A micropropagation system for Carnation (*Dianthus caryophyllus*) - an important ornamental plant

Thesis submitted in partial fulfillment for award of the degree of
Master of Science in Biotechnology

By

Divya Jaggi

Registration No. 301101011

Under the supervision of:

Dr. Manju Anand

Assistant Professor

Department of Biotechnology and Environmental Sciences

Thapar University

Patiala -147004

July-2013

CONTENTS

CHAPTER

ACKNOWLEDGEMENT

At this platform, I am deeply privileged to evince a word of thanks to everyone who played a positive role in the completion of my project. I thank the almighty whose blessings have empowered me to accomplish my work successfully.

Words fail to express my sincere and deep sense of reverence to my advisor Dr. Manju Anand, Deptt. of Biotechnology and Life sciences, who with her endless patience, constant encouragement, constructive criticism and affectionate attitude made me work on the project. Without her assistance & guidance, it would have been indeed difficult for me to shape up this work. Her unflinching courage and conviction will always inspire me, and I hope to continue to work with her noble thoughts. I am highly obliged to her for the cordinal atmosphere in which she guided me throughout the period of work.

I am also extremely indebted to Dr. M.S. Reddy, Head, Deptt. of Biotechnology and Life sciences for providing necessary infrastructure and resources to accomplish my research work. I am also thankful to all the faculty and staff members of the Department of Biotechnology and Environmental Sciences for providing me all the facilities required for the completion of this work.

I feel lacuna of words to express my gratefulness and indebtness to all my colleagues especially for their help, support and understanding. I owe a word of thanks to all the research scholars and lab assistants for their help. The heavenly grace of Almighty has defined and given meaning to my existence. My heart brims with gratitude for my Dad, Mom, sister and brother for their perpetual blessings, inspiration and love.

Divya Jaggi

301101011

CERTIFICATE

This is to certify that the thesis entitled **"A Micropropagation system for Carnation – an important ornamental plant"** submitted by Divya Jaggi (301101011) in partial fulfillment of the requirements for the award of degree of Masters of science in Biotechnology to Thapar Institute of Engineering and Technology (Deemed University), Patiala, is a record of student's own work carried out by her under our supervision and guidance. The report has not been submitted for the award of any other degree or certificate in this or any other University or Institute.

Manju Anand

(Dr.Manju Anand)
Supervisor
Deptt. Of Biotech and Env.Sciences
Thapar Institute of Engg. And Tech,
PATIALA

(Dr. M.S.Reddy)
Head
Deptt. Of Biotech and Env. Science
Thapar Institute of Engg. And Tech,
PATIALA

Dean (Academic Affairs)
Thapar Institute of Engineering & Technology
PATIALA

Abbreviations

BAP	Benzylamino purine
MS	Murashige and Skoog’s medium
CM	Coconut Milk
IAA	Indole-3- Acetic acid
IBA	Indole 3-bnutyric acid
Kn	Kinetin
NAA	Naphthalene Acetic acid
CH	Casein hydrolysate
TDZ	Thidiazuron
µM	Micromolar
mM	Millimolar
2,4-D	2,4-dichloro phenoxy acetic acid
^{o}C	Degree Celsius

ABSTRACT

The present investigation was carried out on an important ornamental plant *Dianthus caryophyllus* belonging to family Caryophyllaceae. The different vegetative parts i.e. nodal explant, shoot apices, stem and leaves were excised from field grown mature plant and thereafter planted on variously supplemented Murashige and Skoog's medium for multiple shoot proliferation, callus induction and *de novo* adventitious root and shoot formation

Dianthus exhibits a good degree of propensity of multiple shoot proliferation from nodal and shoot apex segments. Multiple shoot proliferation from nodal explants was observed on MS medium supplemented with BAP(4.4-8.8µM) or Kn(4.65-9.30 µM)either alone or in conjunction with lower concentrations of NAA(1.47-7.35µM). Out of all the combinations tried, best results were, however, obtained on MS+BAP(4.4µM) where 16-18 shoots were formed from single axillary bud and shoot apex.

The present material had a great organogenic potential as it exhibited a high efficiency of *de novo* adventitious root and shoot formation directly from the stem segment. Profuse growth of lateral roots occurred on the entire surface of stem segment along with adventitious shoot formation on lower concentrations of NAA(7.35µM). These roots were short and bore root hairs profusely. Further incorporation of CM(15%) to the NAA supplemented medium considerably enhanced the rooting where entire segment was covered over with clumps of roots. The roots were thin, white having dense root hairs. A high efficiency of adventitious shoot formation from the stem segment was observed. Stem segments cultured on MS medium supplemented with NAA(1.47-7.35µM) either alone or in conjunction with BAP(4.4µM) or Kn(9.30µM) and MS + BAP(4.4µM) or MS+ Kn(9.30µM) produced adventitious shoots directly on the surface of stem explants without the formation of intervening callus.

The regenerated shoots thus formed were excised and transferred to different root inducing media to form complete plantlets. Among the various growth regulators tested, NAA(7.35µM) showed the best results where rooting occurred. Attempts are underway to establish regenerated plantlets into the soil.

Callusing of the nodal segment, shoot apices, leaf and stem segments occurred immediately after culturing on optimal chemical milieu. Murashige and Skoog's agar gelled medium supplemented with NAA (29.4 µM), with or without CM (15%) or 2,4-D (19.48 µM) + Kn(4.65 µM) turned out to be optimal for initiation and sustained growth of calli from different plant parts. The calli were pale yellow, friable and fast growing. Formation of green friable callus from the leaf explant on 2,4-D (4.87µM) + BAP(0.88 µM) + 2000mg/l casein hydrolysate was the best medium reported.

The callus thus formed from different vegetative parts on the medium were more or less identical in morphology. The calli obtained from all the parts were heterogenous being composed of parenchymatous ovoid, oblong, semicircular cells or those with aberrant shapes. Histogenetic differentiation in the form of tracheids was observed in all the calli. Tracheids occurred singly or in groups and possessed scalariform thickenings on their walls. No organogenetic differentiation could be effected from the calli on the various media tried.

INTRODUCTION

ORNAMENTAL PLANTS – AN OVERVIEW

Plants have formed part of the human existence since time immemorial. This enduring bond between mankind and plants has flowered into a profound human appreciation of plants as objects of beauty and of gardens as works of art. The connection between man and ornamental plants is as old as its civilization. From 1970s onwards there has been a remarkable resurgence in worldwide interest in ornamental plants which resulted in renewed efforts to search for and develop new ornamental plants. Ornamental plants bring aesthetic, physical and psychological enhancements to our surroundings and add economic value to them. In a world that is increasingly becoming urbanized, ornamental plants provide an important link with the natural world (Middleton *et. al*. 2011).

Since ancient times ornamental plants have been an integral part of life with gardens, flowers, and ornamental horticulture being noted in most of our historical references. Garlands of olive leaves were wore by the roman soldiers and lotus blossoms decorated the Egyptian royalty. Backyard growing of flowers dates back to ancient times like the Ramayana and Mahabharata. Ornamental plants are important aesthetically and economically. Aesthetically, they enhance the beauty of the surroundings. They are used as decorative materials in constructing a lawn, park, plaza and various kinds of gardens. Importance of flowers is not restricted to the beautification, decoration or preparation of gajra, garland, veni or bouquets but also have the industrial importance. Some flowers like Rose, Jasmines, Tuberose, Kevda are used for extraction of essential oils which is base for preparation of perfumes, scents or attar. Imanishi *et.al.* (1992) stressed that flowers are indispensable items and influence human feelings substantially. Throughout history flowers have been an instrument for celebrating special occasions of the moment and for expressing emotions. Flowers are unique in that their use is often a means for communicating in ways that word alone cannot convey. 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).

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 sheer number and the variety of ornamental plants is overwhelming. It has been estimated that developed countries in Europe, America and Asia account for more than 90 per cent of the total world trade in floriculture products. Ornamental plants synthesize a variety of biochemical products mainly which are extractable and used as raw material for various scientific investigations (Schmidt *et. al*.2006).

The raising of flowering and non-flowering ornamental plants is a thriving business in the cities and urban areas. A big volume of cut flowers is sold daily in flower shops to form bouquets, corsages, and wreaths. Among the most popular flowers sold are varieties of roses, orchids, chrysanthemums, calla lilies. Among the non- flowering ornamentals, the ferns and asparagus make high sales because they are needed in various flower arrangements.

Some ornamental plants are grown for showy foliage. Their foliage may be deciduous, turning bright orange, red, and yellow before dropping off in the fall or evergreen, in which case it stays green year round. Some ornamental foliage has a striking appearance created by lacy leaves or long needles, while other ornamentals are grown for distinctively colored leaves, such as silvery-gray groundcovers and bright red grasses, among many others(European Commission, General for agriculture and rural development, 2006)

Other ornamental plants are cultivated for their blooms. Flowering ornamentals are a key aspect of many gardens, with many flower gardeners preferring to plant a variety of flowers so that the garden is continuously in flower through the spring and summer. Depending on the types of plants being grown, the flowers may be subtle and delicate, or large and showy, with some ornamental plants producing distinctive aromas which paint a palette of scents in addition to colors (Ley et.al. 2002)

PRODUCTION AND CONSUMPTION OF ORNAMENTAL PLANTS

Floriculture is a major global industry in both developing and developed countries. The world production of floriculture is growing at a rate of 10 per cent per year. There are currently over 50 countries that are active in floriculture production on a large scale. The total area under floriculture in the world, both under protected area and open, is presently estimated to be around 628,972 hectares. In terms of production value, the Netherlands, the United States, Japan, Italy, Germany and Canada are the largest producers of cut flowers and plants. Some of the major developing country producers and exporters of cut flowers and plants are Colombia, South Korea, Kenya, Israel, Ecuador, Poland, Ethiopia,Costa Rica, Thailand, India, China, Zimbabwe, and Mexico. With China (286,068 ha) and India (161,000 ha) having the majority of the world acreage under cut flowers and plants production, the Asia-Pacific region has the major share (75 percent) of the total world area under floriculture production (Starman et.al. 1995).

Europe, the USA and Japan are the major consumers of the floriculture products. The major consumer markets are Germany (22%), the US (15%), France (10%), the UK (10%), the Netherlands (9%), Japan (6%), Switzerland (5%) and Italy (5%). In case of cut flowers, per capita consumption in Japan is the highest, followed by Europe and USA**.** The European Union (EU) consumes over 50 percent of the world's flowers, and includes many countries, which have a relatively high per capita consumption of cut flowers. Germany is the major consumer, followed by the UK, France and Italy (Getu, 2009).

WORLD FLORICULTURE TRADE

Floriculture is a booming industry today. Global trade volume is estimated to be worth more than $100bn per annum. Cut flowers represent the largest segment of the industry followed by flowering pot plants, tree and nursery crops, flower bulbs, and other propagation material. Developed countries in Europe, America and Asia account for more than 90 percent of the total world trade in floriculture products. Germany is the main market for imports and the Netherlands is the world's leading exporter followed by Colombia, Italy, and Israel. Asia-Pacific countries are the main suppliers to Japan and Hong Kong. African and other European countries are the principal suppliers to Europe's main markets, and the supplies to the USA are mainly catered by Colombia and Ecuador.

The global exports of floriculture products stood at about US$ 17 billion in the year 2007. Fresh cut flowers and foliage accounted for around 49.1 percent (US$ 8.31 billion), and live plants, bulbs and cuttings accounted for 50.9 percent (US$ 8.60 billion) of total floriculture products exported in 2007. Roses contribute around 70 percent of the total cut flower industry trade. The Netherlands continues to dominate the world floriculture industry, accounting for 49.6 percent (US$ 8.56 billion) of world floriculture exports in 2007. Colombia is the second largest exporter of floricultural products in the world with a share of around 6.5 percent (US$ 1.12 billion).

Germany (US$ 2.59 billion) was the largest importer, followed by the United Kingdom (US$ 1.89 billion), USA (US$ 1.81 billion), the Netherlands (US$ 1.55 billion), and France (US$ 1.43 billion)(Starman et.al.1995). Europe as a region is the world's leading importer of flowers and foliage. Roses are the most popular flowers imported by Europe. Other flowers imported by Europe include tropical flowers, summer flowers and Orchids. Japan is the largest importer of floricultural products in the Asian region.

