tr>
<td></td>
<td>Glucoverodoxin</td>
<td>Gl-Dtl</td>
</tr>
<tr>
<td></td>
<td>Glucolanodoxin</td>
<td>Gl-Dx</td>
</tr>
<tr>
<td></td>
<td>Glucogitaloxin</td>
<td>Gl-Dx-Dx-Dx-Dx-Dx-Dx-Dx-</td>
</tr>
<tr>
<td></td>
<td>Gitaloxin</td>
<td>Dx-Dx-Dx-Dx-Dx-Dx-Dx-</td>
</tr>
</tbody>
</table>

Acdx = 3-acetyl-β-D-digitoxose; Dtl = β-D-Digitalose; Dx = β-D-Digitoxose; Fuc = β-d-Fucose; Gl = β-D-Glucose.

*Hippeastrum*

The genus *Hippeastrum* belongs to the family Amaryllidaceae. A genus of showy bulbous herbs, native to tropical America, now placed under *Amaryllis*. A few species and hybrids are cultivated in Indian gardens. Some of the species and their numerous hybrids bear beautiful flowers of rich deep crimson, blood red, orange scarlet, white
and pink colour. Several types with striped, mottled and blended corollas have also been evolved. Propagation is done by seeds or offsets. They may be grown in large pots, but do better in beds.

*Hippeastrum equestre* Herb. is a lily-like plant with globose bulbs, strap-shaped leaves and a few fragrant, attractive funnel-shaped flowers borne on erect stem 1-2 ft. high. The bulb is used in Java for poulticing on swellings of the neck and contusions. It is used as indoor plant or cut flowers for decoration purposes. Bulbs of *Hippeastrum* are acrid and poisonous; contain 0.9% of an alkaloid bellamarin, which is identical with lycorine. Pseudolycorine, present both in *Hippeastrum* and *Narcissus*, has antiviral activity. Two other novel alkaloids, i.e., 3-O-demethyltazettine and egonine are also present.
OBJECTIVES

In the present investigation two plants *Digitalis* and *Hippeastrum* were selected and optimization of various *in vitro* steps like callus initiation, maintenance, regeneration, *in vitro* rooting and other related aspects was undertaken. The study included the following aspects:

- Cell culture establishment after identifying suitable explants
- Establishment of medium composition and hormonal factor at different stages of development
- Establishment of suspension culture
- Induction of somatic embryos in solid media
- Study of root regeneration programme
- Acclimatization and transplantation
- Biochemical studies in response to various morphogenetic programmes
- Effect of chemical mutagens (EMS and colchicine) on *in vitro* culture and their further evaluation
- Study of alkaloid profiles of regenerated plants/cultures