In vitro mass cloning of *Dianthus chinensis* – a horticultural important plant

A

Dissertation Report

submitted in partial fulfillment for award of the degree of

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CERTIFICATE

This is to certify that the dissertation entitled **"*In vitro* mass cloning of *Dianthus chinensis* – a horticultural important plant"** submitted by **Kirandeep Kaur (301201007)** in partial fulfillment of the requirements for the award of degree of Masters of Science in Biotechnology to Thapar University Patiala is a record of student's own work carried out by her under my supervision and guidance. The report has not been submitted for the award of any degree or certificate in this or any other University or Institute.

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DECLARATION

I hereby declare that the work presented in dissertation entitled, **"*In vitro* mass cloning of *Dianthus chinensis* – a horticultural important plant"** in partial fulfillment for the award of degree of Master of Science in Biotechnology, is an authentic record of my own work done during the period of six months from January 2014 to July 2014, under the guidance of Dr. Manju Anand, Associate Professor, Thapar University, Patiala. I have not submitted the matter embodied in this dissertation for the award of any other degree or diploma.

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ABBREVIATIONS

BAP	Benzylaminopurine
BMS	Basal Murashige and Skoog’s medium
˚C	Degree Celcius
CM	Coconut Milk
2,4-D	2,4- dichloro phenoxy acetic acid
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
2-ip	2-isopentenyl adenine
Kn	Kinetin
NAA	Naphthalene acetic acid
TDZ	Thidiazurone

ABSTRACT

The present investigation was carried out on a horticultural important plant *Dianthus chinensis* var. *heddiwigii* (China pink) belonging to the family Caryophyllaceae. The different vegetative plant parts viz. shoot apices, axillary buds, stem and leaves were excised from field grown healthy plants and thereafter cultured on variously supplemented Murashige and Skoog's medium for multiple shoot proliferation, *de novo* adventitious shoot and root formation, callus induction and organogenesis.

The leaf segments own a great organogenic potential as they exhibited a high efficiency of direct *de novo* adventitious root and shoot formation. Prolific root regeneration occurred from the entire surface of leaf explant on higher concentrations of NAA (2-4 mg/L) and with the incorporation of CM (15%) to NAA (2-4 mg/L) + Kn (1 mg/L) supplemented medium. The roots were thin, white and bore dense root hairs. A high efficiency of adventitious shoot formation from leaf explant was observed on MS medium supplemented with Zeatin (4 mg/L) or NAA (0.5 mg/L) + BAP (1mg/L) forming 20-25 shoots from the cut ends of leaf explant without the formation of intervening callus. In contrast, stem segments exhibited comparatively a low frequency of direct *de novo* adventitious shoot and root formation. Out of the various growth regulators tested, lower concentrations of IBA (0.5-1 mg/L) promoted direct rooting from the explant. Out of the variously tested cytokinins, only BAP (2 mg/L) was effective in generating shoots from the stem segment forming 20-22 shoots after 8 weeks of planting.

Callusing of the leaf and stem segments occurred immediately after culturing on optimal chemical milieu. Among the various growth regulators tested, optimal callusing of the leaf explants was observed on Zeatin (2 mg/L) or TDZ (4 mg/L) or IBA (2-4 mg/L) or 2,4-D (4 mg/L) + BAP (1 mg/L) supplemented medium. Murashige and Skoog's agar gelled medium supplemented with IBA (2-4 mg/L) or NAA (0.5-4 mg/L) or NAA (2-4 mg/L) + Kn or BAP (1 mg/L) with or without coconut milk (15%) turned out to be optimal for initiation and sustained growth of callus from the stem segments. However, MS medium supplemented with higher concentrations of IBA (2-4 mg/L) was the best medium reported for callus induction from leaf as well as stem segments.

The calli thus formed were green, hard, compact and fast growing. They were heterogeneous being composed of parenchymatous ovoid, oblong, semicircular cells or those with aberrant shapes.

The spectrum of induced differentiation from calli was wide and involved xylogenesis, rhizogenesis and caulogenesis. The tracheids occurred either singly or were grouped together and were mostly elongated having reticulated thickenings on their walls. Best root differentiation from leaf and stem calli occurred on ms medium supplemented with IBA (2-4 mg/L) and NAA (1 and 4 mg/L) where the roots formed were thin, white having dense root hairs. Shoot differentiation from the leaf callus occurred on 2, 4-D (2-4 mg/L) + BAP (1 mg/L) either alone or in conjunction with CM (15%) forming 8-10 shoots after 8 weeks.

Dianthus exhibited a high degree of propensity of multiple shoot proliferation from shoot apices and nodal segments. Multiple shoot formation was observed on MS medium supplemented with BAP or Kn or Zeatin or TDZ (1-4 mg/L) and NAA (0.5 mg/L) + BAP or Kn (1-4 mg/L). Best results were, however, obtained on BAP (4 mg/L) supplemented medium where 70-80 shoots were regenerated from single shoot apex or axillary bud. Once the clusters of shoots were formed, small clumps of 5-6 shoots were excised and transferred onto fresh multiplication medium where the increased drastically forming 140-150 shoots after 4 weeks.

Regenerated shoots formed from different vegetative parts were carefully rescued from the culture vessels and transferred to root inducing media to form complete plantlets. Best rooting response (60%) was observed on BMS medium forming well developed bunch of roots at the base of the stem. The plantlets thus formed were acclimatized and attempts were made to establish regenerated plantlets into the soil.

CHAPTER -1
INTRODUCTION

Over the years, horticulture has emerged as one of the potential agricultural enterprise in accelerating the growth of an economy. This sector has established its importance in improving land use, promoting crop diversification, generating employment and providing nutritional security to the people. There is no doubt that agriculture is transforming itself as a commercial venture through horticulture which encompasses cultivation of a wide range of crops e.g., fruits, vegetables, spices, flowers, ornamentals, plantation and medicinal crops (Lagzi, 2013). As compared to other agri-horticultural crops, floriculture has emerged as a lucrative profession with the much higher potential for returns in recent decades.

Floriculture – An overview

Floriculture is a branch of horticulture concerned with commercial production, marketing, and sale of bedding plants, cut flowers, potted flowering plants, foliage plants, flower arrangements and non-commercial home gardening. It has become an important commercial activity in agriculture. Today floriculture is a fast emerging and highly competitive industry. In a world that is increasingly becoming urbanized, ornamental plants provide an important link with the natural world (Middleton *et. al*. 2011).

Flowers and ornamental plants are used by humans because of their beauty, symbolic significance, color and fragrance, therapeutic and emotional value (Tuyl et al. 2014). Flowers are inseparable from the social fabric of human life. Since the very early civilizations flowers have been part of important cultural and religious customs. Being adorable creation of God, they befits all occasions, be it at birth, marriage or death. Expressions of empathy, sympathy, joy,sorry, recognition, happiness, care, love and acknowledgment are but a few of the characteristics realized with giving flowers (Oppenheim and Fly, 2000). The integration of flowers in daily human life has a long history and substantiates our appreciation for their delicacy and wide variation in possible shapes and colors. Flowers have been of great importance in human cultures and societies throughout the world in all ages being used for art, adornment, decoration, fragrance, medicine, food and floral design.

The importance of flowers has a central place in life of persons belonging to Indian cultural and religious practices. The religious worships do not take place without offering flowers to the God. In addition, women in some parts of the country adorn themselves with flowers

daily. The arrangements of expensive cut flowers are inherent part of decoration in luxury hotels and affluent homes. Importance of flowers is not restricted to the beautification and decoration but also have industrial importance. Some flowers like Rose, Jasmines, and Tuberose are used for extraction of essential oils which is base for preparation of perfumes, scents or attar.

In the past, flowers were not of much economic importance. With the passage of time drastic changes have come about in the life style of people leading to commercialized cultivation of flowers. The science and art of commercial floriculture has been recognized as an economic activity with the potential for generating employment and earning valuable foreign exchange. In several countries of the world, floricultural products are amongst the main export items of agricultural origin. The aesthetic value of flowers and ornamental plants, their use in social events, and high income generating power are attracting modern entrepreneurs to invest money in the floriculture industry. The demand for flowers and ornamental plants for different needs like religious, official ceremonies, parties, house decoration, weddings, funerals, etc, is on the rise. The Government of India has also identified floriculture as a niche area with vast potential for export (Sudhagar, 2013).

Floriculture is a sunrise industry and owing to steady increase in demand of flower floriculture, it has become one of the important commercial trades in Agriculture. Floriculture is increasingly regarded as a viable diversification from the traditional field crops because of higher returns per unit area. The floriculture industry is very dynamic in its varieties and the trade volumes. It comprises cultivation and marketing of a wide variety of plants and planting material: starting from parental products like plant parts and cuttings to the end products for the market like cut flowers (rose, carnation, chrysanthemum, gladiolus tulip, etc.) foliage, potted plants, garden plants, nursery stock(trees), showy leaves, annuals, perennials, flower bulbs and tubers (Martsynovska, 2011).

Floral production and consumption

Floral production has become a booming and highly specialized industry with intensive trading networks on a global scale. The world production of floriculture is growing at a rate of 10 percent per year. There are currently over 50 countries that are active in floriculture production on a large scale. In terms of production value, the Netherlands, the United States, Japan, Italy, Germany and Canada are the largest producers of cut flowers and plants. With China and India having the majority of the world acreage under cut flowers and

plants production in the world, the Asia-Pacific region has the major share (77 percent) of the total world area under floriculture production. Europe, the USA and Japan are the major consumers of floriculture products. Germany is the biggest consumer, followed by UK, France and Italy (Muthukumaran, 2010).

About 305,105 ha area is under flower production in different countries of the world. Flowers grown under protected greenhouses in different countries around the world total 46,008 ha. India has the maximum area under ornamental crops (88,600 ha) followed by China (59,527 ha), Indonesia (34,000 ha), Japan (21,218 ha), USA (16400 ha), Brazil (10285 ha), Taiwan (9.661 ha), The Netherlands (8,017 ha), Italy (7.654 ha), the United Kingdom (6,804 ha), Germany (6,621 ha) and Colombia (4,757 ha).

World Floriculture Trade Scenario

The production of flower crops has increased significantly and there is a huge demand for floricultural products in the world, resulting in growing International flower trade (Sudhagar, 2013). The global exports of floriculture products stood at US$ 17 billion in the year 2007. Fresh cut flowers and foliage accounted for around 49.1% (US$ 8.31 billion), and live plants, bulbs and cuttings accounted for 50.9% (US$ 8.60 billion) of total floriculture products exported in 2007. Developed countries in Europe, America and Asia accounts for more than 90% of the total world trade in floriculture products. The world imports of floricultural products in 2007, stood at US$ 16.7 billion. Germany (US$ 2.59 billion) was the largest importer, followed by the United Kingdom (US$ 1.89 billion), the USA (US$ 1.81Bbillion), the Netherlands (US$ 1.55 billion), and France (US$ 1.43 billion) (Muthukumaran, 2010).

Europe is the largest consumer as well as exporter of floricultural products in the world. Five European countries, viz., the Netherlands, Italy, Belgium, Denmark and Germany, account for over 66 percent of total exports by value of floricultural products in the world. Especially the Netherlands continues to dominate the world floriculture industry; it has been the epicenter for world flower trading. It was estimated that in 2007 over 50 percent (US$ 8.56 billion) of world floriculture exports came from the Netherlands (Ruud et al. 2005).

Indian Floriculture Trade Scenario

Floriculture industry has tremendous potential in India. After liberalization, the Government of India identified this activity as a sunrise industry and accorded it 100 per cent export-

oriented status. India is bestowed with diverse agro-climatic and ecological conditions, which are favorable to grow all types of commercially important flowers generally found in different parts of the world. It also enjoys the best climate in selected pockets for floriculture during winter months (Sudhagar, 2013).

The Indian floriculture industry has been growing at a compound annual growth rate of 25% over the past decade and is currently worth US$ 230 million. It comprises the florist trade, nursery plants, potted plants, bulb and seed production, micropropagation material and extraction of essential oils from flowers. Karnataka, Tamil Nadu, Andhra Pradesh, West Bengal, Maharashtra, Uttrakhand, Uttar Pradesh, Dehli, Haryana, Kerala, Himachal Pradesh and north eastern states are the major flowers growing states in India. Tamil Nadu is the largest loose flower production state, while West Bengal is the leading cut flower production state in India.

Rose is the principal cut flower grown all over the country. Other most important cut flower crops in the country are Gladiolus, Tuberose, Asters, Gerbera, Carnation, Anthurium, Lilium and Orchid (Muthukumaran et al.,2008, Muthukumaran, 2010).

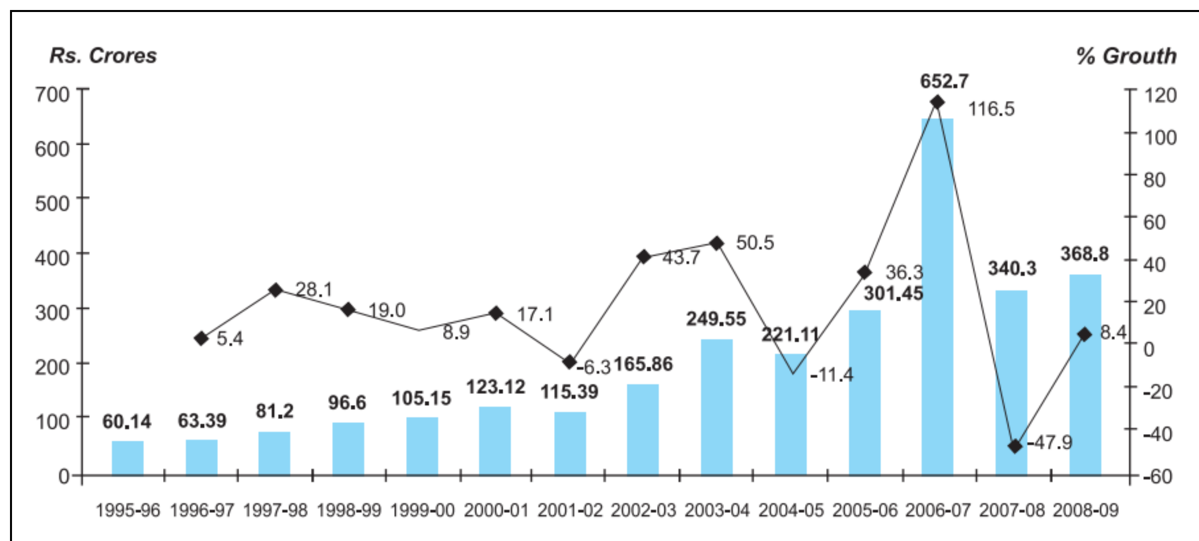

Figure 1: Floriculture exports from India (Muthukumaran, 2010)

India's exports of floricultural products in the year 2007-2008 decreased by 48% to US$ 84.5 million, from US$ 144 million in 2006-2007, and further decreased by 5.18 percent in the year 2008- 2009 to US$ 80.19 million. In the recent years, dried flowers and foliage have been forming a large part of floricultural product exports from India. During 2008-2009, dried flowers constituted over 60% of cut flowers exports and dried foliage constituted

around 99% of total foliage exports from India. Europe continues to be the largest destination for Indian floriculture exports (Muthukumaran, 2010).