Floriculture in India

The growth in Indian floriculture cultivation has been phenomenal in the last few decades. After liberalization, the Government of India identified this activity as a sunrise industry and accorded it 100 per cent export-oriented status. Floriculture is an industry which has tremendous potential in India. The different types of climatic conditions provide for the possibility of growing almost all the major cut flower species of the world, either from tropical, subtropical or temperate climates. The Indian floriculture industry comprises the florist trade, nursery plants, potted plants, bulb and seed production, micro propagation material and extraction of essential oils from flowers.

Karnataka, Tamil Nadu, Andhra Pradesh, West Bengal, Maharashtra, Uttarakhand, Uttar Pradesh, Delhi, Haryana, Kerala, Himachal Pradesh and North Eastern states are the major flower growing states in India. Tamil Nadu is the largest loose flower producing state, while West Bengal is the leading cut flower producing state in India. According to the data compiled by ASSOCHAM(2012), Indian floriculture industry is growing at a compounded annual growth rate (CAGR) of about 30 per cent and is likely to cross the Rs 8,000-crore mark by 2015. Indian floriculture industry is very dynamic in its varieties and trade volumes demonstrating 6-9% of annual growth (Muthukumaran et al.,2008).

Rose is the principal cut flower grown all over the country. One estimate has projected out that rose flower industry in India has a share of about 65 per cent of the overall floriculture industry and in view of international trade it accounts for 75 per cent of the overall global floriculture industry (Talukdar *et al*.,2012). Other most important cut flower crops in the country are Gladiolus, Tuberose, Asters, Gerbera, Carnation, Anthurium, Lilium and Orchid. Important cut flowers exported from India include roses, lilies, carnations and orchids. In the recent years, dried flowers and foliage have been forming a large part of floricultural product exports from India. During, 2008-09 dried flowers constituted over 60 percent of cut flowers exports, and dried foliage constituted around 97 percent of total foliage exports from India. Europe continues to be the largest destination for Indian floriculture exports. However, in the recent years, Indian exports of floriculture products have also been to the Japanese and Australian markets.

Other associated activities of flower growing in India include the dry flower industry and the essential oil industry. India is the fifth largest exporter of dried flowers and second largest exporter of dried foliage in the world accounting for around 7 percent of world exports in dry flowers and foliage. The main export markets for India's dry flower industry are USA, Netherlands, UK and Germany. West Bengal accounts for around 70 percent of the dried flower exports from India. India is the second largest exporter of essential oil in the world after USA. India's exports of essential oil stood at US$ 298.7 million in 2007-08; during the period of April –February 2008-09, export of essential oil stood at US$ 329 million. India is the largest exporter of jasmine oil in the world accounting for over 40 percent of total world exports in jasmine oil (Muthukumaran, 2009)

PLANT TISSUE CULTURE IN ORNAMENTAL PLANTS:

Consumption and use of ornamental plants is increasing in day to day life. To meet the growing demands, propagation of numerous ornamental plants by tissue culture has become accepted commercial practice. The rapid strides achieved in the field of micropropagation of ornamental plants have been made possible by the research accomplishments of myriad dedicated scientists. No discussion of tissue culture propagation of ornamental plants can be presented without giving credit to the work of Morel, often referred to as the "Father of micropropagation." Morel's successful culture of apical and axillary buds of *Cymbidium* paved the way for rapid development of micropropagation technique. A wide range of species are routinely propagated by tissue culture methods that are listed in various publications and reports. Hence *in vitro* and mass propagation is the viable alternative and offers the opportunity to overcome many problems which are inherent: misidentification, genetic as well as phenotypic variability, toxic components and contaminants (Stem, 2009)

The use of *in vitro* propagation can overcome difficulties and can manipulate the phenotypic variation. There has been significant progress in the use of tissue culture to bring ornamental plants into successful commercial cultivation. For the naturally rare and slowly growing plant species, plant tissue culture provides a cost-effective, sustainable and well-controlled means for mass production of disease free planting material. Besides, the callus derived plants exhibit huge genetic variation that could be exploited for developing superior clones/varieties particularly in vegetatively propagated plant species.

Advantages of Micropropagation:

Advantages of *in vitro* propagation are as follow:

1.) Production of many plants that are clones of each other
2.) Production of secondary metabolites.
3.) New and improved genetically engineered plant can be produced.
4.) Plant available all year round (independent of regional or seasonal variation)
5.) Produce disease-free plants
6.) Produces rooted plantlets ready for growth, saving time for the grower when seeds or cuttings are slow to establish or grow.
7.) Only viable method of regenerating genetically modified cells or cells after protoplast fusion.
8.) Useful in multiplying plants which produce seeds in uneconomical amounts, or when plants are sterile and do not produce viable seeds or when seed cannot be stored
9.) Produces more robust plants, leading to accelerated growth

Techniques of Micropropagation

Three basic methods are used to propagate plants in vitro

1) **Multiplication by apical and axillary buds**

- Micropropagation through apical and axillary shoot proliferation is the most common method for commercial production.

Cells of the meristems are uniformly diploid and are least susceptible to genetic changes. Hence most reliable technique for mass propagation since it ensures genetic stability of clones.

2) ***De novo* formation of adventitious shoots**:

New adventitious shoots can develop either

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

or

b) Indirectly from callus cultures obtained from these explants.

Adventitious shoots arise naturally on plant tissues located in sites other than at the normal leaf axil regions. 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, flower parts or indirectly from the callus cultures obtained from these explants.

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

Somatic or nonzygotic embryogenesis

• Involves the formation of bipolar embryos which can develop into fully functional plants under appropriate conditions. These embryoids can develop into fully functional plants under appropriate conditions.

Stages in Micropropagation

Micropropagation involves 4 definite stages. These are as follow:-

Stage- I

Initiation and establishment of aseptic cultures:-

This involves explant isolation, surface sterilization and establishment on an appropriate culture medium. Cultures are initiated from explants of several organs but shoot apices and axillary buds are most often used for commercial micropropagation.

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 & adventitious buds.
- Somatic or nonzygotic embryogenesis directly on the explants or in callus cultures.

Stage- III

Rooting of regenerated shoots in *in vitro* conditions:-

This stage is characterized by preparation of stage II shoots or shoot clusters for successful transfer to the soil. The process may involve:-

- Elongation of shoots prior to rooting.
  Root formation.

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.

- Micropropagated plants are difficult to transplant for two primary reasons:-

    - ❖ A heterotrophic mode of nutrition.
    - ❖ Poor control of water loss.

To overcome these limitations, plantlets should be transplanted into a well drained, sterile growing medium and maintained initially at high relative humidity (90%) and reduced light at 20 to 27oc for the first 10-15 days by keeping them under mist or covering them with clear plastic bags. After spending few days under high humidity the plants should be moved to the green house bench. Transplants should then acclimatized by gradually lowering the relative humidity over 1 to 4 weeks period. Plants are gradually moved to higher light intensities to promote vigorous growth.

REVIEW OF LITERATURE

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 (Jain, 2002). While ornamental plants are produced mainly for their aesthetic value, the propagation and improvement of quality attributes such as leaf types, flower colour and fragrance, longevity and form, and the creation of novel variation are the important economic goals for the expanding ornamental industry(Jain *et.al*.2009). Plant Cell and tissue culture has already contributed significantly to crop improvement and has great potential for the future (Kumar and Kumar, 1996).

During the last few years, micropropagation has emerged as a promising technique for rapid and large scale propagation of selected plants. Micropropagation is infact the miniature version of clonal propagation, which is carried out under aseptic conditions. The technique of micropropagation is based on the concept of totipotency as proposed by Harberlandt. Every cell of the plant body is totipotent i.e. capable of giving rise to a new plant under proper nurture conditions. Ornamental industry has applied immensely *in vitro* propagation approach for large-scale plant multiplication of elite superior varieties. As a result, hundreds of plant tissue culture laboratories have come up, especially in the developing countries due to cheap labour costs. 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 three approaches

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

The first two approaches lead to plantlet formation via organogenesis through production of unipolar shoots which must then be 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 shoots:-

Axillary and apical shoots contain quiescent or active meristems depending upon the physiological state of the explants. 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 high cytokinin concentration, usually develop axillary shoots which can be subdivided into smaller clumps of shoots which in turn can develop similar clusters after subculturing 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. Udom *et.al*.(2009) reported multiple shoot proliferation from mature nodal segment of *Rosa hybrida* on BA(13.2 μM) + NAA(0.022 μM) where the highest no. of shoots per node was formed . Waseem *et.al.*(2009) established a protocol for *in vitro* propagation of *Chrysanthemum* from shoot tips on 0.48 μM IAA where maximum shoot initiation (86.6%) was recorded . Multiple shoot proliferation was also reported in *Rosa rugosa* from nodal segments on MS medium supplemented with BA 2.2 μM, NAA 0.054 μM, GA_3 2.0 μM and 3% sucrose by Xing *et.al*. (2010). *Haouala et. al.(2012)* developed a protocol for *in vitro* propagation of an ornamental plant *Gladiolus grandiflorus* from apical buds on MS supplemented with 2.46 μM IBA and 9.84 μM BA. In *Chrysanthemum,* Nalini (2012) reported multiple shoot proliferation from shoot tips on MS medium supplemented with Kinetin 13.85 μM and IAA 9.6 μM. 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).

Shirdel *et.al*.(2013) reported clonal multiplication of *Rosa canina* from axillary bud and MS medium supplemented with BAP (26.4 µM) was found to be the best medium.

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. Likewise, Ali *et.al*.(2008) reported the cytokinin BAP (4.4 µM) to be most effective in multiple shoot formation 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* . The highest number of shoots (5 shoots/explant) was formed on medium supplemented with 4.4 µM BAP + 1.47 µM NAA.

Multiplication by adventitious buds:-

Many ornamental plants 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.

Indirectly through callus

Dillen *et.al*.(1996) reported shoot regeneration in long term callus cultures derived from mature leaves as explants of *Cyclamen persicum.* Hsia and Korban (1996) reported organogenesis in callus cultures of *Rosa hybridia* and *Rosa chinensis minima*. 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 chinesis.* 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 .

Directly from the explant

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* and high effiecieny of shoot regeneration was reported on an average from 67% of the leaves, with an average of seven shoots per explants. 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*. Organogenesis has also been reported in a number of other ornamental plants. For instance, somatic cells of *Petunia hybrida* (Colijin *et.al*.1979), *Gladiolus*(Roy *et.al*.2006), *Euphorbia tirucalli* (Guohua Ma 2010), *Hygrophila polysperma* (Karats *et.al*.(2013),) can give rise to whole plantlets, thus demonstrating the totipotency of plant cells in an unambiguous terms.

Altvorst *et.al*.(1992) established a protocol of adventitious shoot formation from *in vitro* leaf explants of *Dianthus caryophyllus*. The highest no. of adventitious shoots was obtained on medium containing 4.4 µM BA and 2.205 µM NAA. Watad *et.al*.(1996) established an efficient method for regeneration of complete plant in *Dianthus caryophyllus* on agar-gelled medium, and the no. of shoots increased with increasing thidiazuron (TDZ) concentration (up to 4 mg/l) and naphthaleneacetic acid(7.35 µM). Arici *et.al*.(2009) developed a protocol for *in vitro* micropropagation in carnation and the best shoot regeneration was observed on MS medium with 4.4 µM BAP+ 0.36 µM NAA.

Somatic embryogenesis:-

It involves the formation of a bipolar structure containing both shoot and root meristems, and developing in a manner similar to zygotic embryos. These embryoids can develop into fully functional plants under appropriate conditions.

Tanaka *et.al*.(2000) demonstrated high efficiency of somatic embryogenesis from leaf explants *of Dendranthema grandiflorum (Chrysanthemum)* on MS medium containing 57.08 m*M* IAA + 0.465 m*M* kinetin. No embryogenesis was observed in the absence of kinetin. Kumar *et.al*. (2002) reported heat shock induced somatic embryogenesis in callus cultures of *gladiolus* in the presence of high sucrose. Nakano *et.al*. (2004) reported somatic embryogenesis and plant regeneration from callus cultures of several species of *Tricyrtis*. Mandal *et.al*. (2005) reported direct somatic embryogenesis from ray floret explants of *Chrysanthemum* on MS medium supplemented with 2,4-D and BA. The best sucrose concentration in this medium was found to be 60 g dm^{-3} where 70 % explants responded while 55 % embryogenic response was obtained on medium supplemented with 400 mg dm^{-3} inositol. Emek *et.al*.(2007) reported somatic embryogenesis in *Gladiolus anatolicus* using leaves of *in vitro* grown shoots as explants. The highest rate of callus formation was obtained on MS medium supplemented with NAA(36.75 µM) in darkness and this creamy white and friable calli produced numerous somatic embryos on MS medium supplemented with BA(0.44 µM) within 4 weeks of culturing. Gow *et.al*.(2008) determine influence of various growth regulators on direct embryo formation from leaf explants of *Phalaenopsis* orchids. Karami Omid(2008) developed a protocol for the induction of callus from leaf explants in *Dianthus caryophyllus*. Embryogenic callus was obtained on MS medium supplemented with 9.74 µM 2,4-D and 0.44 µM BA. 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 media supplemented with 4.4 µM BAP for somatic embryogenesis. Xu *et.al*.(2012) established a protocol for somatic embryogenesis from leaf and stem segments of *Chrysanthemum* on MS medium supplemented with 0.26 µM BA and 13.23 µM NAA.

An efficient method has been developed for regeneration of complete plants via somatic embryogenesis in *Dianthus caryophyllus*, an important ornamental plant, 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. Dejou *et.al*.(2008) reported secondary somatic embryogenesis of carnation and the highest rate of secondary embryogenesis occurred on mannitol containing medium. Iantcheva *et.al*. (2008), reported somatic embryogenesis from leaf explants of *Dianthus caryophyllus*. Vigor somatic embryos were obtained on embryo formation medium supplemented with 2.2 µM BAP and 250 mg/l casein hydrolysate. Similarly, Frey *et.al*.(2009)developed a procedure for somatic embryogenesis in carnation. The optimum protocol for the induction of somatic embryogenesis included initiation of callus reported on basal MS supplemented with 3µM 2, 4-D.

OBJECTIVES

The present investigation was carried out on an important ornamental plant - *Dianthus caryophyllus*. 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.

MATERIAL AND METHODS:

Choice of Material:

Dianthus caryophyllus (family –Caryophyllaceae) commonly known as 'Carnation' was selected as an experimental material. Carnation is one among the most popular commercial cut flowers of the world, ranking second in commercial importance next only to rose. Carnation is preferred to roses and chrysanthemums by several exporting countries, on account of its excellent keeping quality, wide range of forms and colours and ability to withstand long distance transportation (Kanwar and Kumar 2009).

Experimental material **:** *Dianthus caryophyllus*

Distribution

The *Dianthus* species are adapted to the cooler alpine regions of Europe and Asia and are also found in the Mediterranean coastal regions. Carnation is not seen in the wild except in some Mediterranean countries. The major breakthrough in the carnation flower industry came with the evolution of the cultivar William Sim in 1938 by William Sim of USA. In India, Sim carnations are reported to have been introduced first by the Maharaja of Patiala at his farm at Dochi. An altitude of 2000-2500m is ideal for carnation cultivation.The most suitable climate for commercial carnation flower production in India prevails in the Nilgiris and Kodaikanal of Tamil Nadu and parts of Himachal Pradesh.

Morphology:

Carnation is a semi hardy herbaceous perennial with thick, narrow, linear and succulent leaves. Leaf blades are simple, entire, linear, glaucous, arranged in pairs, keeled and five nerved and their colour varies from green to greyblue or purple. The stems are hardy, shiny and have one to three angles with tumid joints. Flowers are bisexual and occasionally unisexual. The flower colour varies from white to pink or purple in colour shown in the figure. When grown in gardens, flowers grow between 6 and 8.5 cm in diameter. Petals are broad with frilled margins and calyx is cylindrical with bracts at the base.

Flower colour in carnation is attributed to the presence of two major pigments viz., carotenoids and flavonoids. The carotenoids are responsible for colour ranging from yellow to orange. However, many carnation plants do not have carotenoid pigments. Flavonoids are water soluble pigments such as anthocyanins. The major types of anthocyanins which contribute colour to carnation flowers are the cyanidins which are responsible for red or magenta colour and the pelargonidins which are responsible for orange, pink or brick red colour. The fragrance in carnation flower is predominantly dueto eugenol, α- caryophyllene and benzoic acid derivatives. The level of these compounds increases during flower development (Stem, 2009).

CULTIVATION

i) Soil

Sandy loam soils rich in organic matter content with pH of 5.5-6.5 are most ideal for carnation cultivation. Clay and silt soil can be improved by incorporating organic matter or compost. The soil must be well drained because the crop is highly susceptible to fusarium wilt.

ii) Temperature

Temperature considerably influences growth and flowering of carnation. The optimum night temperature for carnation is 10-11°C during winter and 13-15.5°C in summer. The optimum day temperature range is 18-24°C. High day and night temperatures, especially during flowering of carnation result in abnormal flower opening and calyx splitting.

iii) Light

Growth behaviour of carnation plants is influenced by duration and intensity of light. Genetically carnation is a quantitative long day plant. Long photoperiod usually promotes flowering while short days tend to delay it. Flower quality can be improved by providing long days only for a short period.

iv) Humidity

The plants must be protected from rain and dew during flowering. Wet buds and flowers are susceptible to fungal diseases. For commercial cultivation, the humidity of greenhouse should be maintained at 80-85% during beginning of vegetative growth and 60-65% during full growth stage.

v) Ventilation

Free circulation of air inside the greenhouse is essential. In uncooled greenhouses, vents have to be provided on the sides or roofs, whereas in cooled greenhouses, a fan-pad cooling system will cater to the needs of air circulation. A ventilation of 25-30% of the polyhouse ground area is ideal.

vi) Carbon dioxide

The best quality flowers can be produced when CO_2 concentration in the greenhouse is maintained at 500 - 750 ppm during day time under high light intensity and in a temperature range of 14-15°C. Under favourable conditions, additional carbon dioxide increases the flower production by 10-30%.(M. Jawaharlal *et.al.* 2007)

IMPORTANCE AND USES

Carnations are excellent for cut flowers, bedding, pots, borders, edging, indoors and rock gardens Though cut carnations are traded in the world market year round, they are in particular demand for the Valentine's Day, Easter, Mother's Day and Christmas. They were known as 'Jove's flower' in ancient Rome as a tribute to one of their beloved gods. Miniature carnations are now gaining popularity for their potential use in floral arrangement.

The flower petals of carnation are candied, used as a garnish in salads and for flavouring fruit, fruit salads, etc. They are also used as a substitute for rose petals in making syrup after removing the bitter white base.

Carnations are commercially utilized for extraction of perfume in France and the Netherlands. The volatile oil of carnation contains 40% benzyl benzoate, 30% eugenol, 7% phenylethyl alcohol, 5% benzyl salicylate and 1% methyl salicylate. About 100g of oil is obtained from 500kg of flowers (Muthukumaran, 2009).

Medicinal Importance:

Its flowers due to their aromatic and stimulative properties had been used in tonic cordials to treat fevers. In traditional European herbal medicine, carnation is prescribed to treat coronary and nervous disorders. The flowers are considered to be alexiteric, antispasmodic, cardiotonic, diaphoretic and nervine. The whole plant has been used as a vermifuge in China and as an animal feed in Spain (Stem, 2009).

Carnations contain substances that soothe the nervous system, reduce inflammation and swelling and can help to restore natural hormonal balances in women with nervous conditions associated with hormone imbalances. Carnation oils have therapeutic benefits for the treatment of skin rashes and act like a conditioner for the skin. Flowers have been used in the treatment of a variety of ills, from acting as a stimulant to an antispasmodic. Carnation tea or supplements have been used to relieve stress, anxiety and insomnia in those with hormone imbalances(Ganga *et.al*.2006)

Toxic effects:

The leaves contain triterpenoid saponins which can cause symptoms if sufficient quantities are eaten. Skin irritation can also occur upon skin exposure. The plant is considered to have a low level of toxicity and large quantities would need to be eaten to cause any symptom. Although carnations are a favorite flower for many people, cat owners should not place them around their home because they are poisonous. According to the American Society for the Prevention of Cruelty to Animals (ASPCA), carnations contain toxic properties that can cause adverse effects if ingested by cats or dogs. It may cause pet to have mild dermatitis and demonstrate mild gastrointestinal signs(Frey, 2007).

Glasswares

The glasswares used for culture work comprised of 6”Õ 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, glasswares were thoroughly brushed with alkaline detergent teepol and then washed in running tap water. It was then treated with hot chromic acid (mixture of $K_2Cr_4O_7 + H_2SO_4 + H_2O$) followed by very thorough washing with tap water. All vessels were then inverted in a clean tray and left to dry. Copper distilled water (5-10ml) was then poured into every culture vessel which was tightly plugged. Plugs were made out of absorbent surgical cotton wrapped in muslin. Glassware was then steam sterilized in an autoclave at a pressure of 15 lb\in^2 (121^{0}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^{o}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	
$(NH_4)NO_3$	1650
KNO_3	1900
$CaCl_2.2H_2O$	440
$MgSO_4.7H_2O$	370
KH_2PO_4	170

$FeSO_4,4H_2O$	27.8
Na_2EDTA	37.3

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 supplement, which were used either singly or in combinations :

i. Basal Medium (BM)
ii. BM + NAA(3.6-29.4µM), IAA(2.4-19.2µM) or 2, 4-D(2.4- 19.4µM)
iii. BM + Kn (2.3-18.6µM) + NAA(3.6-29.4µM), IAA(2.4-19.2µM) or 2, 4-D (2.4-19.4µM)
iv. BM + BAP (2.2-17.6µM) + NAA(3.6-29.4µM), IAA(2.4-19.2µM) or 2, 4-D (2.4-19.4µM)

v. BM + BAP (2.2-17.6µM)

vi. BM + Kn (2.3-18.6µM)

vii. BMS + Kn (2.3-4.65µM) + NAA(3.6-29.4µM), IAA(2.4-19.2µM) or 2, 4-D(2.4- 19.4µM)

viii. BMS + BAP (2.2-8.8µM) + NAA(3.6-29.4µM), IAA(2.4-19.2µM) or 2, 4-D(2.4-19.4µM)

ix. BMS + CM (15%) + NAA(3.6-29.4µM), IAA(2.4-19.2µM) or 2, 4-D(2.4- 19.4µM)

x. BMS + CM (15%) + Kn(4.65µM) or BAP (4.4µM) + NAA(3.6-29.4µM), IAA(2.4-19.2µM) or 2, 4-D(2.4- 19.4µM)

Coconut milk (liquid endosperm) when used was extracted from young coconuts and was stored at -4^{o}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^{0}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.

<u>Inoculation</u>

All the experimental manipulations were carried under aseptic conditions in an inoculation chamber fitted with a bactericidal ultraviolet tube (15W, peak emission 2537Ao). 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.

<u>Surface sterilization of inoculum</u>

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 bevistin 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 explants like stem and nodal explants were treated with 0.1% $HgCl_2$ for 3-4 minutes. Similarly explants like leaves were treated with 0.1% $HgCl_2$ for 1-2 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 the cultures were maintained in an air conditioned culture room at a temperature of 25 ± 4oC. All the inoculated cultures were incubated in growth room in controlled conditions at a temperature of 25.0 ± 2° C with a photoperiod of 12 hours per y. Illumination was provided by cool white fluorescent tubes (Philips India Limited, Mumbai) at 50 µmol/m^2/s^1.

RESULTS AND OBSERVATIONS

Nodal segments, shoot apices, leaf and stem segments were excised from field grown mature plant of *Dianthus caryophyllus* and used for experimental work.

Axillary bud and shoot tip culture

Fresh nodal explants each holding one dormant lateral bud were collected from mature field grown healthy *Dianthus caryophyllus* plant. After sterilization, the damaged internodal tissues on both sides were cut off and the nodal segments 3-4 mm in size were cultured on MS medium supplemented with various growth regulators. The axillary shoot proliferation from the cultured explants was remarkably influenced by the type and concentration of the growth regulator used.

Out of various growth regulators tested, best multiple shoot induction occurred on MS medium augmented with BAP (4.4µM) or Kn(9.30 µM). On BAP(4.4µM) supplemented medium, initial bud break occurred after 6 days of inoculation (Figure 1) forming 3-4 shoots from the axillary position after 15 days(Figure 2). These shoots multiplied further leading to the formation of numerous shoots after four weeks (Figure 3). A maximum of 18-20 shoots were formed after 6 weeks. When these microshoots were subcultured on the fresh medium containing BAP(4.4µM) more axillary shoots were initiated from them (Figure 4). Equally good results were obtained when nodal segments were cultured on MS medium supplemented with Kn(9.30 µM) where axillary bud break initiated after 8 days(Figure 5). 5-6 shoots were regenerated after 18 days (Figure 6) leading to the formation of 14-15 shoots after 5 weeks of culturing(Figure7). On subculturing on the fresh medium, the shoots grew continually thereafter forming numerous shoots. It was also observed that an increase in the concentration of BAP or Kn was not effective in inducing shoot formation from the nodal segments.