Application of Plant Tissue Culture techniques to Floriculture

There has been a rapid growth in demand and consumption of floriculture products in recent decades. As population pressure increases and climatic changes produce additional production constraints, the challenge is to use plant tissue culture to develop sustainable and durable horticultural production systems.

Over 100 years ago, Haberlandt envisioned the concept of plant tissue culture and provided the groundwork for the cultivation of plant cells, tissues and organs in culture. At the peak of the plant tissue culture era in the 1980s, in a relatively short time, many commercial laboratories were established around the world to capitalize on the potential of micropropagation for mass production of clonal plants for the horticulture industry (Akin-Idowu et al. 2009).

Most of the ornamental plants raised through seeds are highly heterozygous and show tremendous variations in growth, habit and yield and it becomes difficult to select plant from wide population having the best qualities. In contrast, vegetative propagation gives rise to clones but not disease-free plants. The use of *in vitro* propagation can overcome difficulties and can manipulate the phenotypic variation. For the naturally rare and slowly growing plant species, plant tissue culture provides a sustainable and well-controlled means for mass production of disease free planting material.

Ornamental industry has applied massively *in vitro* propagation loom for large-scale plant development of selected better varieties. Recent progress in tissue culture techniques of plant has opened new possibilities for improvement of ornamental pot plants (Sharma and Agrawal, 2012). Plant tissue culture is now a well-established technology and encompasses much more than clonal propagation and micropropagation. The range of routine technologies has expanded to include somatic embryogenesis, somatic hybridization, virus elimination as well as the application of bioreactors to mass propagation.

Advantages of Micropropagation

Micropropagation offers several distinct advantages not possible with conventional propagation techniques (Thiart, 2003).

- ❖ A large number of clonal plants can be obtained. It is especially useful where plants are difficult to propagate vegetatively, are slow growing or where a single individual with good characteristics needs to be propagated.
- ❖ It is often much faster than conventional means. A single explant can be multiplied into several thousand plants in a very short time.
- ❖ The production of plants in sterile containers that allows them to be moved with greatly reduced chances of transmitting diseases, pests and pathogens.
- ❖ With the use of meristem techniques it is possible to produce certified virus-free plants.
- ❖ Production can be continued all the year round and is more independent of seasonal changes.
- ❖ Less space is required for propagation purposes and for the maintenance of stock plants.
- ❖ New cultivars can be selected from cultures due to mutations of rapidly dividing cells.
- ❖ Plant material needs little attention between subcultures and no labor is required for weeding, spraying, watering, etc.
- ❖ It facilitates the international exchange of germplasm without the inherent risk of spreading pests and diseases

Techniques of Micropropagation

Micropropagation can be achieved by any of the three approaches:

1. Multiplication by apical and axillary buds

The production of plants from apical and axillary buds has proved to be the most generally applicable and reliable method of true-to-type *in vitro* propagation. This is the most widely used method for micropropagation. Shoot tip and axillary bud having preformed meristems usually develop axillary shoots on a high cytokinin concentration. This method ensures genetic stability as cells of shoot meristem are uniformly diploid and are least susceptible to genotypic changes.

2. *De novo* formation of adventitious shoots

Shoots arising from any place other than the leaf axil or shoot apex are termed as adventitious shoots. *In vitro* adventitious shoots can develop

a) Directly from the explants like root, stem, petiole, leaf lamina, flower parts etc. or

b) Indirectly from callus cultures obtained from these explants.

Plants obtained through calli may not be true elites because of high incidence of polyploidy and aneuploidy associated with callus cells & plants obtained from it.

3. Somatic or nonzygotic embryogenesis

It involves the formation of bipolar embryos containing root and shoot axis which can develop into fully functional plants under appropriate conditions.

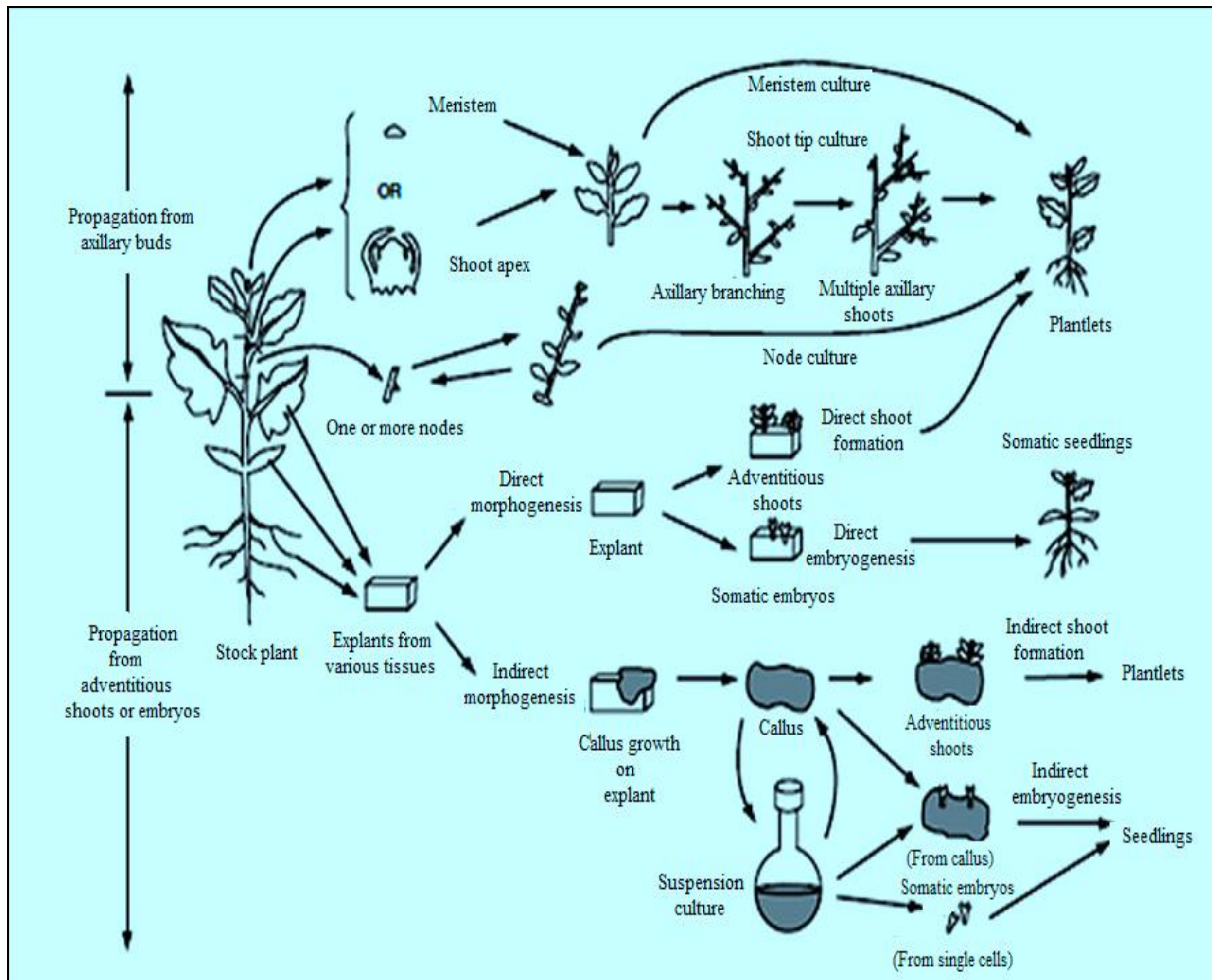

Figure 2: The principal methods of micropropagation (E.F George et al. 2008)

Stages of Micropropagation

Micropropagation involves 4 definite stages

Stage I: Initiation and establishment of aseptic cultures

This involves explant isolation, surface sterilization and establishment on an appropriate culture medium.

Stage II: Shoot multiplication using a defined culture medium

It can be achieved through any one of the following four methods:

- Multiplication through calli obtained from different organs and tissues and their subsequent subculturing leading to organogenesis.
- Multiplication through direct induction of shoots on the explants.
- Multiplication through growth and proliferation of existing apical shoots and axillary buds.
- Somatic or nonzygotic embryogenesis directly on the explants or in callus cultures.

Stage III: *In vitro* rooting of regenerated shoots

Root formation is very important for clonal propagation of plants. The success of any micropropagation protocol depends on the quality and extent of rooting of microshoots.

Stage IV: Transfer of plantlets to natural environment (acclimatization)

The climatic adaptation of a plant when moved to a new environment is known as acclimatization. Plants which are produced under cultural conditions (high humidity, low light, constant temperature) when transferred to field conditions are required to be acclimatized. Survival rate of plants depends upon the efficacy of hardening process.

This is achieved as follows:

- Initially plantlets are maintained at high relative humidity (80%) and reduced light for first 10-15 days in small pots containing potting mixture and covered with perforated polythene bag.

- The plantlets are then transferred to polybags with same potting mixture and kept in growth room for another 15 days.
- Later on plants are transferred to green house bench for 2 weeks and finally to open field conditions.

Rationale and Objectives

The present investigation was carried out on a horticultural important plant *Dianthus chinensis* var. *heddiwigii*. *Dianthus chinensis* is propagated traditionally by seeds, usually resulting in plants exhibiting great variations in growth, vigor and quality. The plant is not amenable to vegetative propagation. Therefore propagation of *Dianthus chinensis* by tissue culture becomes mandatory which offers greater potential to deliver large quantities of elite, high yielding, disease-free, true-to-type healthy stock within a short span of time to meet the demands of horticulture industry.

The main objectives of the present investigation were:

- ❖ To develop a reliable protocol for the rapid and mass scale propagation of plants in short duration of time and space.
- ❖ To obtain genetically pure elites rather than having indifferent population under *in vitro* conditions.

CHAPTER-2
REVIEW OF LITERATURE

In the recent years, floriculture has emerged as a fast growing domain of horticulture. The commercial production of ornamental plants is growing worldwide. Its monetary value has significantly increased over the last two decades and there is a great potential for continued further growth in both domestic and international markets. Ornamental industry has applied immensely *in vitro* propagation approach for large-scale plant multiplication of elite superior varieties About 156 ornamental genera are propagated through tissue culture in different commercial laboratories worldwide (Ahmed et al. 2012).

Plant tissue culture, or the aseptic culture of cells, tissues, organs, and their components under defined physical and chemical conditions in vitro, is an important tool in both basic and applied studies as well as in commercial application. It is based on the concept of totipotency and owes its origin to the ideas of the German scientist, Haberlandt, at the beginning of the 20th century (Thorpe, 2006). *In vitro* propagation or micropropagation is the most widely used discipline of plant tissue culture technology and has become a viable alternative to slow and time consuming methods of conventional propagation. It is in fact the miniature version of conventional propagation which is being carried out under aseptic conditions.

Micropropagation protocols have been developed for a wide range of ornamental plants like *Gladiolus* (Kumar *et.al.* 2002; Emek *et.al*. 2007; Haula *et.al.* 2012), *Rosa* (Rout *et.al*. 2005; Xing *et.al.* 2010; Shirdel *et.al*. 2012), *Chrysanthemum* (Tanaka et.al. 2000; Waseem *et.al.* 2007; Mani *et.al*. 2011), *Dianthus caryophyllus* (Lee *et.al*.1997; Karami *et. al*. 2006; Daniel *et. al*. 2009) and *Orchids* (Gow *et.al*.2008; Chen *et.al.*2004) and many more.

Micropropagation is a complex process which can be achieved by any of the following three approaches:

- Multiplication by axillary and apical buds
- Multiplication by adventitious buds
- Somatic embryogenesis

The first two approaches lead to plantlet formation via organogenesis through production of unipolar shoots which get further multiplied, followed by rooting in a multistage process. In

contrast, somatic embryogenesis leads to the formation of bipolar embryos through steps that are often similar to zygotic embryogenesis.

All the above three techniques have been adopted for the production of ornamental plants under *in vitro* conditions.

Multiplication by apical and axillary buds

Micropropagation through apical and axillary shoot proliferation is the most reliable technique for mass propagation since it ensures genetic stability of clones. A shoot tip and an axillary bud when grown under a high cytokinin concentration usually develop axillary shoots which can be subdivided into smaller clumps of shoots which in turn can develop similar clusters after subculture on fresh medium. This process can go on indefinitely and millions of plants can be raised starting from a single shoot tip or axillary bud. This method ensures genetic stability as the cells of meristems are uniformly diploid and are least susceptible to genotypic changes.

The multiplication rate through this technique varies with genotype and cytokinin requirement has been extremely variable. Priyakumari et al. (2005) established a protocol for micropropagation of *Gladiolus* (Peach Blossom) using axillary buds where maximum shoot proliferation was observed on MS medium fortified with BAP (4 mg/L) + NAA (0.5 mg /L).

Waseem *et.al.* (2009) reported in *vitro* propagation of *Chrysanthemum* from shoot tips on 0.1 mg/L IAA where maximum shoot initiation (86.6%) was recorded. A year later, in 2011, Waseem et al. yet again reported an efficient *in vitro* regeneration of *Chrysanthemum morifolium* plantlets from nodal segments on 0.3 mg/L IAA with 80% shoot initiation. A protocol for *in vitro* propagation of *Rosa hybrida* was established by Attia et al. (2012) using nodal explants. The highest percentage of shoot initiation (85%) was observed on MS medium containing 2 mg/l BAP + 1 mg/l Kn, whereas maximum average number of multiplied shoots (2.7) was produced on MS medium with 3 mg/l BAP + 1 mg/l Kn. Marian *et.al.* (2012) reported *in vitro* propagation of *Aglaonema* using axillary shoots as explants on MS medium supplemented with 1.5 mg/l thidiazuron (TDZ).

Brar *et.al.* (1996) studied the effect of Thidiazuron and Benzylaminopurine on multiple shoot proliferation from axillary buds in different cultivars of *Dianthus caryophyllus*. Number of multiple shoots produced was influenced by the cytokinin type and concentration. Barlo IINora cultivar of carnation produced the highest shoot number on MS medium supplemented

with 8.8 µM BAP. . Kantia et al. (2004) established a protocol for the mictopropagation of ornamental species of *Dianthus,* viz. *Dianthus chinensis, Dianthus caryophyllus and Dianthus barbatus* from shoot tips and nodal segments which were cultured on MS medium supplemented with BAP and NAA. Best response in terms of multiple shoot formation was observed on MS medium supplemented with BAP (1 mg/L) + NAA (0.5 mg/L). Ali *et.al*.(2008) reported the cytokinin BAP (4.4 µM) to be most effective for multiple shoot formation in *Dianthus caryophyllus* from apical and nodal meristems on MS medium +4.4 µM BAP within 7 days of culture. Daniel *et.al.* (2009) reported multiple shoot proliferation in *Dianthus caryophyllus* from shoot tips and maximum multiplication of shoot tips occurred in the presence of 4.4 µM of either BAP or Kn. Kharrazi *et.al*. (2011) evaluated the effect of different plant growth regulators for multiple shoot proliferation from axillary buds in *Dianthus caryophyllus* and reported the highest number of shoots (5 shoots/explant) on medium supplemented with 4.4µM BAP + 1.47 µM NAA.