MS medium supplemented with NAA(1.47 µM) in conjunction with either BAP (4.4µM) or Kn (9.30 µM) was also effective in inducing bud break and forming multiple shoots at the rate of 8-10 shoots per nodal explant after 45 days of culture.

Young healthy shoot apices were collected from field grown mature plants of *Dianthus caryophyllus* and cultured on MS medium supplemented with different concentrations of BAP (4.4 – 17.6 µM) or Kn (4.65 -18.6 µM) either alone or in combination with NAA (1.47- 14.7 µM). Maximum shoot proliferation occurred on BAP(4.4µM) or Kn (9.30 µM) supplemented medium. On this medium, initial bud break was noticed after 10 days (Figure 8) and multiple shoot initiation occurred leading to the formation of 5-6 shoots after 21 days (Figure 9). Shoots multiplied further leading to the formation of 12-15shoots from a single shoot tip after 5 weeks of culturing (Figure 10). On subsequent subculturing on the same medium, the number of shoots increased further(Figure 11).

Response of various growth regulators on shoot proliferation from nodal and shoot apex segments is depicted in table 2 and Figure 12.

Table 2

Hormone conc.	**Type of explant**	**No.of shoots per explant**
BAP(2.2 µM)	Node	1
BAP(4.4 µM)	Node	18-20
BAP(8.8 µM)	Node	9
BAP(17.6 µM)	Node	2
Kn(2.32 µM)	Node	0
Kn(4.65 µM)	Node	2
Kn(9.30 µM)	Node	14-16
Kn(18.6 µM)	Node	4
BAP(2.2 µM)	Shoot tip	0
BAP(4.4 µM)	Shoot tip	3
BAP(8.8 µM)	Shoot tip	15-16

BAP(17.6 µM)	Shoot tip	2-3
Kn(2.32 µM)	Shoot tip	0
Kn(4.65 µM)	Shoot tip	2
Kn(9.30 µM)	Shoot tip	10-12
Kn(18.6 µM)	Shoot tip	3

Rooting of microshoots

Regenerated microshoots were carefully rescued from the bottles in laminar air flow on sterile glass plate. Then each of these shoots was carefully inoculated upright in the MS medium supplemented with or without auxins for root initiation. Shoots were inoculated on BMS medium and MS media with different concentration of IBA, NAA or IAA for root initiation. On NAA (7.35µM) supplemented medium, root initiation occurred at the base of shoot after 4 weeks. The roots were white and bore root hairs and elongated further leading to the formation of complete plantlet (Figure 13).

Attempts are underway to acclimatize these plantlets for their transfer to the field conditions.

Callusing

Callusing of the nodal explants occurred on MS medium supplemented with 14.7 to 29.4µM of NAA. However, best growth of callus occurred on MS + NAA (29.4 µM), with or without CM (15%). Callusing started at the cut ends or along the entire surface of the explant after 6-8 days of culturing (Figure 14) and after 3 weeks the entire segment turned into a mass of yellow soft and friable callus (Figure 15). The callus when subcultured on the same medium proliferated further and showed sustained growth. Callusing from the nodal segment was also observed on MS medium supplemented with NAA (14.7 µM) + Kn (4.65 µM). Likewise lower concentration of NAA (7.35 µM) were not effective in inducing callusing.

Callusing of the nodal explants also occurred on MS medium supplemented with 2, 4-D

(19.48 µM) + Kn (4.65 µM) where callusing started at the cut ends or along the entire surface of the explant (Figure 16) and after 4 weeks the entire segment turned into a mass of yellow soft and friable callus(Figure 17).

For the callusing of shoot apices, out of various growth regulators tested, 2,4-D (19.48 µM) + Kn(4.65 µM), with or without CM (15%) showed the best growth of callus after 3 weeks of culture(Figure 18). The callus when subcultured on the same medium grew rapidly and was capable of sustained growth. The callus was yellow, soft and friable (Figure 19).

<u>Stem Culture:</u>

Direct *de novo* adventitious root and shoot formation :

Stem segments, 3-5mm in length were planted on basal medium supplemented with various additives. Profused rooting from the stem segment was observed on lower concentrations of NAA(3.675 – 7.35 µM). Direct rooting from the stem segment occurred after 28 days (Figure 20), the roots grew further (Figures 21and 22) and the entire segment was covered with the roots after 40days(Figure23). These roots were white and bore dense root hairs. Rooting of the stem segment on NAA(7.35 µM) was invariably accompanied by direct shoot formation from the stem segment (Figure 23). Regeneration of roots declined on higher concentrations of NAA (14.7-29.4µM).

Direct shoot formation from stem segment occurred on MS medium supplemented with either BAP(4.4 µM) or Kn(9.3 µM). On BAP (4.4 µM) supplemented medium, green shoots regenerated from the explant after 10 days of culturing (Figure 24) forming 4-5 shoots after 20 days (Figure 25). These shoots multiplied further (Figure 26) leading to the formation of numerous shoots after 4 weeks of culturing(Figure 27). The shoots elongated forming well developed leaves (Figures 28 and 29).

Similarly prolific multiple shoot formation was observed on MS medium supplemented with Kn(9.3 µM). On MS + Kn(9.3 µM) nearly 5-6 shoots were regenerated after 18 days leading to the formation of 14-15 shoots after 5 weeks of culturing. When these microshoots were subcultured on the fresh medium containing Kn(9.3 µM) more adventitious shoots were initiated from them. Figure 30 depicts the effect of different growth hormones on shoot regeneration from the stem explant.

Callusing

Callusing of the stem segment occurred on MS medium supplemented with NAA (29.4 - 44.1µM). Best growth of callus however occurred on MS + NAA (29.4µM), with or without CM (15%). Callusing started at the cut ends (Figure 31) or along the entire surface after 14-15 days of culturing (Figure 32) and after 4 weeks the entire segment turned into a mass of yellow, soft and friable callus (Figure 33). The callus when subcultured on the same medium proliferated further and showed sustained growth (Figure 34).

Callusing of the stem segment also occurred on MS medium supplemented with 2,4-D(19.48µM) + Kn (4.65µM) where callusing started at the cut ends after 10-12 days of culturing (Figure 35) and after 3 weeks the entire segment turned into a mass of callus (Figure 36), which was capable of sustained growth on subsequent subculturing. The callus was yellow, soft and friable(Figure 37)

Study of callus

The stem callus formed on 2,4-D(19.48µM) + Kn(4.65µM) was extremely friable breaking up into single cells when shaken in water . It was heterogenous comprising cells of different shapes like spheroidal, ovoid, elongated and of different sizes. The cells of the callus invariably lacked chloroplasts. Cells were having prominent nuclei, dense cytoplasm and numerous starch grains. (Figures 38 and 39).

Differentiation

Xylogenesis

Three week old stem callus revealed differentiation of tracheids which occurred singly or in groups forming nodules and possessed scalariform thickening on their walls(Figure 40).

Leaf culture

Callusing

Leaf segments, 4-5 mm in length were excised and were planted on basal medium supplemented with various additives. Among the various growth regulators tested, callusing of the leaf explants occurred on MS medium supplemented with 2,4-D (4.87µM) + BAP(0.88 µM) + 2000mg/l casein hydrolysate after 7-8 days of culturing. Callusing started at the cut ends or whole of the leaf segment callused simultaneously (Figure 41) and within three weeks the entire explants turned into a mass of callus (Figure 42). A 5 week old green and friable leaf callus is shown in figure 43. The callus when subcultured on the same medium proliferated further and showed sustained growth(Figure 44). The callus was soft, green and friable. Callusing from the leaf explant was also observed on IBA(4.92 µM) ; NAA(14.7 µM)+Kn(4.65 µM)+CM (15%); NAA(14.7 µM)+CM(15%) (Figures 45-48). Callus induction along the entire surface of the leaf explants on NAA (14.7 µM)+Kn(4.65 µM)+CM (15%) and MS + NAA(14.7 µM)+CM(15%) medium was compact and greenish white.

Study of callus

The callus was solid and thus had to be teased with needles to study its cell types. Cell types studied in various calli showed their heterogeneous nature with wide variations in size and shapes of cells(Figures 49). Majority of the cells had abundant starch grains in them(Figure 50).

Differentiation

Xylogenesis:

Histogenetic differentiation in the form of tracheids was observed in all the calli. Tracheids occurred singly or in groups and possessed scalariform thickenings on their walls (Figure 51).

No differentiation of roots, shoots or whole plantlets from the calli could be effected under the present cultural conditions employed.

Axillary bud and shoot tip culture

Figure1 Nodal bud break reported after 6 days of inoculation on MS+BAP(4.4 µM)

Figure2 Regeneration of shoots(3-4) from nodal explant on MS + BAP(4.4 µM) after 15 days

Figure3 Formation of numerous shoots after four weeks on MS + BAP(4.4 µM)

Figure4 6 weeks old nodal explant showing multiple shoot formation (18-20) on MS + BAP (4.4µM)

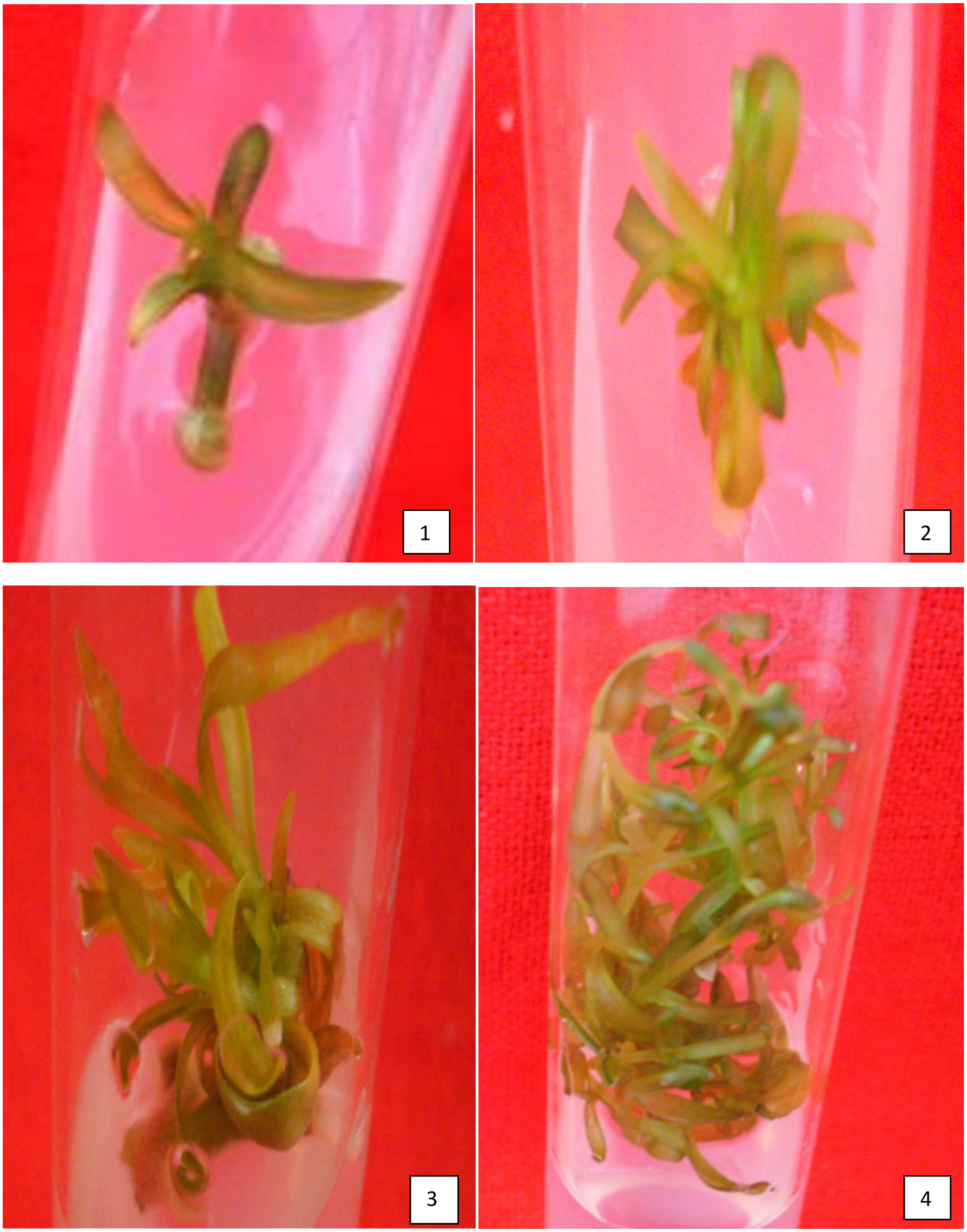

1
2
3
4

Figure 5 Nodal bud break reported after 8 days of inoculation on MS medium supplemented with Kn(9.30 µM)

Figure 6 Development of 5-6 shoots on MS + Kn(9.30 µM)after 18 days of culturing

Figure7 Formation of 14-15 shoots on MS + Kn(9.30 µM) after five weeks of culturing

Figure8 Initial bud break from shoot tip on MS + BAP(4.4µM) after 10 days of culturing

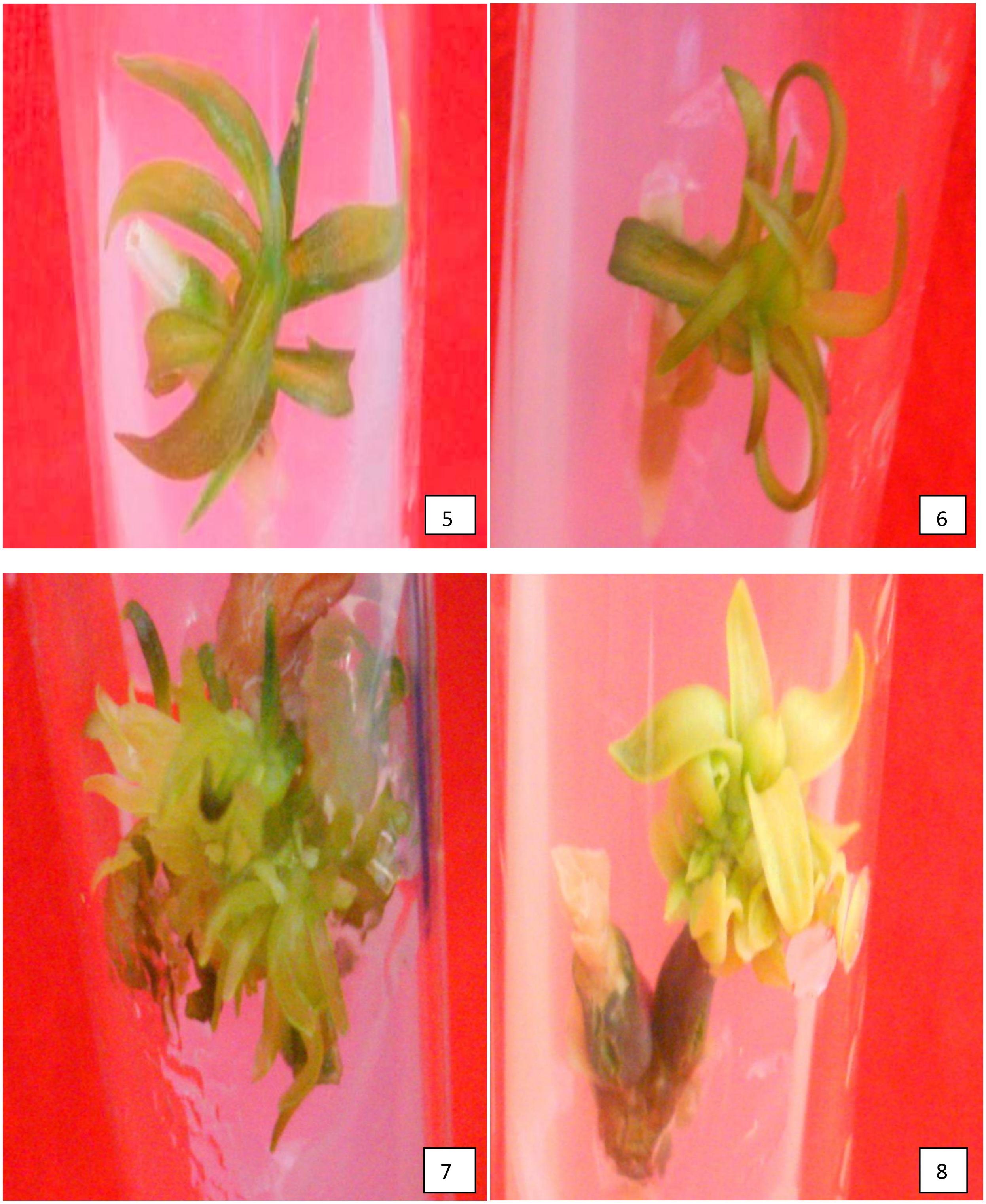
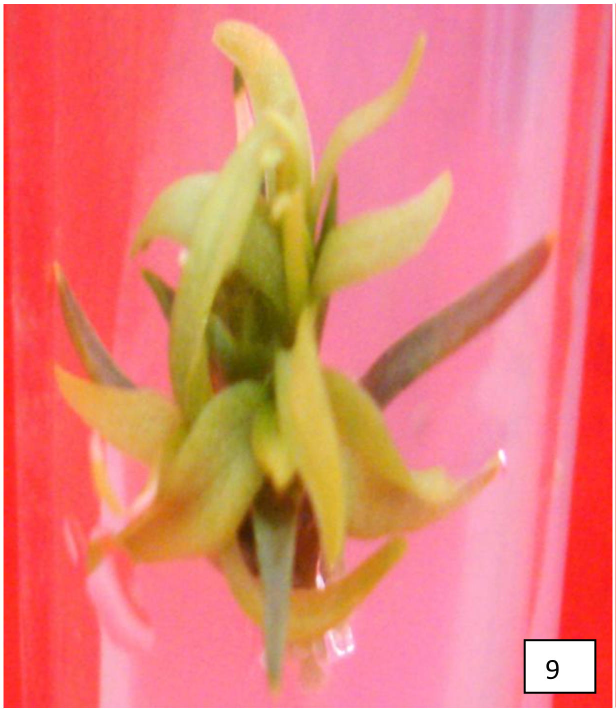

5
6
7
8

Figure 9 Multiple shoots initiation(5-6shoots) from shoot tip on BAP(4.4µM) after 21 days of culturing

Figure 10 Multiple shoot proliferation(12-15 shoots) from shoot tip on MS + BAP(4.4µM) after 5 weeks of culturing

Figure 11 Multiple shoot proliferation(12-15 shoots) from shoot tip on MS + BAP(4.4µM) on subsequent culturing on the fresh medium


9

10

11

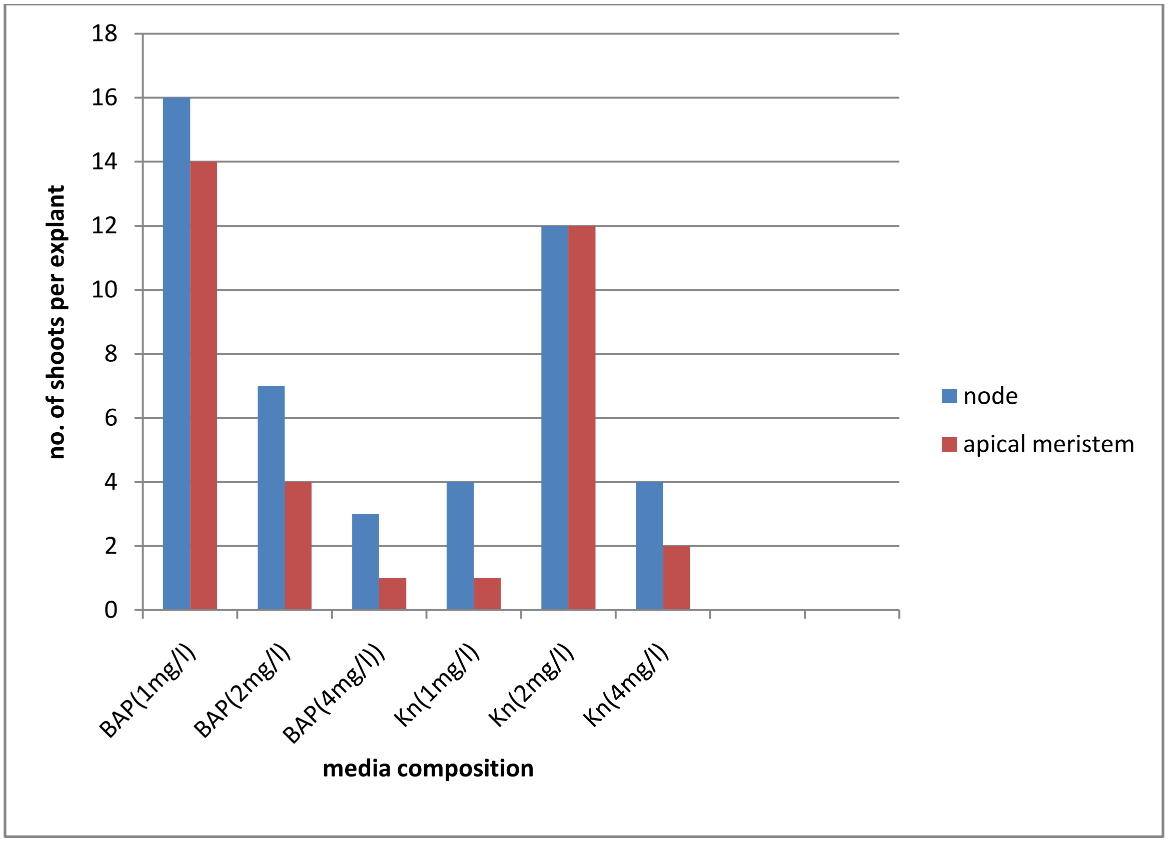


Figure 12 Histogram depicts the response of various growth regulators on shoot proliferation from nodal and shoot apex segment

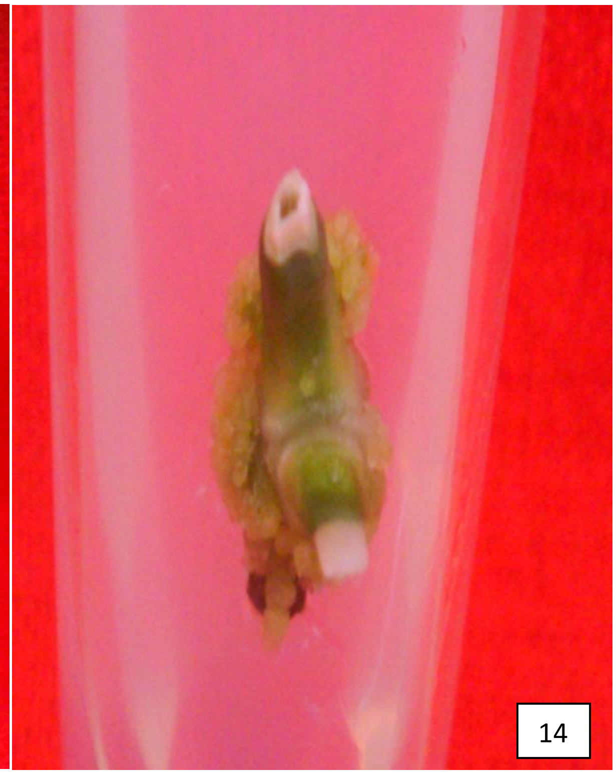
Figure13 Rooting occurring at the base of shoot on MS+ NAA(7.35µM) leading to the formation of complete plantlet

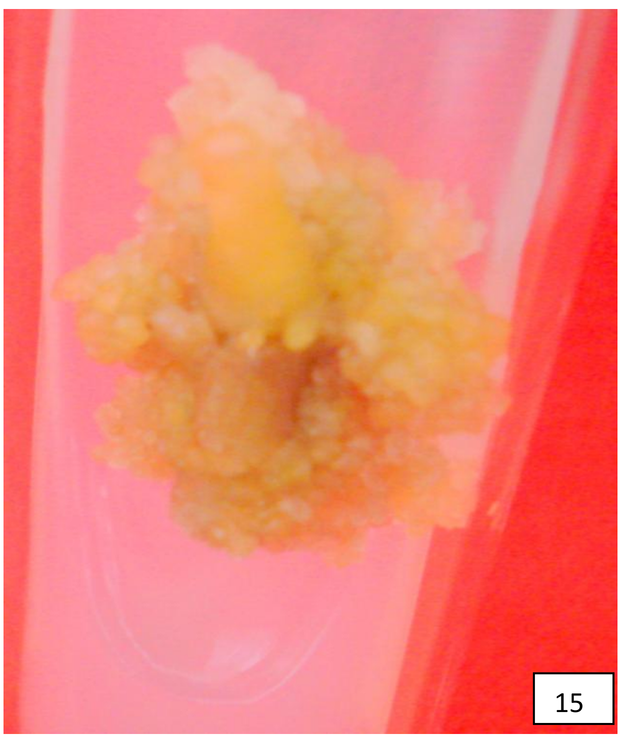
Figure14 Formation of yellow friable callus at the cut ends of the nodal explant on MS + NAA (29.4 µM) after 6-8 days of culturing