Multiplication by adventitious buds

Many ornamental and horticultural species have been successfully propagated *in vitro* by adventitious shoot initiation. New adventitious shoots can develop directly from the explants like root, stem, petiole, leaf lamina and flower parts or indirectly from the calli obtained from these explants. Choice of explants and hormone regime to which the explants are subjected to, are two important factors in the initiation of adventitious shoots.

Directly from the explants

Kantia et al. (2002) established a protocol of high efficiency adventitious shoot bud induction from leaf explants of *Dianthus chinensis* cultured on MS medium supplemented with 6- BAP (13.2 µM) and 1-NAA (7.35 µM). Martin et al. (2003) reported direct shoot regeneration from lamina explants of two commercial cut flower cultivars of *Anthurium andraeanum.*

Chen et al. (2004) developed a protocol of high frequency plant regeneration through direct shoot formation from leaf cultures of *Paphiopedilum* orchids. The three treatments (4.52 µM 2, 4-D, 22.71 µM TDZ, 4.52 µM 2, 4-D plus 4.54 µM TDZ) gave a higher response of mean numbers of shoots per explant with intact leaf explants. Waseem *et.al.* (2009) demonstrated the effect of different auxins on the regeneration capability from leaf discs of *Chrysanthemum*.

Indirectly through callus

Kantia and Kothari (2002) reported adventitious shoot bud formation on BAP (2.2 µM) and 2,4-dichlorophenoxyacetic acid (4.87 µM) supplemented MS medium along with excessive callus formation on the surface of the leaf explants in *Dianthus chinensis.* Smaranda *et.al*.(2005) reported indirect micropropgation of *Chrysanthemum* through callus cultures obtained from stem segments. Kaviani *et.al*.(2011) established a protocol of callus induction and shoot and root formation from leaf explants in *Matthiola incana*.. Ghanati *et.al.* (2012) developed an efficient procedure for the development of callus and shoot formation from different explants especially leaf explants of *Eustoma grandiflorum*. MS medium containing 14.4 µM IAA, 0.465 µM Kn, 22.05 µM NAA was the best medium reported for the induction of callus. Utgikar *et.al*. (2005) established a protocol for micropropagation and callus regeneration in *Dianthus caryophyllus*. Kanwar and Kumar (2009) studied the influence of various plant growth regulators, explants and their interactions on *in vitro* shoot formation from callus in *Dianthus caryophyllus* L. Out of twenty seven shoot regeneration media tested, only 2 mg/l thidiazuron (TDZ) and zeatin alone or in combination with naphthalene acetic acid (NAA) and/or indole acetic acid (IAA) could differentiate calli. The highest average number of shoots was observed with 2 mg/l TDZ and 4.8 µM IAA.

Somatic embryogenesis

Tanaka et al. (2000) demonstrated high efficiency of somatic embryogenesis from leaf explants of *Dendranthema grandiflorum* (*Chrysanthemum*) on MS medium containing 57.08 Mm IAA + 0.465 Mm kinetin. No embryogenesis was observed in the absence of Kn. Kumar et al. (2002) reported heat shock induced somatic embryogenesis in callus cultures of *gladiolus* in the presence of high sucrose. Kim et al. (2004) reported somatic embryogenesis and plant regeneration from in vitro grown leaf explants of different species of Rose. Ilahi et al. (2007) reported rapid clonal propagation of *chrysanthemum* through embryogenic callus formation when nodal explants were cultured on half strength MS medium containing diffrernt growth hormones. Emek et al. (2007) reported somatic embryogenesis in *Gladiolus anatolicus* where leaves from *in vitro* raised shoots obtained from lateral buds were used as explant. The highest rate of callus formation was obtained on MS medium supplemented with NAA (5 mg/L) in darkness and this creamy white and friable calli produced numerous somatic embryos on MS medium supplemented with BAP (0.1 mg/L) within 4 weeks of culturing. Mani *et.al*. (2011) reported indirect somatic embryogenesis in *Chrysanthemum* via

callusing from leaf explant on MS medium containing 4.87 µM 2, 4-D and the best friable calli were subjected to suspension culture in MS medium supplemented with 4.4 µM BAP for somatic embryogenesis.

An efficient method has been developed for regeneration of complete plants via somatic embryogenesis in *Dianthus caryophyllus* from flower bud induced callus by Lee et al.(1997). The embryogenic callus, induced from flower buds, had proliferated without loss of embryogenic competence through periodic subculture on MS medium supplemented with 2, 4-D (0.487 µM). Karami et al. (2006) established an efficient method of somatic embryogenesis of *Dianthus caryophyllus* and studied the effect of sucrose concentrations on somatic embryogenesis. The maximum frequency of embryogenic callus was obtained on the medium containing 9% and 12% sucrose supplemented with 9µM 2, 4-D and 0.8µM BA. Yang et al. (2008) reported the factors affecting somatic embryogenesis in the anther cultures of Dianthus *chinensis.* The genotype of the donor plant was found to be major factor in determining the success rate. Iantcheva et al. (2008), reported somatic embryogenesis from leaf explants of *Dianthus caryophyllus*. Vigorous somatic embryos were obtained on embryo formation medium supplemented with 2.2 mM BAP and 250 mg/l casein hydrolysate. Similarly, Frey et al. (2009) developed a procedure for somatic embryogenesis in carnation on basal MS supplemented with 3µM 2, 4-D.

CHAPTER-3
MATERIALS AND METHODS

Choice of Plant Material

Dianthus chinensis var. *heddiwigii* was selected as an experimental material. It belongs to the angiospermic family Caryophyllaceae. It is commonly known by various names such as Annual Pink, China Pink, Chinese Pink, Dianthus, Indian Pink, Japanese Pink, Pinks and Rainbow Pink. It is an important floricultural crop which grows very well in the temperate climate. It ranks just next to rose in popularity as cut flowers in western countries (Roychowdhury et al**.** 2011). They are loved for their ease in growing, low care requirements and for the wonderful variety of colors and forms.

Figure 3: *Dianthus chinensis* var. *heddiwigii*

Distribution

The species is indigenous to northern China (Gansu, Hebei, Heilongjiang, Henam, Jilin, Liaoning, Nei Mongol, Ningxia, Qinghai, Shaanxi, Shandong and Xinjiang), Korea, Mongolia, Kazakhstan and southeastern Russia and has naturalized in southern China. It is widely cultivated elsewhere in temperate and subtropical areas (Lim, 2014).

Agroecology

In its native range, the species occurs in forest edges, forest grasslands, scrub on mountain slopes, hillside grasslands, dry hillsides, sandy hill summits, valleys, rocky ravines, meadows, streamsides, mountain stream wetlands, rocks, steppes, steppe sands, fixed dunes and seashores. The species require well-drained to slightly alkaline soil and full sun to partial shade (Lim, 2014).

Botany

China pinks are small, glabrous, herbaceous, perennials growing in dense tufts to 30-50 cm high with erect and distantly branched stem. The leaves are green to grayish green, slender, 3-5 cm long and 2-4 mm broad with acuminate apex, tapering base and entire margin. The flowers, 3-4 cm across, solitary or in a few flowered cymes with four ovate bracts and on 1-3 cm pedicels; calyx cylindric with lanceolate 5 mm; fringed petals; and limb bright red, purple-red, pink or white in color. The common name, pink, does not refer to the color of the blooms but to the serrated edges of the blooms (Lim, 2014)

Cultivation and Propagation

Pinks thrive in rich well-drained neutral to alkaline soil in a sunny position from spring to midsummer with moderate water and humidity requirements. They are ideal choice as cut flowers, potted flowering plants, bedding plants, borders and elements in mixed containers. They are easily propagated by seed sown directly in the ground in late March to early April. Seeds may also be planted in the fall in warmer climates, or in containers in a cold frame in cold climates. Propagation by division may be done in the spring when plants are 3-5 inches high. Plants are dug up, divided into 3 or 4 clumps, and replanted.

Edible Plant Parts and Uses

Like most Dianthus, it has a pleasant spicy, floral, clove-like taste and is ideal for decorating or adding to cakes. They also make a colorful garnish to soups, salads, sauces and jams. It is advisable to remove the bitter white base of the petal (Lim, 2014).

Ornamental importance

For centuries, Dianthus has been one of the most sought after plants for the garden due to their charming forms and colors. Dianthus plants are versatile and lend themselves to different uses. There are dwarf and mat-forming varieties that are perfect for edging along a border, as a ground cover, or grown in containers. The medium to tall varieties are grown in a cutting garden with other annuals and perennials, or they look especially nice growing in front of a back-drop of evergreen shrubs (Bell, 2012). Because Dianthus cross-pollinates between species with ease in the wild and in the garden, connoisseurs had an abundance of different plants to select from.

Medicinal importance

Dianthus contains a variety of chemical compounds namely anthocyanins; saponins; volatile oil components – eugenol, phenylethyl alcohol, benzyl benzoate and methyl salicylate; phosphoric acid; vitamin A; alkaloids and flavones.

It has been used for more than 2,000 years in Chinese herbal medicine. The whole plant is a bitter tonic herb that stimulates the digestive and urinary systems and also the bowels. It is also anthelmintic, antibacterial, antiphlogistic, antioxidant, anticancer, diaphoretic, diuretic, emmenagogue, febrifuge and haemostatic. It is used internally in the treatment of acute urinary tract infections (especially cystitis), urinary stones, constipation and failure to menstruate. It is used externally to treat skin inflammations and swellings. The old leaves are crushed and used for clearing the eyesight. The plants are harvested just before the flowers open and are dried for later use (Lim, 2014).

Toxic effects

Dianthus is available as a pill, powder or decoction. Large doses of dianthus can cause long-term contractions of the uterus. Since dianthus can stimulate contraction of the uterus, it should not be taken by women who are pregnant or have recently given birth.

Glassware

The glassware used for culture work comprised of 6” × 1” borosil test tubes, 250ml, 500ml and 1000ml borosil flasks. In addition, other glassware includes graduated measuring cylinder, Petri dishes, beakers and a range of pipettes. Before use, glassware was thoroughly brushed with alkaline detergent teepol and then washed in running tap water. It was then treated with ho chromic acid (mixture of $K_2CrO_4 + H_2SO_4 + H_2O$) followed by very thorough washing with tap water. All vessels were then inverted in a clean tray and left to dry. Copper distilled water (5 to 10ml) was then poured into every culture vessel which was tightly plugged. Plugs were made out of absorbent surgical cotton wrapped in muslin. The glassware was then steam sterilized in an autoclave at a pressure of 15 psi (121˚C) for 15 to 20 minutes.

Culture Media

Murashige and Skoog’s (1962) medium was used as basal medium. Stock solutions of generally 8-10 times major elements, 1000 times minor elements and 100 times organic constituents were prepared. These stock solutions were stored at 4˚C and were mixed in desired proportions only before use. None of the stock solutions were stored for more than 15 days.

Composition of Murashige and Skoog’s medium (1962)

Ingredient	**Amount (mg/l)**
Major elements	
NH_4NO_3	1650
KNO_3	1900
$CaCl_2.2H_2O$	440
$MgSO_4.7H_2O$	370
KH_2PO_4	170
$FeSO4.H_2O\text{-}Na_2EDTA$	40
Minor elements	
$MnSO_4.4H_2O$	22.3
$ZnSO_4.7H_2O$	8.6
H_3BO_3	6.2
KI	8.3
$Na_2MoO_4.2H_2O$	0.25

$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025

Organic constituents

Myoinositol	100
Glycine	2.0
Nicotinic acid	0.5
Pyridoxine HCl	0.5
Thiamine HCl	0.1
Sucrose	20000
Agar-agar	10000

Determined amounts of all the constituents except agar were mixed and volume was adjusted by distilled water. The pH of solution was adjusted to 5.8 ± 0.2 using 0.1N NaOH or HCl depending upon high or low.

Following are some of the supplements, which were used either singly or in combinations:

1. Basal Medium (BM)
2. BMS + NAA, IAA, IBA or 2,4-D (0.5- 4 mg/l)
3. BMS + BAP, TDZ, Kinetin or Zeatin (1- 4 mg/l)
4. BMS + BAP or Kn (1- 4 mg/l) + NAA (0.5 mg/l)
5. BMS + NAA (0.5- 4 mg/l) + BAP or Kn (1 mg/l)
6. BMS + 2, 4-D (2- 4 mg/l) + BAP or Kn (1 mg/l)
7. BMS + NAA or 2, 4-D (2- 4 mg/l) + BAP or Kn (1 mg/l) + CM (15%)
8. BMS with 3% and 4% sucrose + 2, 4-D (2-4 mg/l)

Coconut milk (liquid endosperm) when used was extracted from young coconuts and was stored at -4˚C. Definite aliquots of media were distributed depending upon the capacity of culture vessels. Generally 20- 25ml in test tube, 50ml in 100ml flask and 100ml in 250ml flask was distributed. Test tubes and flasks were plugged with sterile cotton plugs (made of cotton wrapped in muslin cloth) and autoclaved at 15 lb/in^2 (121˚C) for 15 to 20 minutes. Test tubes were placed over racks that tilt the test tubes during cooling and gave slanted surface to the agar media.

Inoculation

All the experimental manipulations were carried under aseptic conditions in an inoculation chamber fitted with a bactericidal ultraviolet tube (15W, peak emission 2537A˚). The floor of the chamber was thoroughly scrubbed with cotton dipped in alcohol. The surface of all the vessels and other accessories such as instruments (spatula, forceps, needles and scalpel etc.), spirit lamp, matchbox, tube containing absolute alcohol etc. were also cleaned with alcohol. The fresh material to be inoculated was kept in a Petri dish covered with a piece of black paper in order to protect it from the harmful effects of ultraviolet rays. Alcohol was then sprayed in the chamber with the help of an atomizer. The chamber was then sterilized with ultraviolet tube kept continuously on for one hour.

Surface sterilization of inoculum

Just like media, plant tissues were disinfected before they were placed over the media. Explants like leaves, stem and nodal explant were taken from plants growing under the *in vivo* conditions.