Figure 15 Formation of yellow friable callus along the entire surface of the nodal explant on MS+ NAA (29.4 µM) after 3 weeks of culturing

Figure16 Callusing started at the cut ends of the nodal explants on MS + 2, 4-D (19.48 µM) + Kn (4.65 µM)


13


14

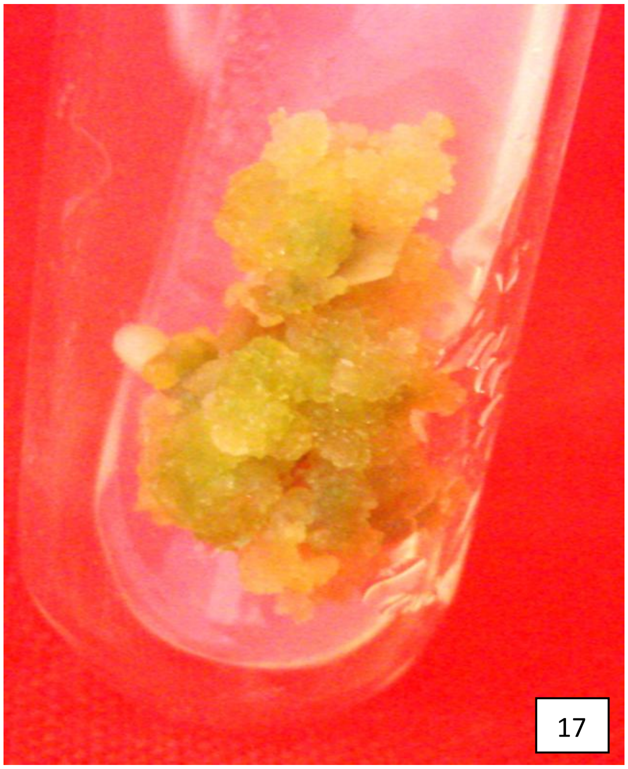

15


16

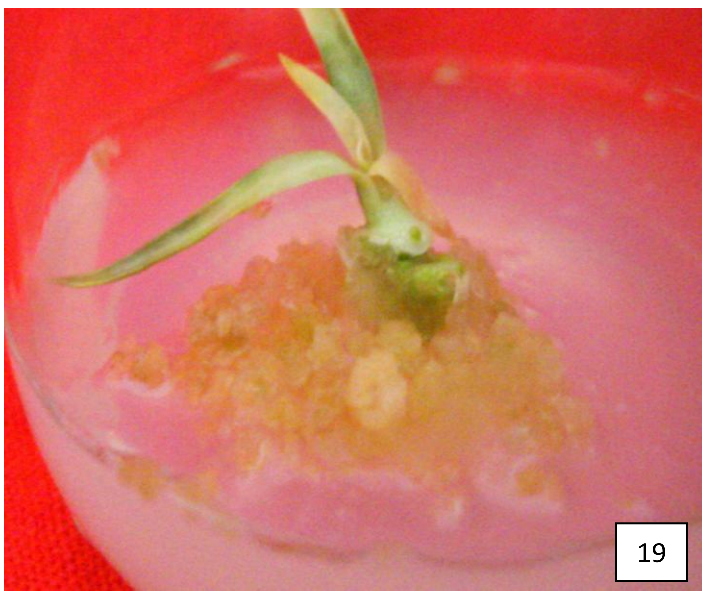
Figure 17 Yellow friable mass of callus along the entire surface of the nodal explant on MS+ 2, 4-D (19.48 µM) + Kn (4.65 µM) after 4 weeks of culturing

Figure 18 Formation of yellow friable callus along the entire surface of shoot tip on MS + 2,4-D (19.48 µM) + Kn(4.65 µM) after 3 weeks of culture

Figure 19 Formation of yellow friable callus after subsequent subculturing on the fresh medium


17


18


19

Stem culture

Figure 20 Direct rooting from the stem segment on MS + NAA(7.35 µM) occurring after 28 days of culture

Figure 21 and 22 Profused growth of lateral roots from the stem segment on MS + NAA (7.35 µM)

Figure 23 Rooting of the stem segment on NAA(7.35 µM) accompanied by direct formation from the stem segment after 40days of culturing


20


21


22


23

Figure 24 Regeneration of green shoots from the stem explant on MS+ BAP (4.4 µM) after 10 days of culturing

Figure 25 4-5 shoots proliferated from stem segment on MS + BAP (4.4 µM) after 20 days of culturing

Figure 26 Regeneration of numerous shoots from the stem segment on MS + BAP (4.4 µM)

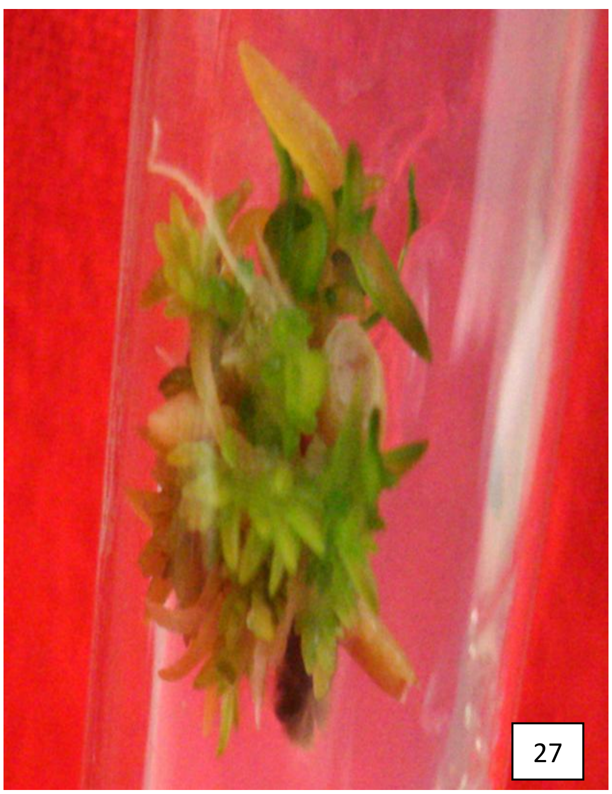
Figure 27 Numerous shoots proliferated further from stem segment on MS + BAP (4.4 µM) after 4 weeks of culturing

Figure 28 Stem segment forming elongated shoots accompanied by well developed leaves on MS + BAP (4.4 µM)


24


25


26


27


28

Figure 29 Stem segment forming elongated shoots accompanied by well developed leaves on MS + BAP (4.4 µM)

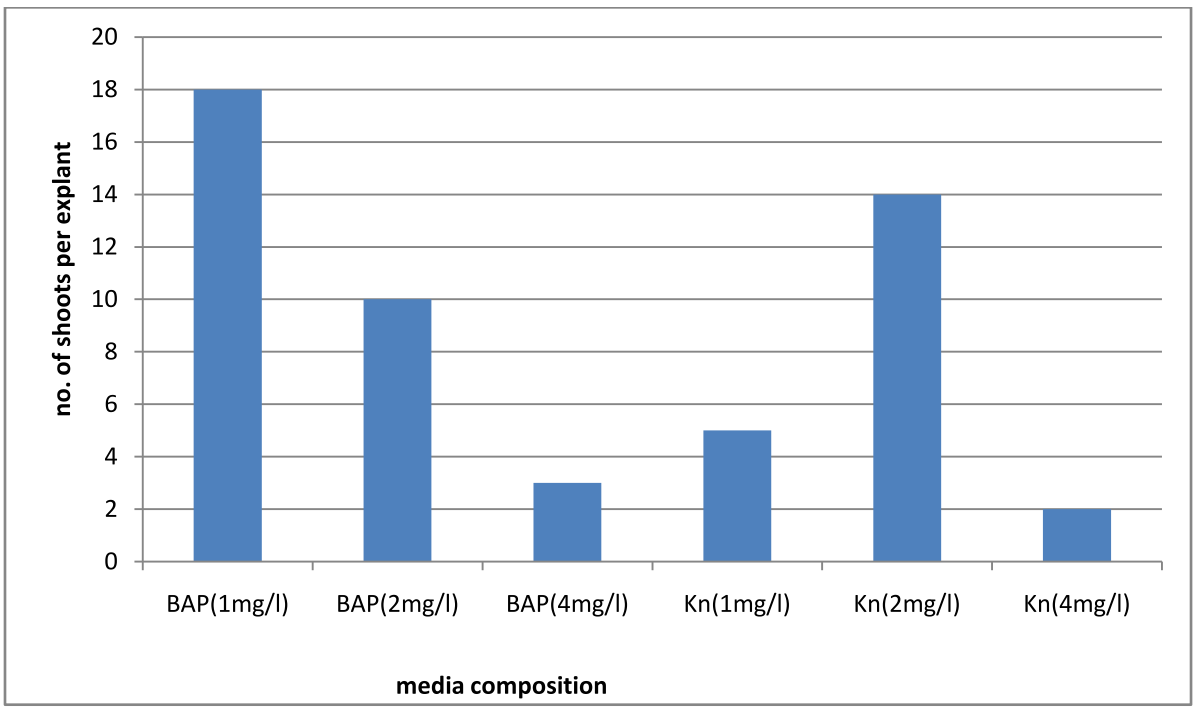


Figure 30 Histogram depicts the effect of different growth hormones on shoot proliferation from the stem explants

Figure 31 Formation of yellow friable callus at the cut ends of the stem segment on MS + NAA (29.4 μM)

Figure 32 Formation of yellow friable callus along the entire surface of the stem segment on MS + NAA (29.4 μM) after 14-15 days of culturing

Figure 33 Yellow friable mass of callus along the entire surface of the stem segment on MS + NAA (29.4 μM) after 4 weeks of culturing

Figure 34 Yellow friable mass of callus when subcultured on the fresh medium proliferated further and showed sustained growth


31

32

33

34

Figure 35 Formation of yellow friable callus at the cut ends after 10-12 days of culturing on MS + 2,4-D(19.48µM) + Kn (4.65µM)

Figure 36 Formation of yellow friable callus along the entire surface of the nodal explant on MS + 2,4-D(19.48µM) + Kn (4.65µM) after 3 week of culturing

Figure 37 Yellow friable mass of callus along the entire surface of the nodal explant On MS + 2,4-D(19.48µM) + Kn (4.65µM) after 5 weeks of culturing


35


36


37

Microscopic study of stem callus

Figure 38 Heterogenous nature of cells with different shapes like spheroidal, circular, ovoid and elongated shown on magnified scale

Figure 39 Cells having numerous starch grains

Figure40 A single tracheid shown on magnified scale with scalariform thickenings on its walls