These were placed in different bottles and covered with net and washed for 30 minutes under running tap water to remove all the adhering dust particles and microbes from the surface. The explants were then washed with liquid detergent (teepol) for another 15 minutes and then washed properly to remove detergent. The explants were then treated with bavistin for another 20-30 minutes to remove fungus and then washed properly to remove fungicide. Under the sterile conditions, the explants were treated with 0.1% $HgCl_2$ solution for 5-10 minutes depending upon the explants. The apical and axillary buds were treated with 0.1% $HgCl_2$ for 3-4 minutes. The explants like stem were treated with 0.1% $HgCl_2$ for 1.5-2 minutes. Similarly explants like leaves were treated with 0.1% $HgCl_2$ for 2-3 minutes. The explants were then thoroughly washed (4-5 washings) with sterilized distilled water to remove traces of $HgCl_2$. Fresh cuts were given to the stem explants after sterilization to remove undesirable or dead portions.

Cultural Conditions

All cultures were maintained in the growth room at a temperature of 25 ± 4˚C at 16 hours illumination (2000 lux) from fluorescent tubes and incandescent bulbs. A photoperiod of 16 hours was maintained with the help of photothermal controller.

In vitro rooting of regenerated shoots

Basal MS medium alone and variously supplemented with IAA, IBA, and NAA was used for induction of *in vitro* rooting. The number of individual shoots that responded for rooting were counted and expressed as percent rooting.

Transplantation

The cultures on rooting medium developed proper root and shoot systems. These plants were transferred to small pots containing soil and organic manure. The pots were covered with perforated polythene bags and kept in growth room initially for 15 days to maintain humidity. The plantlets were then transferred to polybags with same potting mixture and kept in growth room for another 15 days. Later on plants were transferred to green house bench and attempts are underway to transfer these plants to natural field conditions.

CHAPTER-4
RESULTS AND OBSERVATIONS

Nodal segments, shoot apices, leaf and stem segments excised from healthy field grown plants of *Dianthus chinensis* var. *heddiwigii* were used for experimental work and cultured on MS medium containing different growth adjuvants.

Leaf culture

The first, second and third pair of young leaves, 4-5 mm in length, were excised, and after sterilization were planted on variously supplemented MS medium for *de novo* adventitious shoot and root formation either directly from the explant or indirectly through the callus.

Direct *de novo* adventitious root formation

The leaf explants exhibited a high degree of direct root regeneration on MS medium supplemented with different concentrations and combinations of growth regulators although its frequency varied with the nature of the auxin supplied. Prolific root regeneration was observed on MS medium supplemented with different concentrations of NAA (0.5–4 mg/L), where root induction started from the cut ends of the explants after 12 days of culture and within 4 weeks the entire surface was covered with roots (figs. 4-8). The roots were white, thin and bore profuse root hair. The best response was, however, observed at higher concentrations of NAA i.e. 2-4 mg/L, where 27-33 roots were formed on an average with 100% efficiency.

The direct root regeneration response declined on the addition of Kn or BAP (1 mg/L) to NAA supplemented medium as the rooting frequency decreased with an increase in the concentration of NAA (figs. 9-11).

However, the highest frequency of rooting occurred on MS medium supplemented with NAA (2-4 mg/L) + Kn (1 mg/L) + CM (15%). The addition of coconut milk promoted profuse rooting and within 5 weeks of culturing the entire explant was covered with thick growth of roots. The roots were found to emerge after 7-8 days of inoculation reaching maximum of 35 roots per explant having an average length of 1.7 cm with 100% root induction (figs. 12-13). A comparative response of rooting on different concentrations of NAA (0.5-4 mg/L) + Kn or BAP (1 mg/L) and NAA (2-4 mg/L) + Kn or BAP (1 mg/L) + CM (15%) is depicted in figs. 14-15.

No regeneration of roots was observed on higher concentrations of IBA (2-4 mg/L) while lower concentrations of IBA (0.5-1 mg/L) favoured 80% root initiation and excelled out all other growth regulators by having a maximum average root length of 6.7 cm (figs. 16-17). Good rooting was also induced on MS medium supplemented with different concentrations of IAA (0.5-4 mg/L), where the roots were highly branched. Highest numbers of roots (20.3) were formed on 4mg/L of IAA with an average root length of 5.1 cm. The rooting frequency on different concentrations of IAA is depicted in figs. 18-22.

Effect of different concentrations and combinations of various growth regulators on direct *de novo* adventitious root formation from leaf explant is depicted in table 1.

Table 1. (Culture period: 6 weeks)

Growth regulators	Concentration (mg/L)	No. of explants cultured	% of explants showing root formation	Average no. of roots per explants	Average length of roots (cm)
NAA	0.5	10	85.7	14.7	4.1
	1	10	100.0	18.6	4.7
	2	10	100.0	27.1	3.8
	4	10	100.0	33.0	2.0
IAA	0.5	10	100.0	12.0	5.0
	1	10	75.0	15.0	4.4
	2	10	85.7	16.4	4.0
	4	10	100.0	20.3	5.1
IBA	0.5	10	80.0	20.2	6.7
	1	10	80.0	22.8	6.2
NAA + Kn	0.5 + 1	5	75.0	14.2	3.2
	1 + 1	5	80.0	24.0	3.0
	2 + 1	5	60.0	15.0	2.0
	4 + 1	5	40.0	10.0	1.0
	2 + 1 + CM (15%)	5	100.0	32.0	1.6
	4 + 1 + CM (15%)	5	100.0	35.0	1.7
NAA + BAP	0.5 + 1	5	80.0	12.0	3.0
	1 + 1	5	60.0	11.0	3.2
	2 + 1	5	60.0	10.0	1.9
	4 + 1	5	40.0	8.0	0.2
	2 + 1 + CM (15%)	5	100.0	18.0	1.6
	4 + 1 + CM (15%)	5	80.0	21.2	1.4
2, 4-D + Kn	2 + 1	5	20.0	6.0	1.2
	4 + 1	5	40.0	12.0	3.5

Direct *de novo* adventitious shoot formation

Leaf segments were cultured on MS medium augmented with different cytokinins like Kn, BAP, Zeatin and TDZ either alone or in conjunction with NAA for *de novo* adventitious shoot formation. No response was observed on any concentration of Kn tested. Although the frequency of shoot bud induction from leaf explants was almost same on all other cytokinins but MS medium supplemented with Zeatin (4 mg/L) produced highest number of shoots in 20% cultures. Adventitious shoot formation was observed from one of the cut ends of leaf surface after 2 weeks (fig.23) and a maximum of 25 shoots were formed after 8 weeks of culture (fig. 24). On NAA (0.5 mg/L) + BAP (1 mg/L) supplemented medium, shoots arose in clusters from the cut ends of the leaf surface after 4 weeks of culturing (fig. 25) which proliferated further forming numerous well developed green shoots (fig. 26). Lower concentrations of BAP (1 mg/L) and TDZ (1 mg/L) were least effective in generating shoot response from leaf explants as only 5 shoots were formed after 8 weeks of culturing. A little growth of callus was more or less simultaneous with the shoot development.

Effect of different concentrations and combinations of growth regulators on direct *de novo* adventitious shoot formation from leaf explant is shown in table 2.

Table 2. (Culture period: 8 weeks)

Growth regulators (mg/L)	No. of explants cultured	% of explants showing shoot formation	No. of shoots per explant	Length of shoots (cm)
BAP (1mg/L)	5	20.0	2.0	0.5
Zeatin (4 mg/L)	5	20.0	25.0	3.0
TDZ (1 mg/L)	5	20.0	5.0	1.0
TDZ (2 mg/L)	5	20.0	18.0	2.0
NAA (0.5 mg/L) + BAP (1mg/L)	5	40.0	18.0	1.2
Kn (1-4 mg/L)	-	-	-	-

Callus induction from leaf explants and organogenetic differentiation

Among the various growth regulators tested, optimal callusing of the leaf explants was observed on Zeatin (2 mg/L) or TDZ (4 mg/L) or IBA (2-4 mg/L) and 2,4-D (4 mg/L) + BAP (1 mg/L) supplemented medium. On Zeatin (1-2 mg/L) supplemented medium, 100% of the explants callused at the cut ends after 8 days of inoculation. The callus grew further (fig. 28) and the entire explant turned into a mass of green and compact callus after 5 weeks (fig.

29). Effect of different growth regulators on callus induction and growth from leaf explants is shown in table 3.

Study of callus

The leaf callus was extremely hard and compact and could not be easily broken into single cells or groups when placed in water. The callus was teased forcibly with the help of thin strong needles. Microscopic examination of callus indicated its heterogeneous nature showing cells of various sizes and shapes. The cells were round, oval and elongated each having numerous starch granules scattered throughout the cytoplasm (fig. 30).

Histogenetic differentiation was observed in the form of tracheids which occurred either singly or in groups and possessed reticulate thickenings on their walls (fig. 31).

Organogenetic differentiation from the callus

Rhizogenesis

Root differentiation occurred in 100% of callus cultures on IBA (2-4 mg/L) after 4 weeks of culture. Initially a few roots were formed (fig. 32) but with further proliferation of callus more and more roots were organized (fig. 33). The roots were thin, long and had numerous root hairs. Likewise root differentiation also occurred from leaf callus on 2, 4-D (2-4 mg/L) + BAP (1 mg/L) with or without coconut milk as depicted in table 3.

Caulogenesis

Shoot differentiation occurred on 2, 4-D (2 mg/L) + BAP (1 mg/L) in 50% of callus cultures after 5 weeks where 3-4 shoots differentiated from callus (fig. 34). With the passage of time there was steady increase in the number of shoots forming 8 shoots after 8 weeks (fig. 35). Equally good results were obtained on 2, 4-D (4 mg/L) + BAP (1 mg/L) + CM (15%) where ten shoots were formed in 25% of cultures. Root differentiation also occurred immediately after the formation of shoots. Effect of different growth regulators on caulogenesis is depicted in table 3.

Table 3. (Culture period: 8 weeks)

Growth regulators (mg/L)	% of explants induced callus	Degree of callus formation	Rhizogenesis		Caulogenesis	
			% of rhizogenesis	No. of roots per culture	% of caulogenesis	No. of shoots per culture
Zeatin (1 mg/L)	100.0	++++	-	-	-	-
Zeatin (2 mg/L)	100.0	+++	-	-	-	-
Zeatin (4 mg/L)	80.0	+++	-	-	-	-
TDZ (1 mg/L)	100.0	++	-	-	20.0	2.0
TDZ (2 mg/L)	75.0	++++	-	-	20.0	2.0
TDZ (4 mg/L)	100.0	++	-	-	20.0	1.0
IBA (2 mg/L)	80.0	+++	100.0	14.0	-	-
IBA (4 mg/L)	100.0	++++	100.0	16.0	-	-
2,4-D (2 mg/L) + BAP (1 mg/L)	100.0	+++	25.0	6.0	50.0	8.0
2,4-D (4 mg/L) + BAP (1 mg/L)	100.0	+++	40.0	8.0	-	-
2,4-D (2 mg/L) + BAP (1 mg/L) + CM (15%)	100.0	+++	-	-	-	-
2,4-D (4 mg/L) + BAP (1 mg/L) + CM (15%)	100.0	++++	50.0	10.0	25.0	10.0

++ = moderate callus growth

+++ = good callus growth

++++ = excellent callus growth

Leaf culture

Fig. 4: Direct root regeneration from leaf explants on NAA (0.5 mg/L) supplemented medium after 6 weeks of culturing.

Fig. 5: Leaf explant showing root initiation on NAA (1 mg/L) supplemented medium.

Fig 6: Leaf segment on MS + NAA (2 mg/L) supplemented medium showing root regeneration.

Fig. 7: 6-weeks-old leaf culturte showing profused rooting on MS + NAA (4 mg/L).

Fig. 8: Effect of different concentratins of NAA (0.5-4 mg/L) on direct root regeneration from leaf explant.

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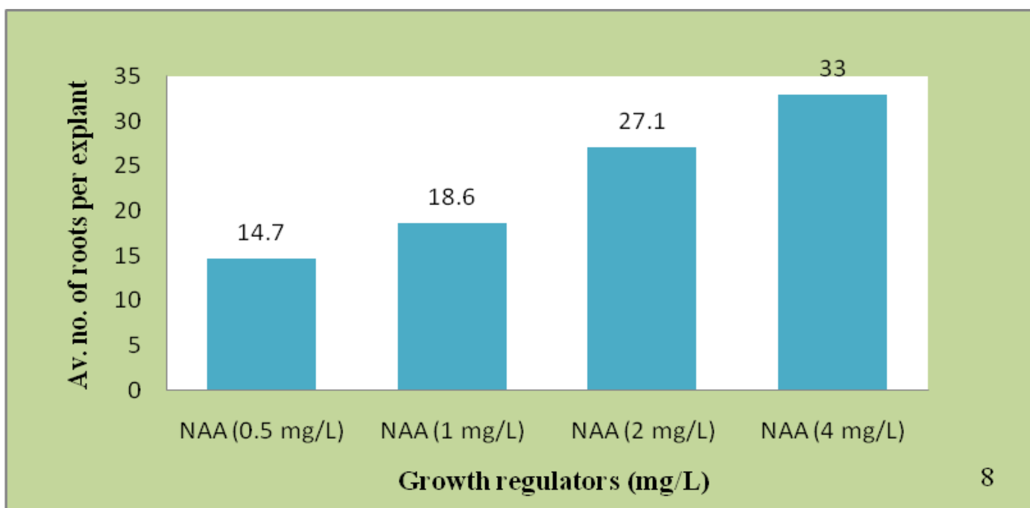
Av. no. of roots per explant
35
30
25
20
15
10
5
0
14.7
18.6
27.1
33
NAA (0.5 mg/L)
NAA (1 mg/L)
NAA (2 mg/L)
NAA (4 mg/L)
Growth regulators (mg/L)
8

Fig. 9: Direct root regeneration from leaf explant on MS + NAA (1 mg/L) + BAP (1 mg/L) after 6 weeks of culturing.

Fig.10 Leaf explant showing root initiation on on MS + NAA (2 mg/L) + BAP (1 mg/L) after 6 weeks of culturing.

Fig.11 Leaf segment on on MS + NAA (4 mg/L) + BAP (1 mg/L) showing root regeneration after 6 weeks of culturing.

Fig 12 6 weeks-old-leaf culture showing profuse rooting on MS + NAA (2 mg/L) + Kn (1 mg/L) + CM (15%)

Fig.13 Leaf explant showing root regeneration on MS + NAA (4 mg/L) + Kn (1 mg/L) + CM (15%).