38

39

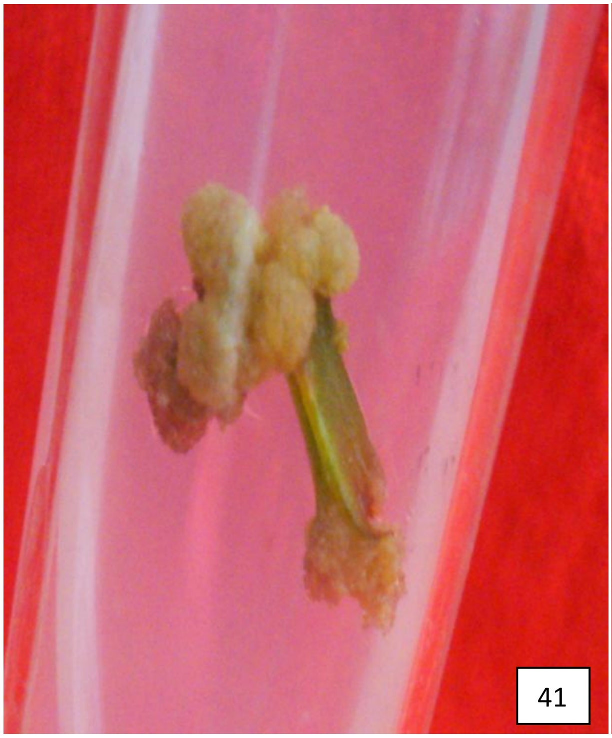
40

Leaf culture

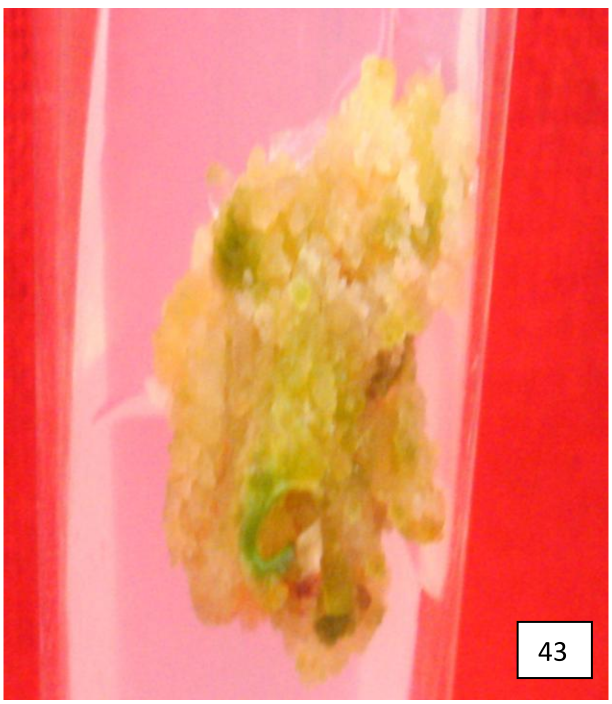
Figure 41 Formation of green friable callus on 2,4-D (4.87µM) + BAP(0.88 µM)+ 2000mg/l casein hydrolysate from leaf after 8 days of culturing

Figure 42 and 43 3weeks and 5weeks old leaf culture showing callusing along the entire surface of leaf explants on MS+2,4-D (4.87µM) + BAP(0.88 µM)+ 2000mg/ l casein hydrolysate respectively

Figure 44 A mass of soft, green friable callus when subcultured on the same medium shows development of green structure that are not inferred yet


41


42


43

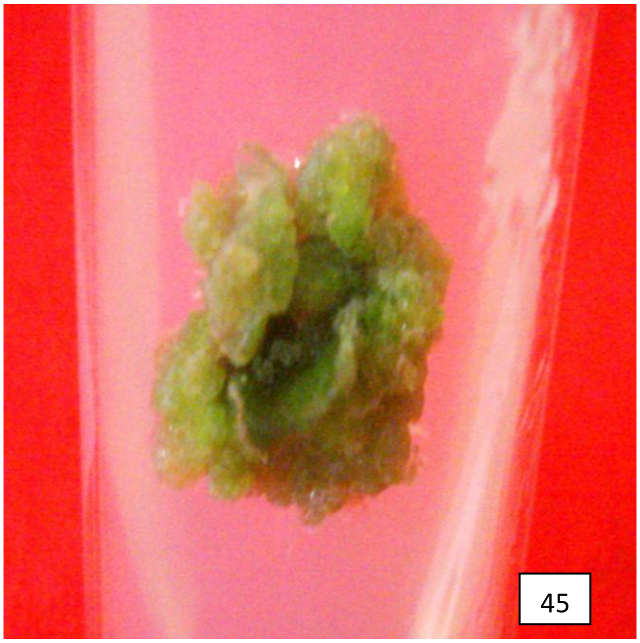

44

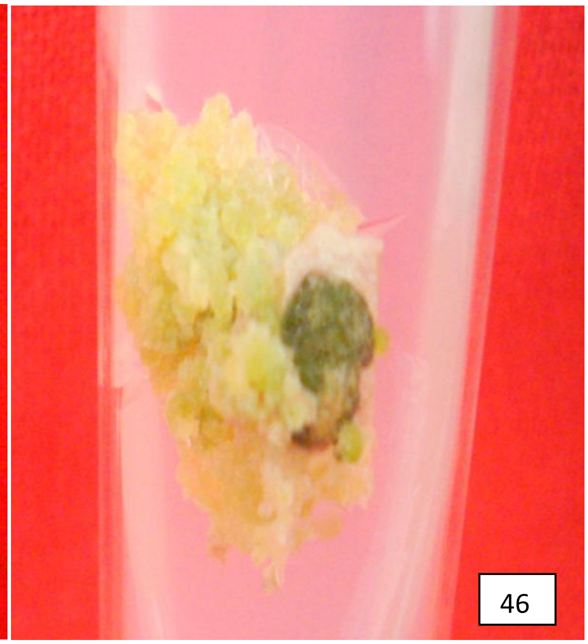
Figure 45 Formation of green friable callus on IBA(4.92 µM) from leaf explants after 28 days

Figure46 6 weeks old leaf culture showing callusing at the cut ends on IBA(4.92 µM)

Figure 47 Callus induction along the entire surface of the leaf explants on NAA (14.7 µM)+Kn(4.65 µM)+CM (15%);

Figure 48 5weeks old leaf culture depicting compact greenish white callusing at the cut ends on MS + NAA(14.7 µM)+CM(15%)


45


46


47


48

Microscopic study of the leaf callus

Figure 49-50 Heterogenous nature of cells of green and friable leaf callus

Figure 51 A tracheid possesseing scalariform thickenings on its walls

49

50

51

DISCUSSION

The present investigation was undertaken on an important ornamental plant *Dianthus caryophyllus* with an aim to develop an efficient, reliable and reproducible protocol for its clonal propagation under *in vitro* conditions.

High efficiency of adventitious shoot bud formation and plant regeneration was observed from different explants excised from field grown mature plant of *Dianthus caryophyllus* like nodal segments, shoot apices and stem segments in the present investigation.

Micropropagation through enhanced axillary branching is a reliable technique for clonal propagation as it enables to retain the clonal fidelity and prevents somaclonal variation in the cultures unlike the callus tissue. In the present investigation multiple shoot proliferation was achieved using shoot tip and nodal segment. Cytokinins used in plant cell culture regulate cell division, stimulate axillary and adventitious shoot proliferation, regulate differentiation and stimulate protein and enzyme activity (Gross and Parthier, 1994). The axillary shoot proliferation from the cultured explants was remarkably influenced by the type and concentration of the growth regulator used. For shoot proliferation growth regulators especially cytokinins are one of the most important factors affecting the response (Sterk *et.al.* 2003, Mishra *et.al.*2005, George *et.al.*2008, Warrier *et.al.* 2010). A wide range of cytokinins like Kn, BAP have been employed in shoot proliferation (Bhojwani and Razdan, 1982 Viji *et.al.*2010). However a wider survey of literature suggested that BAP is the most reliable and effective cytokinin for shoot proliferation. A number of plants such as *Dianthus*(Pareek *et.al.*2004), *Rosa indica* (Hameed *et.al.*2007), *Rosa canina* (Shirdel *et.al.*2013), *Gladiolus grandiflorus (Haula et.al.2012), Dianthus caryophyllus (Brar et.al.1996;* Daniel *et.al.*2009) have been successfully multiplied using BAP.

In the present investigation, axillary shoots were induced from the nodal segments and shoot apices on MS medium supplemented with various growth adjuvants in different combinations. For nodal explants best results were obtained on BAP(4.4µM), where a maximum of 18-20 shoots were formed after 6 weeks of culturing. Equally good results were obtained when nodal segments were cultured on MS medium supplemented with Kn(9.30 µM) where 14-15 shoots were formed after 5 weeks of culturing. Similar results were also reported by other workers in *Rosa hybrida* (Udom *et.al*.2009), *Chrysanthemum* (Waseem *et.al.*2009), *Rosa rugosa (*Xing *et.al*. 2010), *Chrysanthemum* (Nalini 2012).

Multiple shoot proliferation from shoot tips was achieved on MS medium supplemented with different concentrations of BAP (4.4 – 17.6 µM) or Kn (4.65 -18.6 µM) either alone or in combination with NAA (1.47- 14.7 µM). Maximum shoot proliferation occured on BAP(4.4µM) or Kn (9.30 µM) supplemented medium formation of 12-15shoots from a single shoot tip after 5 weeks of culturing. Our results are in agreement with other workers who have reported the synergistic effect of BAP or Kn either alone or in conjunction with NAA for multiple shoot proliferation from shoot tips and nodal segments in *Dianthus caryophyllus*(Ali *et.al*.(2008), Daniel *et.al.*(2009), Kharrazi *et.al*.(2011)). Ali *et.al*.(2008) reported effective multiple shoot formation when cultured on MS medium fortified with BAP (4.4 µM) from apical and nodal meristems within 7 days of culture. Similarly, Kharrazi *et.al*.(2011) evaluated the effect of different plant growth regulators for multiple shoot proliferation from axillary buds in *Dianthus caryophyllus* . The highest number of shoots (5 shoots/explant) was formed on medium supplemented with 4.4 µM BAP + 1.47 µM NAA.

Many ornamental plants 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. The present material has a great organogenic potential as it exhibited a high efficiency of *de novo* adventitious root and shoot formation directly from the stem segment. During the present investigation profuse growth of lateral roots occurred on the entire surface of stem segment along with adventitious shoot formation on lower concentrations of NAA(7.35µM). These roots were short and bore root hairs profusely. Further incorporation of CM(15%) to the NAA supplemented medium considerably enhanced the rooting where entire segment was covered over with clumps of roots. The roots were thin, white having dense root hairs.

A high efficiency of adventitious shoot formation from the stem segment was observed. Stem segments cultured on MS medium supplemented with NAA(1.47-7.35µM) either alone or in conjunction with BAP(4.4µM) or Kn(9.30µM) and MS + BAP(4.4µM) or MS+ Kn(9.30µM) produced adventitious shoots directly on the surface of stem explants without the formation of intervening callus.

Likewise Chen *et. al.* (2001) reported direct de novo adventitious bud formation from the stem internode explant of *Adenophora triphylla* on MS medium supplemented with BAP (2.22 - 35.51 µM) and NAA(0.54 µM).. Archana *et al.* (2002)reported adventitious bud regeneration from the entire leaf surface of *Dianthus chinensis* on BAP (13.2 µM) and NAA (3.675 µM).

Martin *et .al*.(2003) reported adventitious shoot regeneration from lamina explants of two commercial cut flower cultivars of *Anthurium andraeanum* and high efficieny of shoot regeneration was reported on an average from 67% of the leaves, with an average of seven shoots per explants .

Induction of roots at the base of in vitro grown shoots is essential and indispensable step to establish tissue culture derived plantlets to the soil. The most effective auxins for rooting are IBA and NAA (Perik., 1987; Uddin *et. al*.2005). In the present case however, NAA proved better than IBA for the induction of adventitious roots from the base of regenerated shoots. Likewise Ali *et.al*.2008 reported best rooting response on MS medium containing NAA(7.35 µM).

In the present work, callusing of the nodal segment, shoot apices, leaf and stem segments occurred immediately after culturing on optimal chemical milieu. Murashige and Skoog's agar gelled medium supplemented with NAA (29.4 µM), with or without CM (15%) or 2,4-D (19.48 µM) + Kn(4.65 µM) turned out to be optimal for initiation and sustained growth of calli from different plant parts. Addition of coconut milk considerably enhanced the callus growth when in conjunction with auxins and cytokinins. The calli were pale yellow, friable and fast growing. Formation of green friable callus from the leaf explant on 2,4-D (4.87µM) + BAP(0.88 µM) + 2000mg/l casein hydrolysate was the best medium reported. Utgikar *et.al*. (2005) established a protocol for micropropagation and callus regeneration in *Dianthus caryophyllus.* Kumar *et.al*.(2009) reported callus induction and *in vitro* shoot bud formation from callus. The highest callus induction was observed on MS medium supplemented with 2,4-D(9.74 µM)+ BAP(4.4

µM). Likewise Kaviani *et.al*.(2011) established a protocol of callus induction and root formation of *Matthiola incana* on MS media containing NAA(3.675 µM) and Kn(2.3 µM)+ NAA on leaf microcuttings. Ghanati (2012) developed an efficient procedure for the development of callus from different explants especially leaf explants of *Eustoma grandiflorum*. MS medium containing IAA(14.4 µM)+ Kn(0.46 µM)+ NAA(22.05 µM) was the best medium reported for the induction of callus.