Fig14 Effect of different concentrations and combinations of NAA and Kn with or without the addition of CM (15%) on direct root regeneration from leaf explant

Fig.15 Effect of different concentrations and combinations of NAA and BAP with or without the addition of CM (15%) on direct root regeneration from leaf explant

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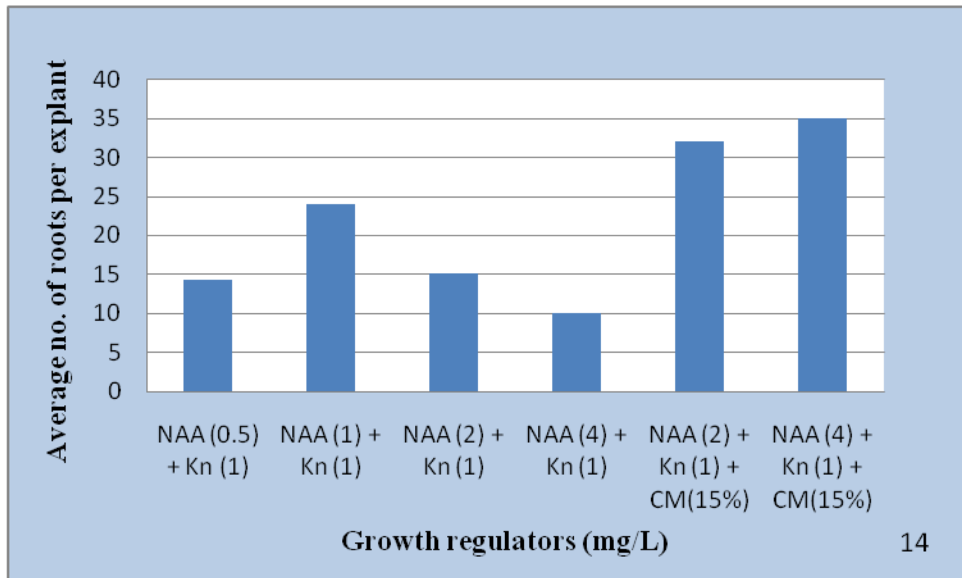
Average no. of roots per explant
40
35
30
25
20
15
10
5
0
NAA (0.5) + Kn (1)
NAA (1) + Kn (1)
NAA (2) + Kn (1)
NAA (4) + Kn (1)
NAA (2) + Kn (1) + CM(15%)
NAA (4) + Kn (1) + CM(15%)
Growth regulators (mg/L)
14

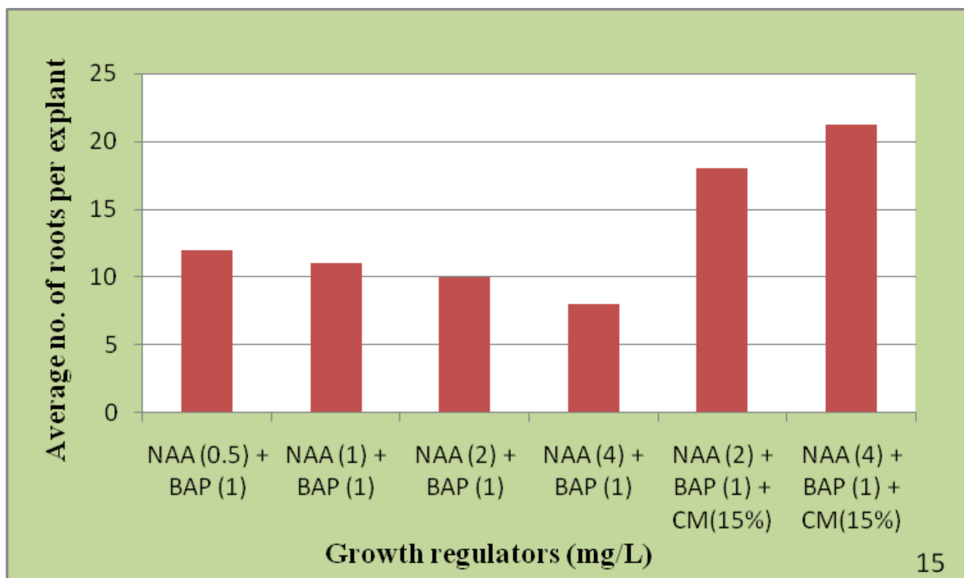
Average no. of roots per explant
25
20
15
10
5
0
NAA (0.5) + BAP (1)
NAA (1) + BAP (1)
NAA (2) + BAP (1)
NAA (4) + BAP (1)
NAA (2) + BAP (1) + CM(15%)
NAA (4) + BAP (1) + CM(15%)
Growth regulators (mg/L)
15

Fig 16 Direct regeneration of long, thin roots from leaf explant on MS + IBA (0.5 mg/L) medium after 2 weeks of culturing.

Fig.17 Leaf explant showing direct root regeneration on IBA (1 mg/L) supplemented medium after 2 weeks of culturing.

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Fig.18 Regeneration of roots from leaf explant on MS + IAA(0.5 mg/L) after 6 weeks of culturing.

Fig. 19 Direct root regeneration from leaf explant on MS + IAA (1 mg/L) after 6 weeks of culturing.

Fig. 20 Adventitious root formation from leaf segment on MS + IAA (2 mg/L) after 6 weeks of culturing.

Fig. 21 Leaf explant showing regeneration of roots from leaf explant on MS + IAA (4 mg/L) after 6 weeks of culturing.

Fig. 22 Effect of different concentrations of IAA on the number of roots formed from the leaf explant.

18 19 20 21

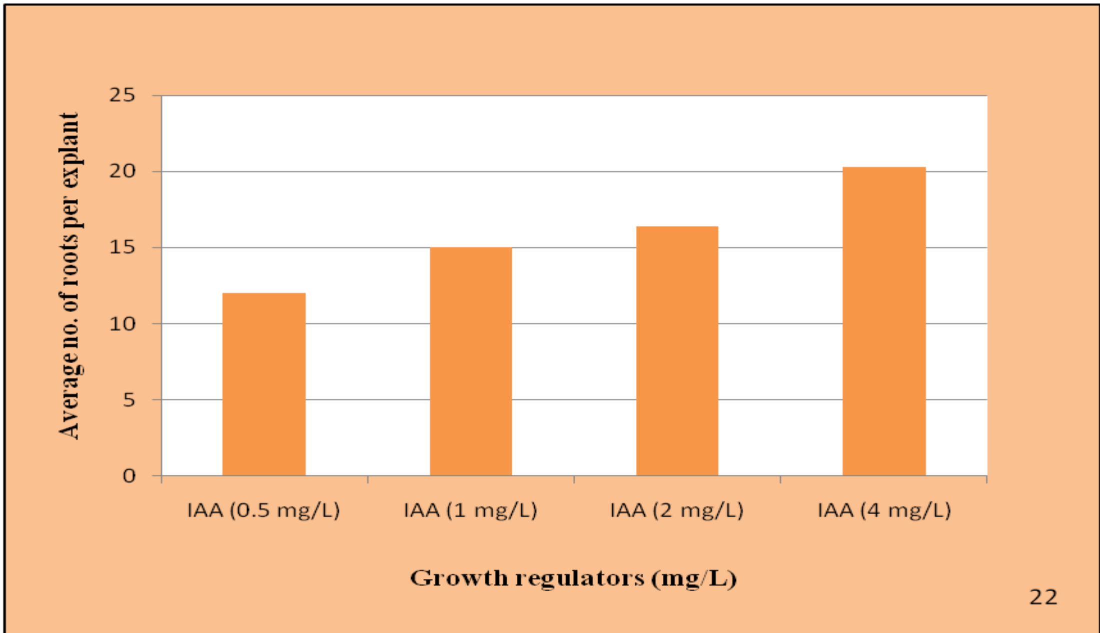

Fig. 23 Direct shoot regeneration from leaf explant on Zeatin (4 mg/L) supplemented medium after 2 weeks of culturing.

Fig. 24 Direct shoot regeneration from leaf explant on Zeatin (4 mg/L) supplemented medium after 8 weeks of culturing.

Fig. 25 Leaf explant on NAA (0.5 mg/L) + BAP (1 mg/L) supplemented medium showing shoot regeneration after 5 weeks of culturing.

Fig. 26 Leaf explant on NAA (0.5 mg/L) + BAP (1 mg/L) supplemented medium showing shoot regeneration after 8 weeks of culturing.

Fig. 27 Histogram showing effect of different growth regulators on direct shoot formation from leaf explant.

Fig. 28 Initiation of callus from the cuts ends of leaf explant on Zeatin (1 mg/L) supplemented medium after 8 days of culturing.

Fig. 29 A mass of green hard compact callus formed after 5 weeks of culturing on Zeatin (1 mg/L) supplemented medium.

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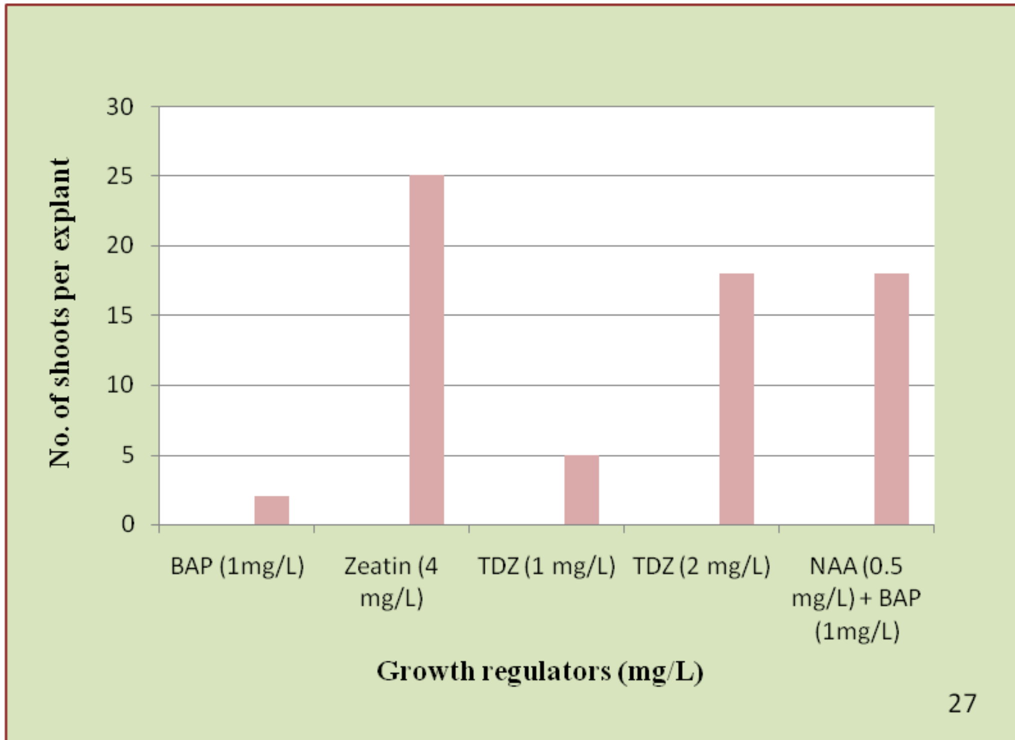
No. of shoots per explant
30
25
20
15
10
5
0
BAP (1mg/L)
Zeatin (4 mg/L)
TDZ (1 mg/L)
TDZ (2 mg/L)
NAA (0.5 mg/L) + BAP (1mg/L)
Growth regulators (mg/L)
27

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Fig. 30 Microscopic examination showing clusters of starch granules in the leaf callus.

Fig. 31 Group of tracheids isolated from leaf callus shown on magnified scale with reticulate thickening on their walls.

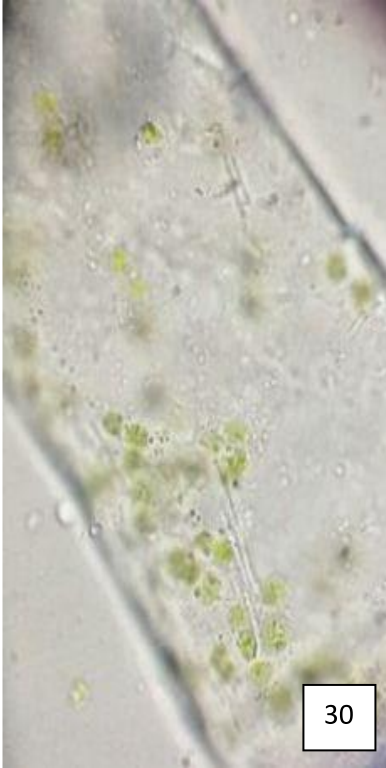
Fig. 32 Regeneration of roots from leaf callus on IBA (4 mg/L) supplemented medium after 5 weeks of culturing.

Fig. 33 Regeneration of numerous roots from leaf callus formed on IBA (4 mg/L) supplemented medium after 8 weeks of culturing.

Fig. 34 Initiation of shoot differentiation from leaf callus on MS + 2,4-D (2 mg/L) supplemented medium after 5 weeks of culturing.

Fig. 35 Differentiation of numerous shoots from leaf callus cultured on MS + 2,4-D (2 mg/L) supplemented medium after 8 weeks.

Fig. 36 Regeneration of clusters of shoots from leaf callus cultured on MS + 2,4-D (4 mg/L) + CM (15%) supplemented medium after 8 weeks of culturing.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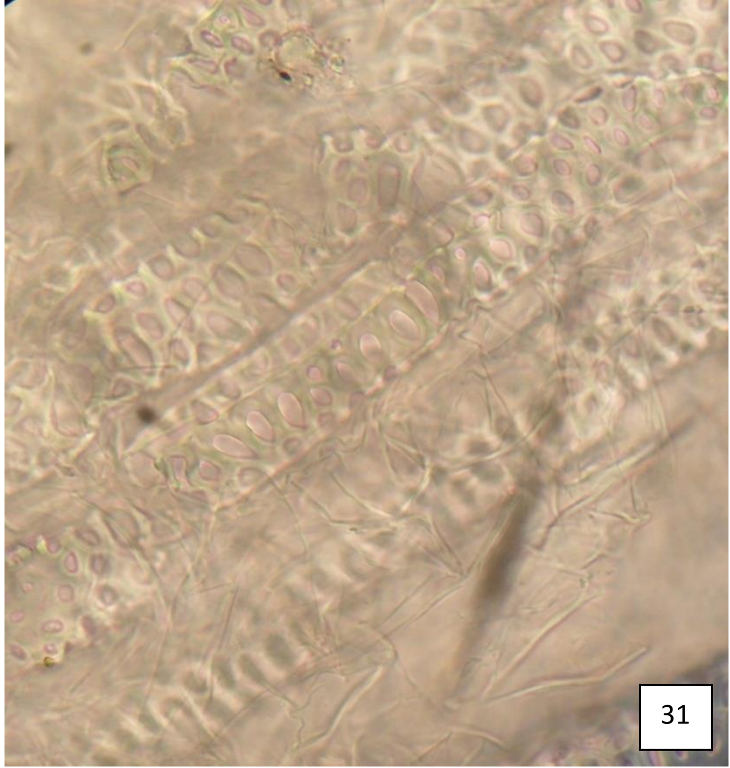
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Axillary bud and shoot tip culture

Shoot tips with youngest leaves attached and fresh nodal segments each holding one dormant lateral bud were cultured on MS medium supplemented with various growth regulators. The nodal segments were trimmed down to 1 cm long prior transferring to the medium. For each treatment five replicates were taken and different parameters including shoot initiation percentage, number of shoots formed per explant and length of shoots were studied respectively.

Effect of growth regulators on shoot proliferation from nodal explants

Out of various growth regulators tested, MS medium fortified with BAP (4 mg/L) produced the highest number of multiple shoots with maximum shoot initiation percentage. The initial bud break occurred after 7 days of inoculation (fig. 37) on BAP (4 mg/L) supplemented medium, forming 5-6 shoots in course of two weeks (fig. 38). The shoots multiplied prolifically after fourth week (fig. 39) and a maximum of 72.2 shoots were formed in 8 weeks of culturing (fig. 40). Once the clusters of shoots were formed, small clumps of 5-6 shoots were excised and transferred onto fresh multiplication medium. One week after the subculture, the number of shoots doubled which further increased drastically to form 140-150 shoots after 4 weeks of sub culturing (figs. 41-42).

Likewise, higher concentrations of Kn (2-4 mg/L) were also effective in generating prolific multiple shoot formation where axillary bud break was noticed after 8 days of culturing forming 5-6 shoots after 2 weeks in 80% of cultures (figs. 43-44). The shoots multiplied thereafter (fig. 45) and increased in number inexhaustibly forming nearly 58 shoots per explants after 8 weeks (fig. 46). An increased rate of multiplication was observed when the shoot clusters obtained at the end of one multiplication cycle were divided into smaller clusters of 5-6 shoots and replanted on fresh multiplication medium. The shoots increased in length forming well developed green, healthy shoots bearing long thin leaves (fig. 47).

MS medium supplemented with BAP or Kn(1 mg/L)) in conjunction with NAA (0.5 mg/L) was also effective in inducing bud break and forming shoots at a rate of 47 and 21 per nodal explant after 8 weeks of culturing as depicted in table 4 and figs. 48-49.

Equally good results were also obtained when nodal explants were cultured on lower concentrations of Zeatin and TDZ (1 mg/L) where the earlier bud break was noticed after 4

days and a maximum of 51 and 30 shoots per explant were formed respectively as depicted in figs. 50-58 and table 4.

Effect of different concentrations and combinations of growth regulators on shoot proliferation from nodal explants is depicted in table 4.

Table 4.

Growth Regulators	**Concentration (mg/L)**	**% of explants showing shoot formation**	**No. of shoots per explant**		**Length of shoots (cm)**
			4W	**8W**	
BAP	1 2 4	66.6 80.0 100.0	14.0 19.8 28.4	22.0 32.0 72.2	2.0 2.2 2.0
Kn	1 2 4	40.0 80.0 80.0	12.0 16.4 22.0	35.2 42.0 58.0	3.2 3.5 4.0
Zeatin	1 2 4	100.0 80.0 75.0	12.8 17.0 15.0	51.0 42.5 30.0	2.1 1.8 1.4
TDZ	1 2 4	80.0 66.6 60.0	12.2 10.0 8.2	30.6 20.0 14.2	1.5 2.0 1.0
BAP + NAA	1 + 0.5 2 + 0.5 4 + 0.5	100.0 100.0 80.0	18.7 15.0 11.0	47.0 42.5 36.0	2.2 2.5 2.2
Kn + NAA	1 + 0.5 2 + 0.5 4 + 0.5	40.0 33.3 33.3	7.2 4.0 2.0	21.0 12.0 6.0	4.0 3.8 3.0

Effect of growth regulators on shoot proliferation from shoot tip explants

Young shoot apices were cultured on MS medium supplemented with different concentrations of BAP, Kn, Zeatin or TDZ (1-4mg/L) either alone or in combination with NAA (0.5 mg/L). It was observed that high concentration of BAP (4 mg/L) excelled out all other growth regulators in generating maximum response at a rate of 80.2 shoots per explant in 80% cultures after 8 weeks of culture period (figs.59-60). The number further increased to 110-120 shoots after 4 weeks of sub culturing on the same medium (fig. 61).

Followed by BAP, the shoot tips cultured on MS medium supplemented with intermediate and low concentrations of zeatin (2 mg/L) and TDZ (1 mg/L) respectively developed multiple shoots at a high frequency of 80% and forming 40 shoots per explants.

Unlike high shoot multiplication rate from nodal explant, Kn was noticed to be less effective in generating multiple shoot response from shoot tip explants. The maximum multiplication rate was achieved on high concentration of Kn (4 mg/L) forming 25.6 shoots per explant with 60% shoot initiation efficiency (fig. 62). The shoots grew continually thereafter forming numerous shoots when sub cultured on fresh medium (fig. 63).

Effect of different concentrations and combinations of growth regulators on shoot proliferation from shoot tip explants is shown in table 5.

Table 5. (Culture period: 8weeks)

Growth Regulators	**Concentration (mg/L)**	**% of explants showing shoot formation**	**Average no. of shoots per explant**		**Average length of shoots (cm)**
			4W	**8W**	
BAP	1	66.6	5.0	7.0	1.4
	2	60.0	18.0	22.2	1.8
	4	80.0	42.6	80.2	2.0
Kn	1	20.0	6.0	12.0	1.0
	2	33.3	8.6	22.0	4.0
	4	60.0	10.0	25.6	2.2
Zeatin	1	40.0	12.0	20.0	1.4
	2	80.0	22.0	40.5	1.8
	4	66.6	12.6	35.0	1.8
TDZ	1	80.0	10.0	40.0	1.0
	2	33.3	9.8	20.0	2.0
	4	50.0	5.0	12.2	2.5
BAP + NAA	1 + 0.5	60.0	5.2	15.0	2.5
	2 + 0.5	40.0	4.2	11.2	2.2
	4 + 0.5	40.0	4.0	10.0	1.8
Kn + NAA	1 + 0.5	40.0	5.0	12.0	2.0
	2 + 0.5	40.0	3.2	11.0	2.5
	4 + 0.5	20.0	2.0	6.0	2.8

The length of shoots as well as shape and size of the leaves were also affected by the nature of the cytokinin used as shown in table 6.

Table 6.

Growth regulators	Length of shoots	Appearance of leaves
BAP	Short (1.8-2 cm)	Very small leaves
Kn	Long (4-4.5 cm)	Long, thin, slender, grass like leaves
Zeatin or TDZ	Intermediate (2-3cm)	Long, broad, swollen leaves

Axillary bud and Shoot tip Culture

Fig. 37	Axillary bud break from nodal explant on BAP (4 mg/L) after 7 days of inoculation.
Fig.38	4-5 shoots proliferated from the nodal explant after 2 week on BAP (4 mg/L).
Fig. 39	Multiple shoot proliferation from nodal explant after 4 weeks of culturing on BAP (4 mg/L) supplemented medium.
Fig. 40	Proliferation of numerous shoots on BAP (4 mg/L) supplemented medium after 8 weeks of culturing.
Fig. 41 & 42	Formation of uncountable shoots on BAP (4 mg/L) supplemented medium after 2 and 4 weeks of subculturing.

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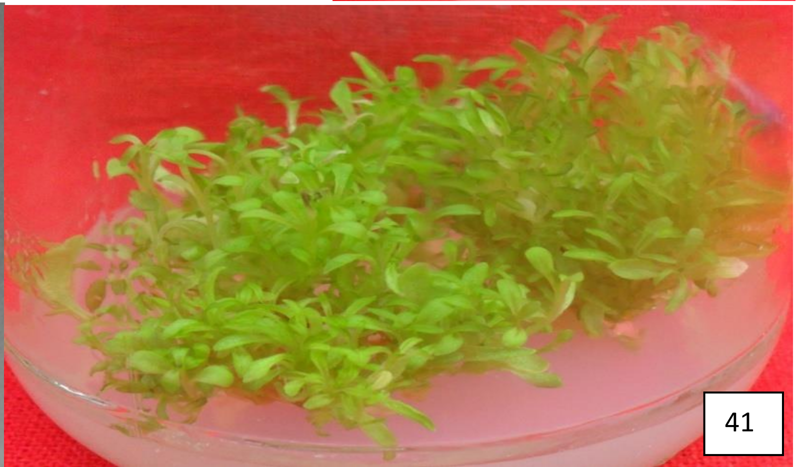
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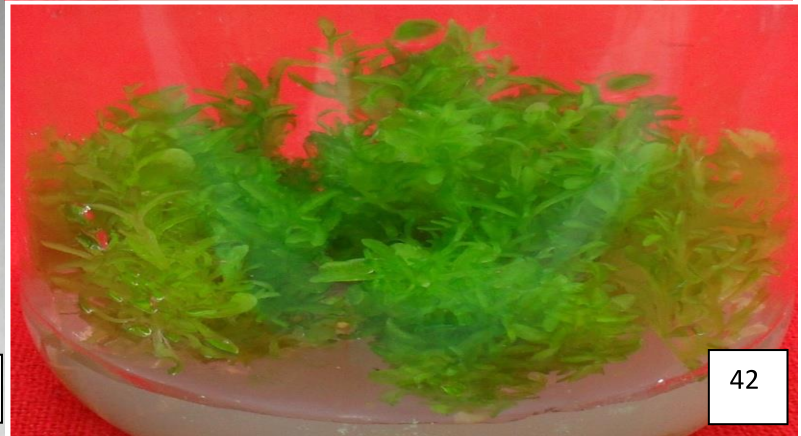
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Fig. 43 Axillary bud break from nodal explant on Kn (4 mg/L) supplemented medium after 8 days of inoculation.

Fig.44 4-5 shoots proliferated from the nodal explant after 2 weeks on Kn (4 mg/L).

Fig. 45 Multiple shoot proliferation from nodal explant after 4 weeks of culturing on Kn (4 mg/L) supplemented medium.

Fig. 46 Further proliferation of multiple shoots on Kn (4 mg/L) supplemented medium after 8 weeks of culturing.

Fig. 47 Formation of uncountable number of shoots on 4 mg/L Kn after 4 weeks of subculturing.

Fig. 48 & 49 Effect of different concentration and combinations of growth regulators on multiple shoot proliferation from nodal explant.

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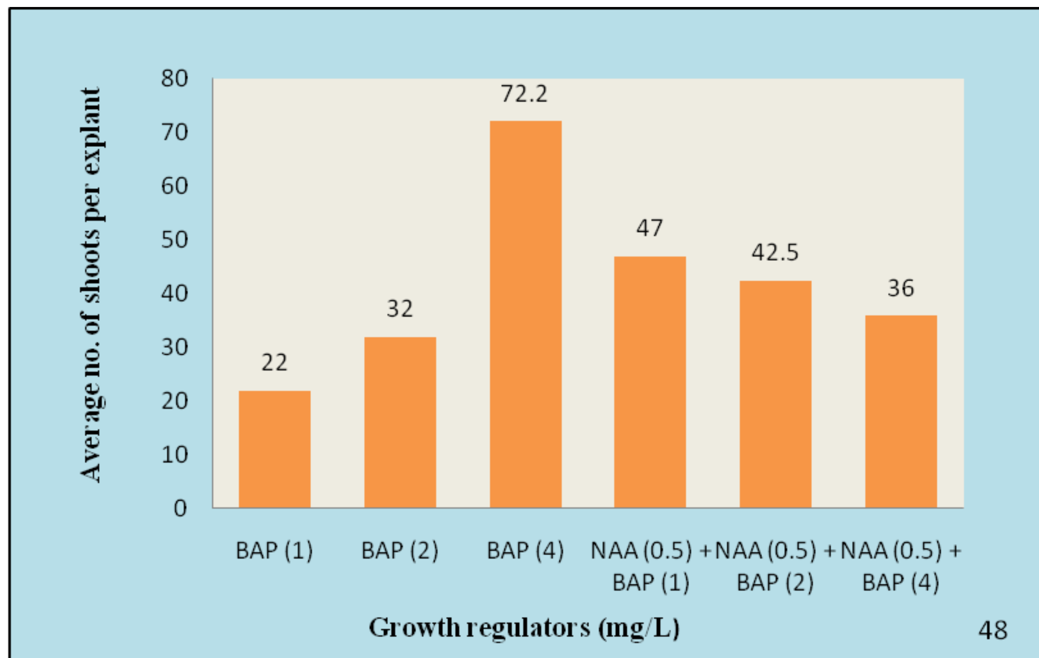
80
70
60
50
40
30
20
10
0
Average no. of shoots per explant
22
32
72.2
47
42.5
36
BAP (1)
BAP (2)
BAP (4)
NAA (0.5) + BAP (1)
NAA (0.5) + BAP (2)
NAA (0.5) + BAP (4)
Growth regulators (mg/L)
48

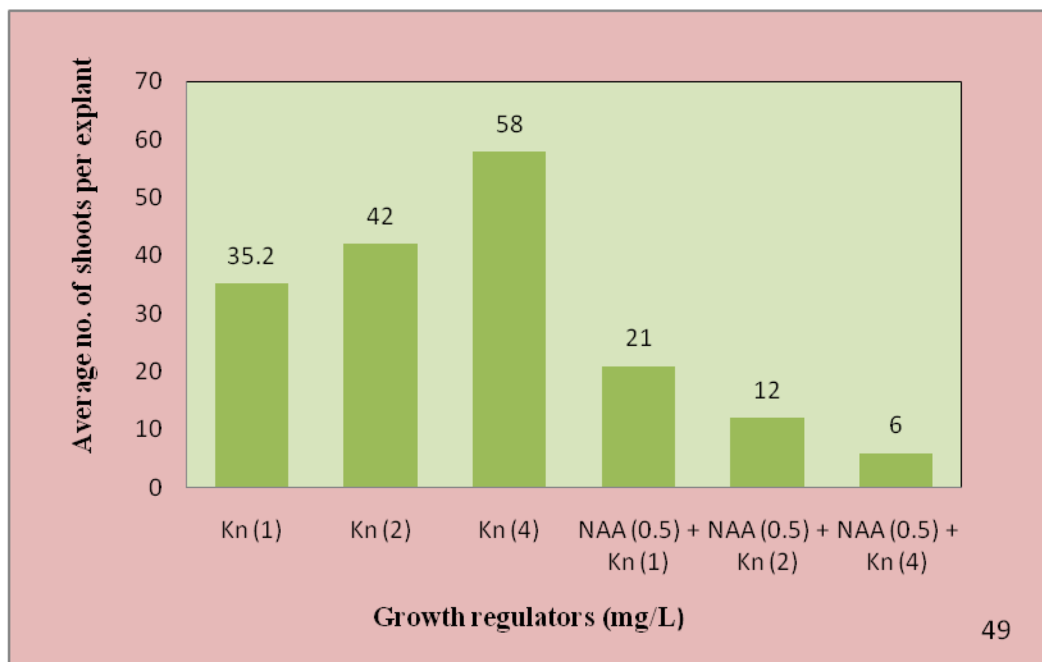
70
60
50
40
30
20
10
0
Average no. of shoots per explant
35.2
42
58
21
12
6
Kn (1)
Kn (2)
Kn (4)
NAA (0.5) + Kn (1)
NAA (0.5) + Kn (2)
NAA (0.5) + Kn (4)
Growth regulators (mg/L)
49

Fig. 50 Nodal bud break after 4 days of inoculation on Zeatin (1 mg/L) supplemented medium.