Addition of CH to the 2, 4-D supplemented medium proved good for callus growth.
In the present study synergistic action of CH with 2, 4-D was demonstrated for the initiation and growth of callus from leaf explants. Moreover synergistic action of CM with NAA was demonstrated for the initiation and growth of callus. Likewise synergistic action of CM with NAA favoured callus growth in *Mesembyanthem (*Mehra and Mehra 1972) and *Dimorphotheca*(Anand and Mehra 1983). It is quite clear that CM make up the deficiency of certain essential nutrients whose synthesis is impaired or suppressed in the normal tissue. The growth rate of callus was greatly activated by the addition of Kn to the auxin and CM supplemented medium. A definite interaction existed i.e. auxin, coconut milk and kinetin.

Both stem and leaf calli showed variability in cell shape. According to Steward *et al*. (1963) "even in the most uniform environment, the two daughter cells from a single clone were rarely identical". A similar observation was made in carrot, endive parsley, lettuce and spinach callus by Kant and Hildebrandt (1969) and in *Pterotheca falconeri* by Mehra and Mehra (1971).

The callus thus formed from different vegetative parts on the medium were more or less identical in morphology. The calli obtained from all the parts were heterogenous being composed of parenchymatous ovoid, oblong, semicircular cells or those with aberrant shapes. Histogenetic differentiation in the form of tracheids was observed in all the calli. Tracheids occurred singly or in groups and possessed scalariform thickenings on their walls. Tracheids were seen in the early stages of callus formation. Firstly only few tracheids could be seen which multiplied with the active proliferation of the callus. It seems there is a correlation between cell division and vascular differentiation. This contention gets support from other reports which suggests that cell division must precede the formation of vascular elements and that no vascular differentiation occurs in the absence of cell division. No organogenetic differentiation could be effected from the calli on the various media tried.

It is concluded that in the present work, a reliable and reproducible protocol has been established to investigate the differentiation and redifferentiation responses of cells of various organs of *Dianthus caryophyllus* to varied and diverse chemical milieu by using all the three techniques of micropropagation. It has not been possible to induce direct shoot organogenesis from callus cultures. It is opined that cells in plant are undoubtedly totipotent but some vital hormonal and nutritional factor or their combination for differentiation could not be discovered by us during the stipulated period of the project.

LITERATURE CITED

- Ahmed H. A. (1986). *In vitro* regeneration and propagation of meristem apices of *Chrysanthemum*. Kerteszetiegyetem Kozlemenyei, 50: 199-214.
- Ali A., Afrasiab H., Naz H., Rauf M. and Iqbal J.(2008) An efficient protocol for in vitro propagation of carnation (*Dianthus caryophyllus*). Pak. J. Bot., 40(1): 111-121.
- Altvorst V., Koehorst H., bBruinsma T., Dons J. 1994, Improvement of adventitious shoot formation from Carnation leaf explants. Plant cell and tissue organ culture. 37: 87-90.
- Anand M. and Mehra P. 1983. Callus induction, growth and differentiation in *Dimorphotheca* ecklonis. Phytomorphology 32:197-204.
- Annadana S., Rademaker W., Ramanna M., Udayakumar M., de Jong J. (2000). Response of stem explants to screening and explant source as a basis for methodological advancing of regeneration protocols for chrysanthemum. Plant Cell, Tissue and Organ Culture, 62: 47-55
- Atak C., Celik O. (2009). Micropropagation of *Anthurium andraeanum* from leaf explants. Pak. J. Bot., 41(3): 1155-1161.
- Baman and Pal(1999). Effect of nitrogen on growth and flowering of Chrysanthemum (*Chrysanathemum morifolium*), Chandrama. Hort. Journal 12(1)- 51-60.
- Bhojwani S. and Razdan M. (1982). Plant cell Tissue culture theory and practice. Elsevier Sci. Publ. Amsterdam, 1-520.
- Brar M.(1996). Effect of Thidiazuron and Benzylaminopurine on in vitro shoot proliferation of carnation (*Dianthus caryophyllus* L.) In vitro 31(3, Part 2): 61A.
- Casas J., Olmos E., Piqueras A.(2010). In vitro propagation of Carnation (*Dianthus caryophyllus* L.), Protocols for in vitro propagation of ornamental plants. Volume 589, Pp. 109-116.
- Chen T., Tsung J., Chang W.(2004). Plant regeneration through direct shoot bud formation from leaf cultures of *Paphiopedilum orchids*. Plant cell Tissue and Organ Culture 76: 11-15.
- Colijin C., Kool A. and Nikamp H. 1979. Induction of root and shoot formation from root meristems and shoot tips of *Petunia hybrid*. Protoplasma 99:335-340.

- Danial G., Yousif A.,Omar M.(2009). Clonal propagation of *Dianthus caryophyllus* L. through tissue culture. J.Duhok Univ. Vol 12. No.1(Special Issue), Pp 91-95.
- Datta S. K., Chakrabarty D., Saxena M., Mandal A. K. A., Biswas A. K. (2001). Direct shoot regeneration from florets of *chrysanthemum* cultivars. Indian Journal of Genetics. 61: 373-376.
- Emek Y., Erdag B.(2007). *In vitro* propagation of *Gladiolus anatolicus*(boiss.) stapf, Pak. J. Bot.,(39(1): 23-30.
- Farsi M.,Yazdi T., Qasemiomran V.(2012), Micropropagation of *Anthurium andreanum* cv. Terra, African Journal of Biotechnology, Vol 11(68), pp. 13162-13166.
- Frey and Janick(1991), Organogenesis in Carnation, J. Amer Soc. Hort. Sci. 116(6): 1108-1112.
- Gow, W. P., Chen, J. T., and Chang, W. C. 2008. Influence of growth regulators on direct embryo formation from leaf explants of *Phalaenopsis* orchids. *Acta Physiol. Plantarum* 30: 507-512.
- Guohua M., Jaime A., Teixeira S., Wu G.(2011), Direct adventitious shoot formation from apical shoot explants of Euphorbia tirucalli, J Plant Growth Regul. 30: 114-116
- Haberalandt G. 1902. Kultuvrsuchemit isollieten pflanzenzellenn. Situzunsber. Akad. Wiss. Wien. , Math-Naturwiss. K1, Abt. 1. 111: 69-92.
- Haouala and Salhi(2012). Axillary budding and rooting of Gladiolus (*Gladiolus grandiflorus* Hort.) in salt stress conditions, Afr. J. Hort. Sci. ,6:101-110.
- Hussey G. 1978, The application of tissue culture to the vegetative propagation of plants. Sci.Prog. 65: 185-208.
- Iantcheva A., Vlahova M., Atanassova B., Atanassov A.(2005). Plant regeneration via direct organogenesis and somatic embryogenesis of two new Bulgarian spray *Carnation* cultivars, Biotechnol. & Biotechnol Eq.19.
- Jawaharlal M., Ganga M., Padmadevi R., Jegadeeswari V., Kartikeyan S. A technical guide on carnation.

- Kantia A., Kothari S. (2002), High efficiency adventitious shoot bu formation and plant regeneration from leaf explants of *Dianthus chinensis* L. Scientia Horticulturae 96: 205-212.
- Karami O., Deljou A., Kordestani G. (2007). Secondary somatic embryogenesis of Carnation (*Dianthus caryophyllus* L.). Plant Cell Tissue and Organ culture 92:273-280.
- Karats M., Aasim M., Cinar A., Dogan M. (2013). Adventitious shoot regeneration from leaf explants of dwarf hygro (*Hygrophilis polysperma* Roxb.). The Scientific World Journal, Vol. 2013.
- Kharrazi M., Nemati H., Tehranifar., Bagher A., Sharifi A.,(2011). *In vitro* culture of Carnation (*Dianthus caryophyllus* L.) focusing on the problem of Vitrification, J. Biol. Eviromental Sciences, 5(13), 1-6.
- Lee, Min M., Nam K., Kyoung E., Hi S., Park Y.,(1997). Biochemical character stics of S-adenosylmethionine decarboxylase from Carnation(*Dianthus caryophyllus* L.)petals, J.Plant Biol., 40(2), 80-88.
- Middleton et.al.2011. Sources of new ornamental plants : the importance of heritage plants and plants relicts from historic place an old gardens. SAJAH, ISSN 0258-3542, Vol.26,2 :55-67.
- Mizutani T., Tanaka T. (1994). Study on the floret culture of Higo-*chrysanthemum*. Proceedings of the School of Agriculture, Kyushu Tokai University, 13: 9-14.
- Murashige, T. 1974. Plant propagation through tissue cultures. Ann. Rev. Plant Physiol 25: 135-166.
- Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Plant Physiol 15: 473-497

  Muthukumaran K.(2008). Indian floriculture industry and challenges.
- Naderi R., Jalali N., Mesbah B., Mirmasoumi M.(2012). Estimate of callus induction and somatic embryogenesis in *Cyclamen*, International Journal of Agriculture and Crop Sciences, 4-11,Pp 699-702.

- Nalini R. (2012). Micropropagation of Chrysanthemum(*Chrysanthemum morifolium*) using shoot tip as explants. Internatioal Journal of Food, Agriculture and Veterinary Sciences, Vol.2(2),pp. 62-66
- Nikbakht A., Kafi M., Mirmasoumi.(2005). Micropropagation of Damask Rose(*Rosa damascene* Mill.),International Journal of Agriculture and Biology,1560-8530-4: 535-538.
- Omid K. (2008). Induction of embryogenic callus and plant regeneration in Carnation (*Dianthus caryophyllus* L.). Journal of biological Sciences.
- Pragya, Singh S., Misra R., Ranjan J.,(2012). *In vitro* shoot regeneration from cormel derived calluS of *gladiolus* and biohardening of plantlets, Indian Journal of Biotechnology, Vol 11, pp-99-104.
- Qaoud H. (2013). Adventitious shoot formation and plant regeneration from leaf explants of carnation(*Dianthus caryophyllus* L.). Vol. 12(21), pp. 3244-3249.
- Rezaee F., Ghanati F. and Baroujeni L. (2012). Micropropagation of Lisianthus (*Eustoma grandiflora* L.) from different explants to flowering onset. Iranian Journal of Plant Physiology 3 (1), 583-587.
- Salehi H. (2006) Can a general shoot proliferation and medium be used for a number of carnation cultivars? African Journal of Biotechnology Vol. 5(1), pp. 025-030.
- S.E. Arici and N.K. Koc(2009) Regeneration and agrobacterium-mediated transformation studies in carnation(*Dianthus caryophyllus* L. cv. Turbo). African Journal of Biotechnology Vol. 8 (22), pp. 6094-6100.
- Shaheenuzzaman M., Haque M., Karim M., Noor Z. (2011). In vitro shoot proliferation and development of micropropagation from leaf discs of Gladiolus, J. Bangladesh Univ. 9(1): 21-26.
- Shirdel M., Azar A., Matloobi M., Nahandi F.(2012). Effect of nodal position and growth regulators on in vitro growth of Dog Rose (*Rosa canina*) Journal of Ornamental and horticulture plants. 2251-6433.
- Shogandha 2010. Overview of floriculture sectur
- Tanaka Y., Kastumoto Y., Brugliera F., Mason J. (2004). Plant Cell Tissue and Organ Culture, 80, 1-24.

- Udom N., Kanchanapoom K., Kamnoon K.,(2009). Micropropagation from cultured nodal explants of rose (*Rosa hybrid* L. cv. 'Perfume Delight', Songklanakarin J. Sci. Technology31(6), 583-586.
- Utgikar and Srinivas(2005). Micropropagation and callus regeneration studies in Carnation(Dianthus caryophyllus L.). Jagdande, N.D., 12:432-435.
- Watad A., Ahroni A., Zuker A., Shejtman H., Nissim A., Vainstein A. (1996). Adventitious shoot formation from carnation stem segments, Scientia Horticulturae 65: 313-320.
- Waseem K., Khant M., Jaskant Q., Jilani M., Sohail M. (2009). Effect of different auxins on the regeneration capability of *Chrysanthemum* leaf discs. International Journal of agriculture and biology. 11-4: 468-472.
- Xing W., Bao M., Qin H., Ning G. (2010). Microopropagation of Rosa rugosa through axillary shoot proliferation, Acta Biologica Cracoviensa Series Botanica 52/2: 69-75.
- Yih C., Saleh N., Zaman F. (2010) *In vitro* multiplication of the rare and endangered slipper orchid,*Paphiopedilum rothschildianum*. African Journal of Biotechnology Vol, 9(14), pp. 2062-2068
- Yantcheva A, Vlahova M., Atanassov A. (1998) Plant Cell Rep., 18, 148-153