Fig. 51 Shoot proliferation from nodal explant after 4 weeks on the same medium.

Fig. 52: Multiple shoot proliferation from nodal explant after 8 weeks on Zeatin (1 mg/L) supplemented medium.

Fig. 53: Formation of numerous shoots after 4 weeks of subculturing Zeatin (1 mg/L).

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Fig. 54 Nodal bud break after 4 days of inoculation on TDZ (1 mg/L) supplemented medium.

Fig. 55-56 Multiple shoots proliferation from nodal explant after 4 and 8 weeks on TDZ (1 mg/L) supplemented medium.

Fig. 57 Proliferation of multiple shoots after 4 weeks of subculturing on TDZ (1 mg/L) supplemented medium.

Fig. 58 Effect of different concentrations on TDZ and Zeatin on multipole shoot proliferation from nodal explant.

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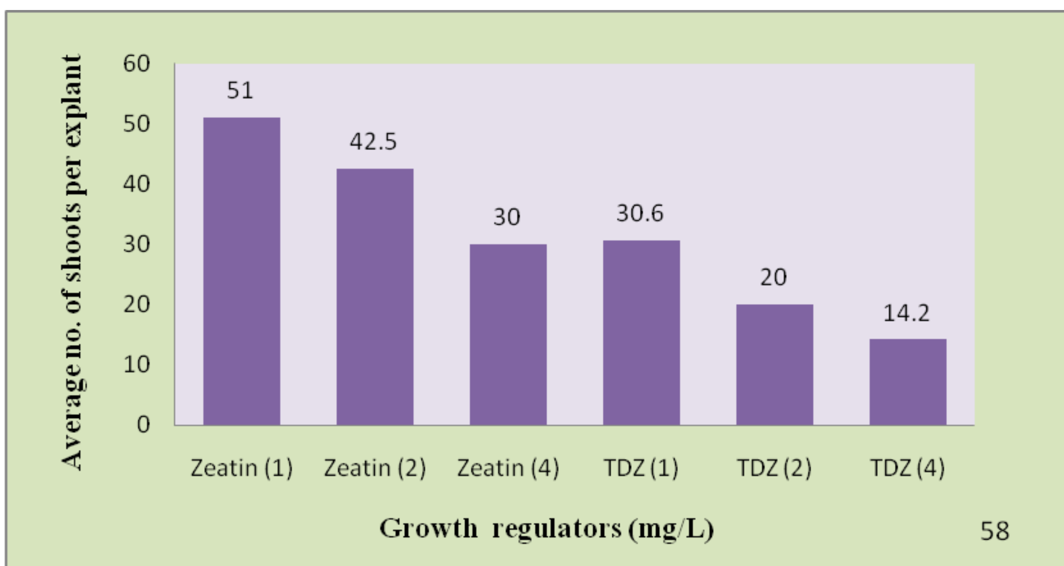
Average no. of shoots per explant
60
50
40
30
20
10
0
51
42.5
30
30.6
20
14.2
Zeatin (1)
Zeatin (2)
Zeatin (4)
TDZ (1)
TDZ (2)
TDZ (4)
Growth regulators (mg/L)
58

Fig. 59 Bud break from shoot tip after 7 days of inoculation on BAP (4 mg/L) supplemented medium.

Fig. 60 Proliferation of multiple shoots from shoot tip after 8 weeks on BAP (4 mg/L).

Fig. 61 Formation of numerous shoots from shoot tip after 4 weeks of subculturing on BAP (4 mg/L) supplemented medium.

Fig. 62 & 63 Multiple shoot proliferation from shoot tip on Kn (4 mg/L) supplemented medium after 4 and 8 weeks respectively.

Fig. 64 Proliferation of multiple shoots from shoot tips on Zeatin (2 mg/L) supplemented medium.

Fig. 65 8-weeks-old shoot tip showing multiple shoot formation on MS + TDZ (1 mg/l) .

Fig. 66 Response of various concentrations and combinations of growth regulators on multiple shoot proliferation from shoot tip explant.

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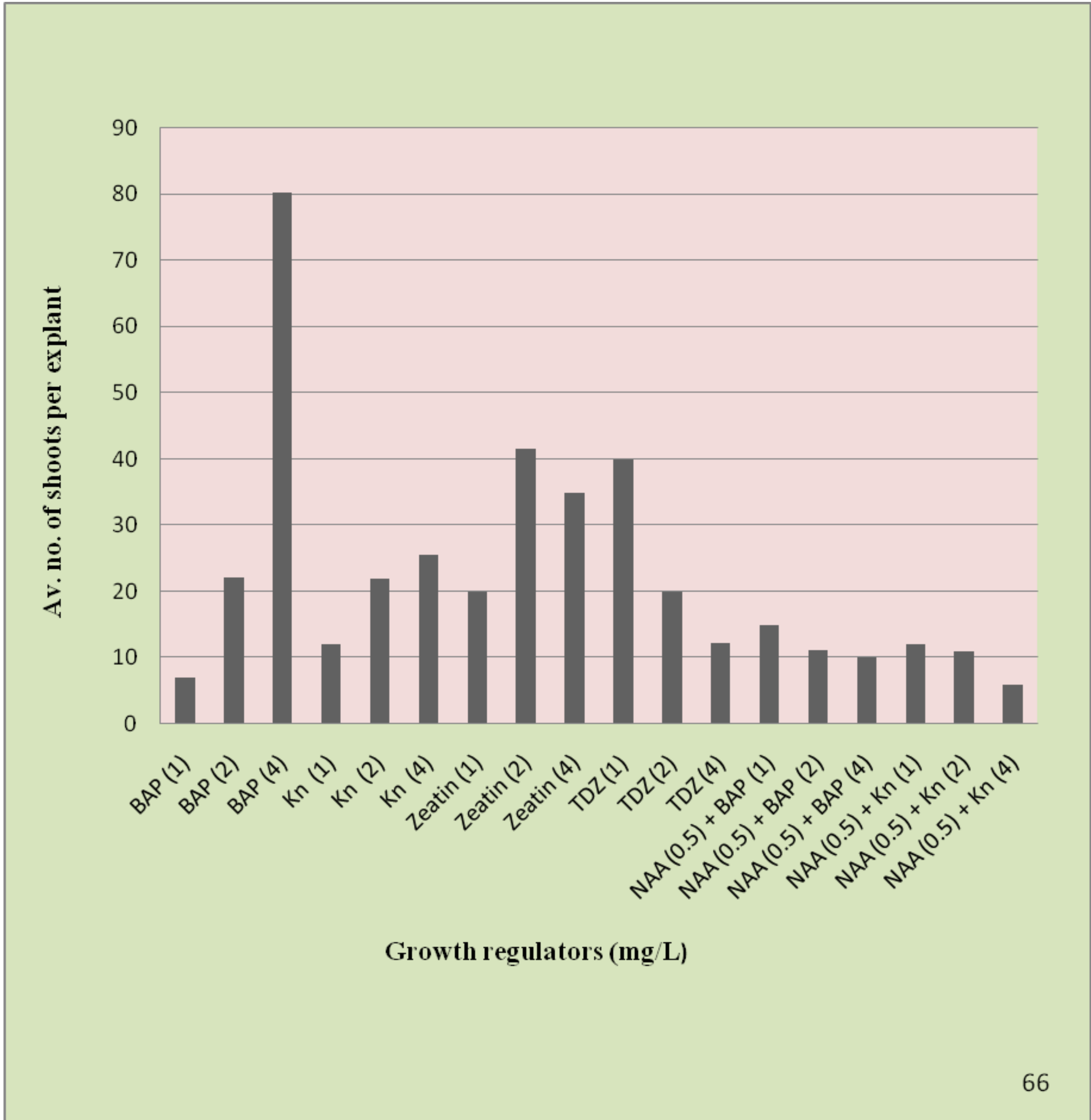

90
80
70
60
50
40
30
20
10
0
Av. no. of shoots per explant
BAP (1)
BAP (2)
BAP (4)
Kn (1)
Kn (2)
Kn (4)
Zeatin (1)
Zeatin (2)
Zeatin (4)
TDZ (1)
TDZ (2)
TDZ (4)
NAA (0.5) + BAP (1)
NAA (0.5) + BAP (2)
NAA (0.5) + BAP (4)
NAA (0.5) + Kn (1)
NAA (0.5) + Kn (2)
NAA (0.5) + Kn (4)
Growth regulators (mg/L)
66

Stem culture

Stem segments were excised from field grown plants of *Dianthus chinensis*. After sterilization, the damaged stem tissues on both sides were cut off and stem segments 4-5 mm in length were cultured on variously supplemented MS medium.

Direct *de novo* adventitious root and shoot formation

Among the various auxins tried, *de novo* adventitious root formation from stem segments occurred only on lower concentrations of IBA (0.5-1 mg/L). The roots were long, thick, bore root hairs and were few in number (fig. 67)

Out of the variously tested cytokinins, only MS medium supplemented with BAP (2 mg/L) was noticed to be effective in generating shoots from the stem segment. Initially 4-5 shoots initiated from the cut end after 4 weeks of culturing and the number increased enormously to 20-22 shoots within 8 weeks (fig.68)

Callus induction from stem segments and organogenetic differentiation

For callus induction, stem segments were cultured on MS medium supplemented with different concentrations of NAA, IBA, IAA and 2,4-D (0.5-4 mg/L), BAP or Kn (1 mg/L) either alone or in combination with each other. Best growth of callus occurred on IBA (2-4 mg/L) supplemented medium where callusing occurred in 100% of the cultures. The callusing initiated at the cut ends of the stem segment after 2 weeks of culturing (fig. 69) and within 5 weeks the entire explant turned into a mass of hard, compact and green callus (fig. 70). Likewise synergetic action of NAA (2-4 mg/L) with Kn or BAP (1 mg/L) with or without the incorporation of CM were also effective in the initiation and sustained growth of callus.

Stem segment failed to show callus induction on MS supplemented with 2, 4-D (2-4 mg/L) either alone or in conjunction with BAP or Kn (1 mg/L) .Effect of different concentrations and combinations of growth regulators on callus induction from stem segment is depicted in table 7.

Study of callus

The stem callus was hard and compact and the microscopic examination revealed its heterogenous nature. The cells were oval, round and elongated each with numerous starch

grains scattered throughout the cytoplasm. Microscopic observation of 4 week old callus revealed differentiation of tracheids, which occurred singly or in groups and possessed reticulate thickenings on their walls (fig. 71).

Organogenetic differentiation from the callus

Rhizogenesis

Root differentiation occurred in 100% of callus cultures on IBA (4 mg/L) supplemented medium where root differentiation occurred after 5 weeks (fig. 72). Likewise profuse root differentiation also occurred on NAA (2-4mg/L) + BAP or Kn (1 mg/L) with or without the addition of CM (15%) as depicted in figure 73 and table 7. The roots were thin, white and bore profuse root hairs.

Table 7.

Growth regulators	Concentration (mg/L)	% of explants induced callus	Degree of callus formation	Rhizogenesis	
				% of rhizogenesis	Mean no. of roots per culture
NAA	0.5	100.0	++	87.5	8.3
	1	100.0	++	100.0	12.75
	2	66.6	+++	66.6	17.0
	4	88.8	+++	88.8	19.0
IBA	2	100.0	++++	75.0	5.8
	4	100.0	++++	100.0	12.0
NAA + BAP	2 + 1	40.0	+++	40.0	7.0
	4 + 1	40.0	+++	40.0	7.0
	2 + 1 + CM (15%)	66.6	+++	40,0	8.0
	4 + 1 + CM (15%)	100.0	+++	40.0	20.0
NAA + Kn	2 + 1	50.0	+++	50.0	13.0

++ = moderate; +++ = good; ++++ = excellent callus growth

Caulogenesis

Solid culture

Excellent shoot differentiation occurred when stem calli raised on IBA (2 mg/L) supplemented medium was transferred to BAP (4 mg/L). Initially 6-7 shoot/ callus mass were formed after 15 days of culturing (fig. 74) which further proliferated forming large clusters

consisting of an average of 37.5 shoots per flask after 6 weeks in nearly 75% cultures (figs. 75-76).

Liquid culture

Callus raised on solid IBA (2 mg/L) supplemented medium when transferred to liquid MS medium containing BAP (4 mg/L) exhibited excellent shoot organogenesis. Initially emergence of few shoot regenerants was observed after 14 days and the entire mass of callus differentiated into clusters of shoots forming an average of 25 shoots per flask after 6 weeks in 66.6% cultures as shown in figures 77-78 and table 8.

Table 8.

Growth regulators (mg/L)	No. of explants cultured	% of caulogenesis	Mean no. of shoots per callus	Mean length of shoots (cm)
BAP (4 mg/L) gelled medium	8	75.0	37.5	6.0
BAP (4 mg/L) liquid medium	8	66.6	25.0	2.2

Rooting of microshoots and acclimatization

Regenerated shoots formed from different vegetative parts were carefully rescued from the culture vessels and were inoculated upright on rooting medium which comprised of BMS medium alone or supplemented with the auxins namely IAA or NAA (4 mg/L). Best root initiation occurred on BMS medium after 15 days of culturing where 8-9 healthy roots emerged in nearly 60% of cultures The roots multiplied further forming a bunch of thin white roots (fig.79). Development of roots from microshoots also occurred on NAA or IAA (4 mg/L) supplemented medium, where 3-4 very thin roots appeared after 10 days of culturing in 40% of the cultures. Figure 80 shows a complete plantlet with well developed root and shoot system.

Effect of BMS medium and auxins on root induction is shown in table 9.

Table 9.

Growth regulators (mg/L)	No. of cultures	% of explants forming roots	Mean no. of roots	Mean length of roots (cm)
BMS (hormone free)	5	60	12.0	9.0
NAA (4 mg/L)	5	40	9.5	4.0
IAA (4 mg/L)	5	40	6.0	3.5

For acclimatization, plantlets were carefully rescued from the culture bottles, washed under running tap water to remove traces of agar sticking to it. They were initially transferred to the plastic cups containing potting mixture of soil: vermicompost (1:1) covered with perforated polythene bags and were kept inside the growth room for 2 weeks (figs. 81-82). The plantlets were further shifted to polybags containing same potting mixture and were kept inside the growth room for another 2 weeks. The plants were monitored and watered regularly and hardened plantlets were then shifted to green house.

Attempts are underway to transfer acclimatized plantlets to the natural field conditions.

Stem Culture

Fig. 67 Direct regeneration of numerous long roots from stem explant on MS + IBA (1 mg/l) after 11 days of culturing.

Fig. 68 Direct regeneration of numerous shoots from stem explant on MS + BAP (2 mg/l) after 5 weeks of culturing.

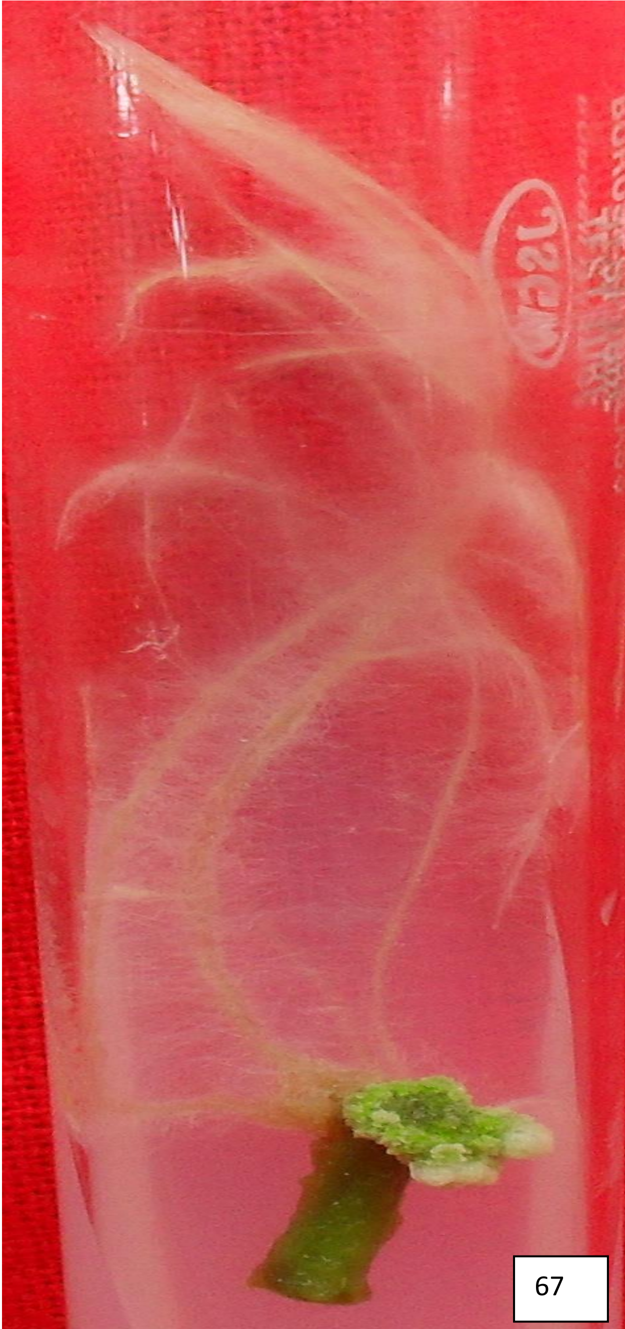
Fig. 69 Callus induction from cut end of stem segment on IBA (4 mg/L) supplemented medium after 2 weeks of culturing.

Fig.70 Whole of the stem segment transformed into a mass of green callus after 5 weeks of culturing.

Fig.71 Group of tracheids isolated from stem callus shown on magnified scale showing reticulate thickening on the walls.

Fig.72 Differentiation of roots from stem callus on IBA (4 mg/L) supplemented medium after 5 weeks of culturing.

Fig. 73 Regeneration of roots from stem callus formed on MS + 2, 4-D (2mg/L) + Kn (1 mg/L) + CM (15%) after 5 weeks of culturing.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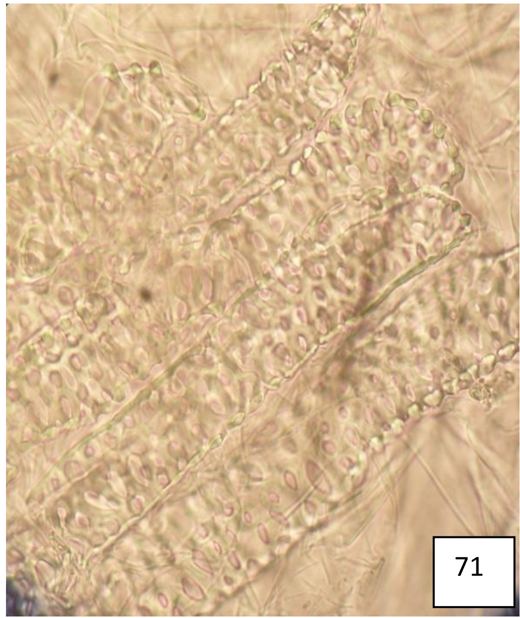
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Fig. 74	Regeneration of shoots from stem callus raised on IBA (2 mg/L) when transferred to BAP (4 mg/L) agar gelled supplemented medium after 2 weeks of culturing.
Fig.75 & 76	Further proliferation of shoots after 4 and 6 weeks respectively on BAP (4mg/L) supplemented medium.
Fig. 77	Differentiation of green leafy shoots on liquid MS supplemented with BAP (4mg/L) after 2 weeks.
Fig.78	Further growth and proliferation shoots in large number.

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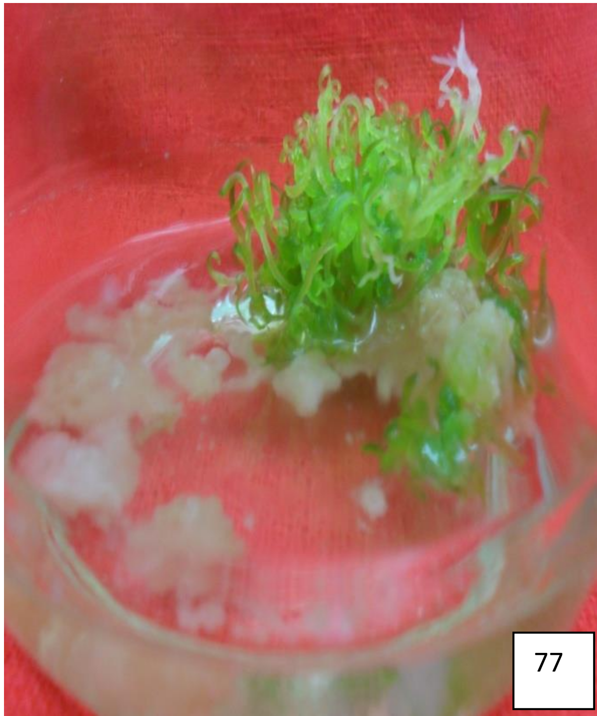
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Fig. 79	Cluster of roots formed at the base of shoots on basal MS medium after 2 weeks.
Fig. 80	An isolated complete plantlet showing well developed root and shoot systems.
Fig. 81&82	Plantlets in plastic cups containing potting mixture under growth room conditions.

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PRECISION SCALE
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CHAPTER-5
DISCUSSION

The present investigation was undertaken on a horticulturally important plant *Dianthus chinensis* var. *heddiwigii* with a view to develop an efficient, reliable and reproducible protocol for its mass clonal propagation under *in vitro* conditions. The different vegetative plant parts viz. shoot apices, axillary buds, stem and leaves were excised from field grown healthy plants and thereafter cultured on variously supplemented Murashige and Skoog's medium for multiple shoot proliferation, *de novo* adventitious shoot and root formation, callus induction and organogenesis.

Axillary shoot proliferation, used primarily to clone selected individuals, has much potential as supporting technology for micropropagation of ornamental plants. *In vitro* shoot formation from nodal segments and shoot tips is useful in producing true to type plants and in maintaining genetic stability of the plants. Type and concentration of growth regulators as well as the different combinations in which they are used, significantly effect the axillary shoot proliferation. Cytokinins form a major class of growth regulators and are considered as a major factor affecting shoot proliferation from the explants (Sterk *et al.* 2003, Mishra *et al.,* 2005, George *et al*., 2008, Warrier *et al.,* 2010). A wide range of cytokinins like Kinetin, BAP, Zeatin and TDZ has been employed in shoot proliferation (Bhojwani and Razdan, 1983, Viji *et al*., 2010).

In the present investigation, multiple shoot proliferation from nodal segments and shoot apices of *Dianthus chinensis* was observed on MS medium containing BAP, Kn, TDZ and Zeatin either alone or in combination with NAA. Best proliferation from nodal segments and shoot apices occurred on BAP (4 mg/L) supplemented medium. Uncountable number of multiple shoots was formed after subsequent subculturing without any decline in the growth thereafter. Other cytokinins like kinetin, Zeatin and TDZ were not equally, but also effective in generating multiple shoots from nodal segments and shoot apices

The effectiveness of cytokinin especially BAP in promoting axillary shoot proliferation is well documented in many ornamental plants e.g. *Rosa indica* (Hameed et.al.2007), *Rosa canina* (Shirdel *et.al.*2013), *Rosa hybrida* (Bayanati et al. 2013), Gladiolus *grandiflorus*

(Priyakumari et al. 2005; Haula et.al.2012 ;), *Dianthus caryophyllus (*Brar et.al.1996*;* Daniel *et.al.*2009; Ali et al., 2008) and *Chrysanthemum morifolium* (Waseem et al. 2009,2011).

Presently, cytokinins (BAP or Kn) in combination with lower concentrations of auxins (NAA) also effectively induced shoot proliferation from nodal explants and shoot tips. Data under discussion is well supported by observations made by Pareek et al. (2004) who also employed the combination of cytokinin BAP with auxin NAA for inducing bud break, growth and multiple shoot proliferation from shoot tips and nodal explants of *Dianthus chinensis*. Best reponse in terms of multiple shoot formation was observed on MS medium with NAA (0.5 mg/L) + BAP (1 mg/L) forming 10-15 shoots in four weeks. In contrast to this single report in which the formation of a few shoots have been reported, we have been able to induce mass production of shoots (80-100) under the present cultural conditions employed.

Direct *de novo* adventitious shoot formation from the organs is also regarded as the most reliable method for clonal propagation of ornamental plants as it upholds genetic uniformity among the progenies unlike those regenerated from callus tissue. In our study on *Dianthus chinensis*, a range of auxins either alone or in combination with cytokinins were employed for *de novo* adventitious shoot formation from leaf and stem segments. Out of all growth regulators tried, zeatin as a lone growth regulator and synergistic action of BAP (1 mg/L) with NAA (0.5 mg/L) were most pronounced in forming healthy green shoots from leaf explants. MS containing 2mg/L BAP yielded significant number of shoots from stem segments. A wider survey suggests that BAP is the most reliable and effective cytokinin employed for shoot formation. Similar results showing the importance of BAP with NAA in forming good number of adventitious shoots have been studied in *Dianthus chinensis* (Kantia et al. 2002) and *Petunia hybrida* (Qaoud et al. 2010).

There are variable reports regarding the potentiality of differentiation of plant tissues in culture but none of the observations in the litrerature reported direct differentiation of roots from the leaf and stem explants of *Dianthus chinensis*. The current work reports for the first time direct root regeneration from leaf as well as stem segments when cultured on NAA, IAA or IBA either alone or in combination with Kn or BAP. Profuse rooting, however, occurred on incorporation of CM (15%) to NAA and Kn supplemented medium. The roots formed were white in color and bore profuse root hairs.

Majority of the plant tissues growing *in vitro* require exogenous hormones in the nutrient medium for dedifferentiation. The reaction of an isolated tissue to auxin depends upon its

endogenous auxin level at the time of excision and its genetic capacity for its synthesis. In the present work, the MS medium was supplemented with various concentrations of different auxins either alone or in conjunction wth Kn or BAP for optimal callusing of stem and leaf segments. The higher concentrations of IBA were reported to be best for callus induction both from leaf and stem segments. The callus formed was green, hard, compact and fast growing. The calli comprised of heterogenous population of cells exhibiting a wide variations in cell sizes and geometric shapes. In the present study, histogenetic differentiation was observed in the form of tracheids from calli showing reticulate thickenings on their walls. They were either loosely scattered or grouped together.

The present investigation demonstrated high organogenic potential of *Dianthus chinensis*, as it exhibited high efficiency shoot formation and plant regeneration from leaf and stem calli under *in vitro* conditions. Leaf and stem segments cultured on MS medium supplemented with higher concentrations of 2, 4-D or IBA (2-4 mg/L) exhibited excellent shoots differentiation from the callus. Stem callus produced on IBA (2 mg/L) when transferred to BAP containing solid or liquid media showed high frequency shoot induction. Earlier Kantia et al. (2002) have used combination of BAP and 2, 4-D for shoot bud induction along with excessive callus formation from leaf explants of *Dianthus chinensis*. A perusal of literature reveals similar observations in *Gladiolus* (Emek *et al. 2007;* Shaheenuzzaman *et al. 2011), Gerbera jamesonii* (Aswath *et al. 2002;* Altaf *et al. 2009)* and *Dianthus caryophyllus* (Jain et al. 2001).

Induction of root from basal end of *in vitro* regenerated shoot is the crucial factor for the success of any micropropagation protocol. Auxins play a vital role in inducing roots at the base of microshoots. In the current study ofn *Dianthus chinensis*, BMS alone or supplemented with auxins namely NAA and IAA were used for rooting of microshoots. The best response was observed on auxin free BMS medium where 8-9 roots were formed followed by NAA and IAA. Both the observations are well supported by references from the literature in *Dianthus caryophyllus* (Jain et al. 2001;Ali et al. 2008), *Dianthus* (Pareek et al. 2004), Anthurium (Farsi, et al. 2012) and *Chrysanthemum morifolium* (Waseem et al. 2009).

In vitro propagation system provides a method for the rapid production of plants but its ultimate success depends upon the successful transfer and establishment of these plants in the field conditions. Plants produced under *in vitro* conditions under controlled high humidity, diffused light and constant temperature need to be acclimatized because transferring of these

plants from *in vitro* to *ex vitro* conditions is the most traumatic experience for them. Direct transfer of tissue culture raised plants to field conditions is not possible due to high mortality rate as the plants kept under controlled environmental conditions have heterotrophic mode of nutrition and uncontrolled loss of water. It is therefore necessary to transfer the plants to field through various hardening stages to increase the survival percentage.

The plantlets thus formed were carefully rescued and acclimatized through various hardening stages upto green house bench. Attempts are underway to transfer acclimatized plantlets to the natural field conditions.

Conclusion

The present work which is an attempt to investigate the dedifferentiation and redifferentiation responses of various cells of *Dianthus chinensis* corroborates the concept of totipotency that every cell of the plant body is totipotent and can express its hidden morphogenetic potentialities with diverse chemical milieu. It is concluded that a successful micropropagation protocol for mass cloning through multiple shoot proliferation and *de novo* adventitious shoot formation directly from different explants and through intervening callus phase has been achieved in *Dianthus chinensis*.

CHAPTER-6

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